Signal transduction via multiple BMP receptor complexes

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1. Introduction

The communication between different cells in the human body through defined signalling networks, conserved through evolution, is crucial for the development from a single cell in the early embryo to the fully developed human being. Perturbation of that network causes pathological conditions, resulting in defective development of an organism, often leading to premature death or physiological disability and disease.
Communication between different cell types is either mediated through direct cell-cell interaction, cell contact with extracellular matrix components or by direct binding of secreted soluble factors to specific receptors at the plasma membrane. Among these factors are cytokines, hormones, neurotransmitters as well as growth factors.
These receptors can be grouped into families, based on the way in which they generate or modulate the intracellular signals that give rise to the particular functional responses. Among these receptors phosphorylation and dephosphorylation events as well as protein-oligomerization and activation of transcription factors play central roles.
Moreover, the activity of a given receptor can be modulated by other signalling pathways in a variety of ways, generating the flexibility required of such a complex system.

The control of diverse cellular processes in response to a plethora of extracellular stimuli implies a high specificity in that system. This fine tuning is achieved by counteracting activators and inhibitors thus balancing different pathways. A variety of signal transduction pathways induced by many factors create a complex network whose complexity is potentiated by crosstalks of different pathways. To understand such complexity first the particularities have to be examined and understood, starting from this to get a picture of the whole entity.

The aim of this thesis was to investigate the effects of BMP receptor oligomerization on BMP2 mediated signal transduction, to explore the phosphorylation status of the BMP type II receptor and to identify associated proteins and their involvement in signal transduction.
Therefore, the function and signal transduction of BMP2 will be described in the following.

1.1 BMP2

Bone morphogenetic proteins (BMPs) were initially identified by their ability to induce ectopic bone and cartilage formation in vivo (Urist, 1965). All BMPs transmit signals by binding to their respective type I and type II receptors. Ligand binding induces a signal transduction cascade which includes receptor phosphorylation, conformational changes of the receptors and trans-phosphorylation of signal transducing molecules, Smads, phospho-ERK and p38 (Canalis et al., 2003). Finally, this cascade leads to the activation of transcription of a variety of specific target genes. In the following, the variety of modulation and the different processes involved will be described in more detail.

1.1.1 TGF beta superfamily

Bone morphogenetic proteins are members of the transforming growth factor β (TGF-β) superfamily. Members of that family are regulating a large variety of biologic responses in many different cells and tissues during embryonic development and postnatal life. Besides the BMPs, TGF-βs, Growth and differentiation factors (GDFs), Activins, nodal and anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), belong to that group of secreted factors. Members of that family are highly conserved during evolution from mammals to *Xenopus*, *Caenorhabditis elegans* to *Drosophila*. The *Drosophila* decapentaplegic (DPP) and the human BMP2/4 show 75 percent amino acid identity in the mature part and can compensate for each other in cellular assays. So far, more than 30 BMP family members have already been described (Balemans and Van Hul, 2002).

1.1.2 BMP2 structure and function

BMP2 is secreted as a large precursor protein, containing the amino-terminal signal sequence, a pro-domain and the carboxy-terminal mature peptide that is released upon furin-mediated cleavage inside the cell. Structurally, all members of the TGF-β superfamily show a seven (to nine) cystine-knot motif. One of these cystine residues is used for covalent inter-chain disulfide bridging, and the others are involved in an intramolecular ring formation, known as the cystine knot configuration. The cystine knot is a folding motif that forces exposure of hydrophobic residues to the aqueous surrounding, and prevents the molecule from assuming a globular protein structure. Instead, it drives the molecules to undergo dimerization, resulting in highly stable dimeric proteins with a butterfly-shape structure (Scheufler et al., 1999). The cystine knot structural motif is not unique to the TGF-β superfamily and is found in other cytokine families, such as NGF and PDGF, which together constitute a large superfamily of cystine knot proteins.

The backbone fold of homodimeric BMP2 (Scheufler et al., 1999) is very similar to that of BMP7 (Griffith et al., 1996) and TGF-β (Schlunegger and Grutter, 1992), (Daopin et al., 1992), even if the sequence similarity is very low.
BMP2 exhibits two different types of surface-epitopes to interact with the respective receptors, the wrist and the knuckle epitope. Whereas the wrist epitope is a large epitope, spanning the interface of the BMP2 dimer binds BRI, the knuckle epitope recognizes BRII and ActRII with low affinity (Kirsch et al., 2000a). Recently, hot spots of receptor interaction have been identified and will contribute to better understanding receptor-ligand interactions as well as for structure based drug design (Keller et al., 2004).

Originally, the BMPs were identified by their ability to induce bone and cartilage formation (Urist, 1965). BMPs induce endochondral bone formation and regulate osteoblast differentiaton (Reddi, 1994). BMP2 exerts its biological activity on many cell types, as monocytes, epithelial, mesenchymal and neuronal cells (Balemans and Van Hul, 2002).

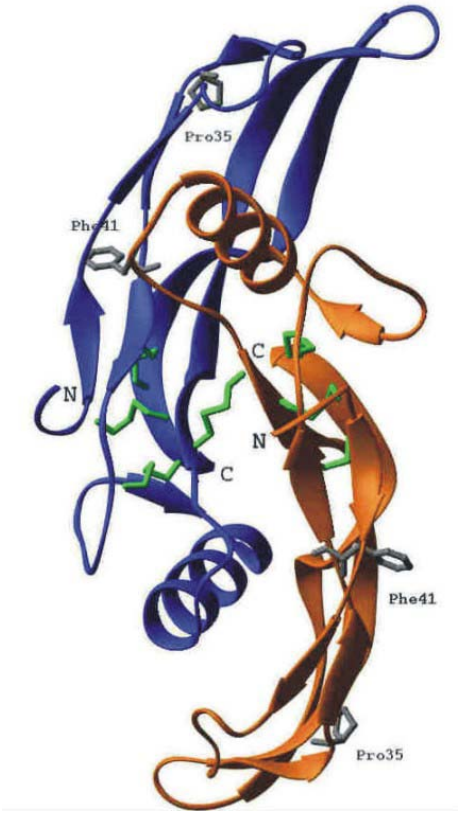


Figure 1.1 Structure of BMP2
BMP2 represents a dimer of two identical monomers, here depicted in blue and orange, respectively. β-sheets and α-helices are indicated. The cystine-bridge connecting the two monomers and the cystine bridges of the cystine knot motif are depicted in green (Scheufler et al., 1999).

1.1.3 Processes that are regulated by BMP2

BMPs play important roles during development and in the adult. BMPs regulate proliferation, differentiation, chemotaxis as well as apoptosis. Besides they influence and control important processes as neurogenesis, gastrulation, mesoderm patterning, haematopoiesis, left-right asymmetry and development of a multitude of organs, as kidney, gut, lung, skin and teeth (Hogan, 1996). In the adult organism, BMPs are responsible for the maintenance and repair of bone (Balemans and Van Hul, 2002).

Disruption of the BMP signal transduction pathway can lead to pathological processes like cancer syndromes or vascular disorders (e.g. Primary Pulmonary Hypertension). These diseases will be discussed in Chapter 1.7 in further detail.

Several BMP knock outs in mice have contributed to the knowledge of BMP action at different stages of development. The severity and the multiplicity of the defects indicates the importance of BMPs.

These experiments point out that BMP activity during embryonic development is tightly regulated and both loss and gain of functions result in severe defects (Botchkarev, 2003).

gene targeted	Phenotype	Reference
BMP2	Embryonic lethality (E9,5) due to defects in amnion and heart development	(Zhang and Bradley, 1996)
BMP4	Early embryonic lethality due to the defect in gastrulation and mesoderm formation	(Winnier et al., 1995)
BMP7	Die shortly after birth from renal failure, also show defects in eye development (microphtalmia) and polydactyly	(Dudley et al., 1995), (Luo et al., 1995)
BRIa	Early embryonic lethality due to the defect in gastrulation and mesoderm formation	(Mishina et al., 1995)
BRIb	Viable, multiple skeletal defects and female infertility	(Yi et al., 2000; Yi et al., 2001)
BRII	BMPR-II in transgenic mice is lethal at an early embryonic stage, homozygous mutant embryos fail to form organized structures and lack mesoderm	(Beppu et al., 2000)
Smad1	Embryonic lethality (E9,5-10,5) due to defects in extra-embryonic tissues (allantois, placenta)	(Lechleider et al., 2001), (Tremblay et al., 2001)
Smad4	Embryonic lethality (E7,5-8,5) due to defects in mesoderm formation, gastrulation, and extra-embryonic tissues	(Sirard et al., 1998), (Yang et al., 1998)
Smad5	Die between E9,5 and E11,5, show numerous defects in developing amnion, gut, heart and nervous system	(Chang et al., 1999), (Yang et al., 1999)
Smad6	Viable, defects in cardiovascular system (formation of cardiac valves, elevated blood pressure)	(Galvin et al., 2000)
Noggin	Die at E18,5 due to abnormalities in nervous system and skeletal development	(Brunet et al., 1998), (McMahon et al., 1998)
Noggin/ Chordin	Embryonic lethality due to defects in forebrain development	(Bachiller et al., 2000)

Tab 1.1 Phenotypes of mice with knock outs of BMPs or related genes
Summary of phenotypes and defects of mice lacking BMPs or proteins in that pathway. According to Botchkarev (Botchkarev, 2003).

1.2 BMP inhibitors

1.2.1 Extracellular inhibitors of BMPs

The local concentration of BMPs controls and regulates important developmental processes in *Drosophila* and *Xenopus* (Dale, 2000), (Vogt and Duboule, 1999), (Day and Lawrence, 2000) (Cornell and Eisen, 2000). Therefore, a fine balance regulating the presence and availability of soluble factors in the extracellular medium is necessary. Local BMP inhibitors support these regulation processes acting as ligand binding traps, sequestering the ligand. Besides that, the action range of BMP is defined through interaction with proteoglycans of the matrix. (Ohkawara et al., 2002). These extracellular inhibitors form three distinct families, the noggin, chordin-sog and the DAN-cerberus family, which are defined by the spacing between cystine residues (Garcia Abreu et al., 2002).

Noggin-family:

Noggin is a dominant inhibitor of BMPs (BMP2/4/7) and involved in a variety of developmental processes during embryogenesis. Besides that, Noggin is expressed in the adult as well. It is essential for cartilage morphogenesis and joint formation (Zimmerman et al., 1996). Groppe et al. reported the crystal structure of the antagonist Noggin bound to BMP7, which showed that Noggin inhibits BMP signalling by blocking the molecular interfaces of the binding epitopes for both type I and type II receptors, showing that Noggin functions by sequestering its ligand in an inactive complex. The scaffold of the 222 amino acid protein Noggin contains a cystine knot topology similar to that of BMPs. Thus, they concluded that ligand and antagonist could have evolved from a common ancestral gene (Groppe et al., 2002).

Sog/Chordin family

Chordin is a large BMP antagonist (BMP2/4/7) of 120kDa, which is highly expressed in the speman organizer (Sasai et al., 1994). It regulates the dorsal-ventral axis formation during gastrulation, but it is expressed in developmental and adult stages (Millet et al., 2001). Being a key developmental protein, Chordin dorsalizes early vertebrate embryonic tissues by binding to ventralizing bone morphogenetic proteins and sequestering them in latent complexes (Piccolo et al., 1996), (Millet et al., 2001). Another level of regulation consists in processing of vertebrate Chordin or its *Drosophila* counterpart, Sog, by astacine metalloproteases like Xolloid-BMP-1/Tolloid, respectively, which subsequently releases an active BMP (Millet et al., 2001). It has been shown in *Drosophila*, that that cleavage could generate novel BMP inhibitory activity or Sog could even promote BMP action (Yu et al., 2000). Tsg is a BMP-binding protein that forms ternary complexes with BMP and Chordin. It does not block BMP signalling, instead it is antagonizing the residual anti-BMP activity of

Chordin, leading to a BMP peak signal (Oelgeschlager et al., 2000; Oelgeschlager et al., 2003).

CRs (cystine-rich domains), originally identified in Chordin, define a novel protein module for the binding and regulation of BMPs. This sequence of 70 amino acids, which is abundantly expressed in the ECM is directly binding to BMPs and inhibiting their action. These modules may function in a variety of extracellular inhibitors to block BMP action (Larrain et al., 2000).

Connective-tissue growth factor (CTGF) is binding to BMP4 and TGF-β1 (Abreu et al., 2002). When injected into *Xenopus* embryos it can bind to BMP4 via its CR domain and antagonize the BMP4 actions via an mechanism similar to Chordin (Abreu et al., 2002). Binding and inhibition were shown in cellular and in vitro assays as well. On the contrary, CTGF can bind to TGF-β1, but functions as a chaperone to facilitate the TGF-β -receptor interaction.

Chordin-like1 (CHL1) (Nakayama et al., 2001), is also called neuralin-1 (mouse) (Coffinier et al., 2001) and ventroptin (chick retina), antagonizes BMP2 and BMP4 (Sakuta et al., 2001) as well as weakly TGF-β2 (Nakayama et al., 2001). It is a 333 amino acid protein containing three CRs and interacts directly with BMPs 2/4/5/6/7 and GDF5 in a competitive manner to prevent binding to the type I BMP receptor ectodomain. As Chordin, it is able to induce secondary axes after mRNA injection in *Xenopus* embryos (Coffinier et al., 2001).

CHL2, a novel chordin-like protein, is structurally most homologous to CHL1. It is expressed preferentially in chondrocytes of developing cartilage and osteoarthritic joint cartilage and interacts directly with BMPs 2/4/5/6/7 and GDF5 in a competitive manner to prevent binding to the type I BMP receptor ectodomain. In the ALP assay in C2C12 cells, it blocks BMP induced ALP production (Nakayama et al., 2004).

Crossveinless-2 (CV2) is a novel inhibitor of BMP function. It inhibits BMP2 and BMP4 dependent osteogenic differentiation and chondrogenic differentiation of ATDC5 cells. Due to 5 CR (Chordin-like cystine-rich) domains it belongs to the group of Chordin and Sog (Binnerts et al., 2004).

Follistatin, a 35kDa protein, is expressed in the Spemann's organizer, in the blastophore during gastrulation. Identified as an inhibitor of Activin, it also binds to and inhibits BMPs (BMP2/4/7/15) (Iemura et al., 1998), (Otsuka et al., 2001). In contrast to Noggin and Chordin, it is not preventing ligand-receptor binding but forming a trimeric complex with BMP and the type I receptor. Thus, the inhibiting mechanism has to be different from the so far described ones. A recently described novel follistatin-like protein can simultaneously bind to and inhibit BMP2 and Activin (Tsuchida et al., 2000).

DAN/cerberus family

This family of proteins includes DAN, gremlin, cerberus and Caronte, all of which can neutralize not only BMP activity but also several other factors.

DAN (differential screening-selected gene aberrant in neuroblastoma), encodes a transcription factor homologous to a mouse tumor suppressor gene (Enomoto et al., 1994).
Cerberus (head-inducing factor) has been identified as BMP (BMP2/4/7), nodal and Wnt inhibitor (Bouwmeester et al., 1996; Piccolo et al., 1999). Cerberus is a secreted glycoprotein that forms dimers when expressed in mammalian cells and binds BMP2 with high affinity.
Another secreted cystine-knot protein that antagonizes BMP is Caronte (Rodriguez Esteban et al., 1999). To control left-right asymmetry in the chick embryo a complex network of antagonistic molecular interactions between Activin, FGF8, Lefty-1, Nodal and BMPs, that cooperate is necessary (Rodriguez Esteban et al., 1999).
Protein related to DAN and cerberus (PRDC) is a novel, secreted protein with a cystine knot structure, that is directly binding to BMP2/4 and inhibiting their actions (Sudo et al., 2004).
Gremlin resembles an antagonist of bone morphogenetic protein (BMP2/4/7) signalling, which is expressed in the neural crest (Hsu et al., 1998). Hsu et al. showed that all family members are secreted proteins and that they act as BMP antagonists in embryonic explants. They also provided support for the model that Gremlin, Cerberus, and DAN block BMP signalling by binding BMPs, preventing them from interacting with their receptors.
Coco, is a maternal BMP4, Activin, TGF-β and Wnt inhibitor, whose role is to ensure proper ectodermal patterning during gastrulation (Bell et al., 2003).
SOST (Sclerostin), an inhibitor of BMP6 and BMP7 (Kusu et al., 2003), (Balemans and Van Hul, 2002), is expressed exclusively by osteocytes in mouse and human bone and inhibits the differentiation and mineralization of murine preosteoblastic cells. It does not inhibit ALP production in C2C12 cells and BMP-induced Smad-phosphorylation (Van Bezooijen et al., 2004). The gene was cloned from families with sclerosteosis (Brunkow et al., 2001).
Ectodin, a newly identified inhibitor, shows 37% homology to SOST and is intensely expressed in developing ectodermal organs, including teeth, vibrissae, and hair follicles. It inhibits the activity of BMP2, BMP4, BMP6, and BMP7 in mouse pre-osteoblastic MC3T3-E1 cells (Laurikkala et al., 2003).

1.2.2 Pseudoreceptors for BMPs

BAMBI (BMP and Activin membrane bound inhibitor) is a pseudoreceptor which extracellularly bears a ligand binding domain but intracellularly lacks a kinase domain. (Onichtchouk et al., 1999). Nma is the mammalian homologue to BAMBI, which was originally identified in *Xenopus* (Tsang et al., 2000). Due to the ability to stably interact with various BMP and TGF-β receptors, thus preventing the formation of functional signalling complexes, it is causing abrogation of BMP- as well as TGF-β- and Activin-mediated signalling.

1.3 Receptors of the TGF-β superfamily

1.3.1 Type I receptors

The members of the TGF-β superfamily bind to serine/threonine kinase receptors (Massague, 1992), (Lin and Lodish, 1993). Only GDNF, not closely related to BMP and TGF-β binds Ret, a receptor tyrosine kinase (Trupp et al., 1996). The receptors of the TGF-β superfamily can be grouped into three families, the type I, type II and type III receptors, according to their size (Wrana et al., 1992; Wrana et al., 1994). Type I receptors exhibit a molecular weight of about 50kDa, show extracellularly a ligand-binding domain, a single transmembrane domain and a cytoplasmatic serine/threonine kinase domain. N-terminal of this kinase they have a glycin/serine (SGSGSG) rich region, the so called GS-box (Wrana et al., 1994). Phosphorylation of that region activates the TβRI kinase to transmit signals (Souchelnytskyi et al., 1996), (Wieser et al., 1995), (Wrana et al., 1994). TβRI activation can be mimicked by the mutation of Thr-204, N-terminal of the GS-box, to aspartic acid, which results in a construct, that signals in the absence of ligand, due to a conformational change (T204D) (Wieser et al., 1995), (Attisano et al., 1996), inactivation is achieved by exchanging the critical lysine of the kinase to arginine (K232R) (Carcamo et al., 1994), (Chen and Weinberg, 1995). An 8 amino acid region between the β-sheets 4 and 5 of the kinase domain, the exposed L45-loop, has shown to be the recognition site for Smads, the signal transducer molecules (Feng and Derynck, 1997).

1.3.2 Type II receptors

The type II receptors, who are important for ligand recognition and complex formation as well as signal transmission exibit a similar structure to the type I receptors. They do not posses a GS-box and are more glycoslyated, thus showing a molecular weight of about 75kDa. Exceptions are the BRII-LF and wishful thinking (wit), a *Drosophila* type II receptor, both having a long C-terminal extension (Aberle et al., 2002). Further details will be discussed later. Type II receptor kinases are thought to be constitutively active and phosphorylating itself, although the regulation of this process remains unclear. After ligand binding and subsequent activation they phosphorylate multiple residues in the GS-box of the type I receptor (Wrana et al., 1994). This process has to be tightly regulated and many proteins serve as check points. Moreover the type II receptors could regulate type I receptor independent pathways via associated proteins.

1.3.3 Accessory receptors

The type III receptor, also called betaglycan, is a specifically TGF-β and Inhibin binding protein (Lewis et al., 2000). Betaglycan presents TGF-β/Inhibin directly to the signalling receptor, forming a high affinity ternary complex with TβRI and TβRII (Lopez-Casillas et al., 1991; Lopez-Casillas et al., 1994; Lopez-Casillas et al., 1993). This property supports binding of TGF-β2 to TβRII. As demonstrated, TβRII shows only low intrinsic affinity for TGF-β2 ligand. Instead, TβRIIB was identified being the high affinity receptor for TGF-β2 (Rotzer et al., 2001). The membrane anchored proteoglycan exhibits extracellularly a N-terminal TGF-β binding domain and a short cytoplasmic tail with no discernible signalling entity. Chen and co-workers have shown that betaglycan interacts intracellularly with β-arrestin2, which is involved in internalization of receptors. Binding of β-arrestin2 to betaglycan is triggered by phosphorylation of the receptor on its cytoplasmic domain, mediated by the TβRII kinase (Chen et al., 2003). The ectodomain of betaglycan can be released from cells as a soluble proteoglycan. It exhibits antagonistic effects through binding and sequestrating the ligand. Thus, betaglycan can act as a positive or negative regulator of TGF-β signalling. (Lopez-Casillas et al., 1994).

Endoglin (CD105) is a transmembrane glycoprotein, highly expressed on endothelial cells, exhibiting intracellularly structural similarities with betaglycan (Cheifetz et al., 1992). It interacts with TGF-β1 and 3, Activin A as well as BMP2 and BMP7 but requires coexpression of the respective ligand binding kinase receptor (Barbara et al., 1999). In contrast to betaglycan it does not facilitate ligand–receptor interaction, moreover it might serve to recruit other proteins to the signal transducing complex.

Although in the human genome 42 open reading frames encode for cytokines of the TGF-β superfamily, only 12 corresponding receptors (7 type I and 5 type II) can be found (Shi and Massague, 2003).

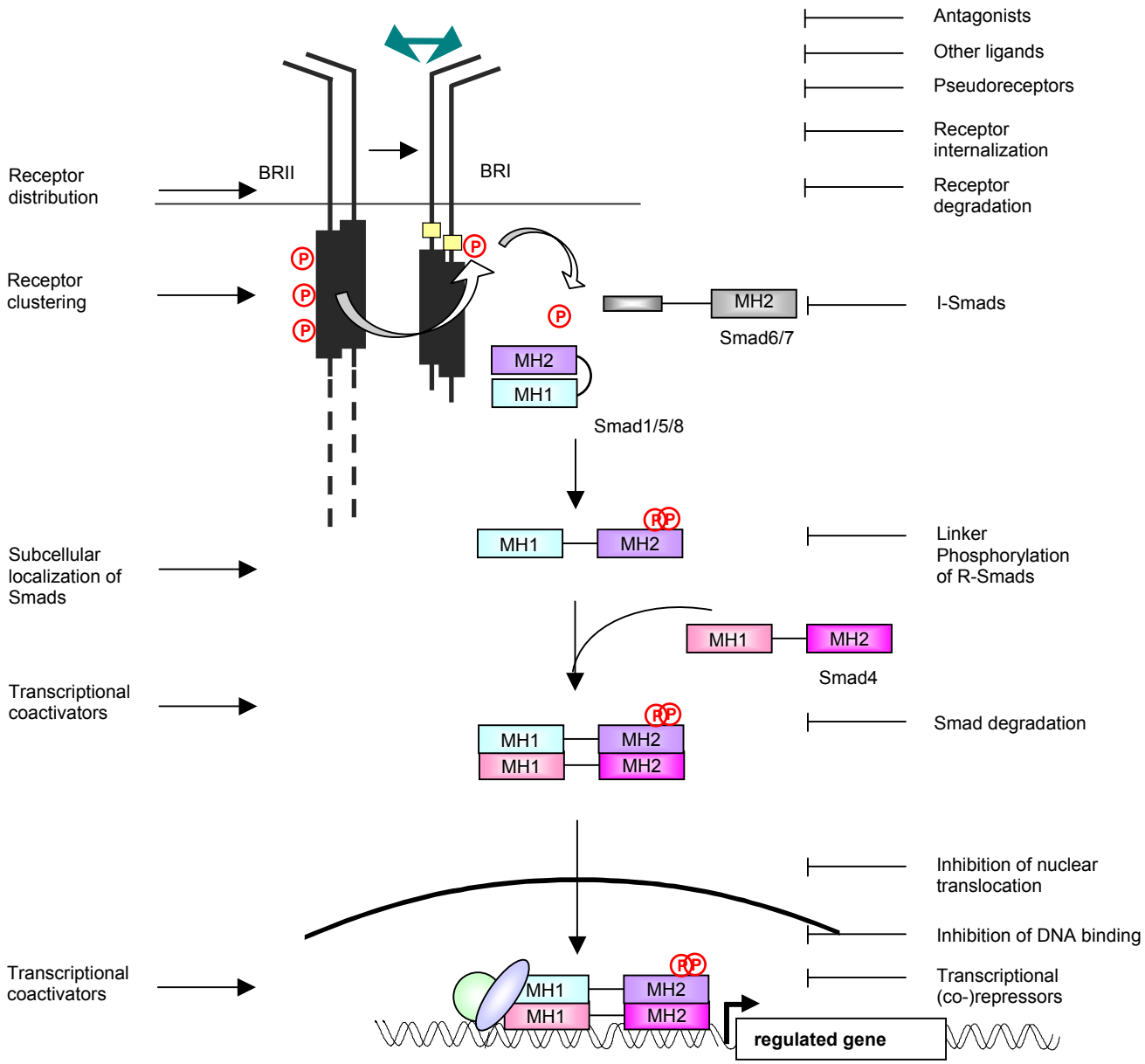


Figure 1.2 BMP2 mediated signal transduction via the Smad-pathway and levels of inhibition
Here the BMP-induced Smad-pathway and the different levels of modulation are indicated. Specific mechanisms of regulation at nearly all levels have been observed. Activating mechanisms are depicted on the left hand and inhibiting or downregulating molecules on the right. Modified and adapted according to ten Dijke et al. and Lutz et al. (ten Dijke et al., 2000), (Lutz and Knaus, 2002).

1.3.4 The BMP receptors

For more than 30 BMPs, only 3 type I and 3 type II receptors are known. For the activation of BMP signalling, binding of the ligand to at least one type I and one type II receptor is necessary (Yamashita et al., 1994), (ten Dijke et al., 1996). BMP binds with high affinity to the respective type I receptor. So far ALK3/BRIa, ALK6/BRIb and, with lower affinity, ALK2/ActRI are described (Ebisawa et al., 1999), (Macias-Silva et al., 1998), (ten Dijke et al., 1994a), (ten Dijke et al., 1994b). Referring to the type II receptors: there exist three type II

receptors which bind to BMPs, the BRII, ActRII (Donaldson et al., 1992) and ActRIIB (Mathews et al., 1992); (Yamashita et al., 1995). The two different Activin type II receptors (ActRII and ActRIIB) are products of alternative splicing events as well. Here, an alternatively spliced segment in the cytoplasmic juxtamembrane region is included (Attisano et al., 1992).
For the BRII two alternative splice variants have been described (Rosenzweig et al., 1995), (Kawabata et al., 1995), (Liu et al., 1995), (Nohno et al., 1995). The genomic organization of *hBMPR2* consists of 12 exons, whereas exon12 represents the BRII-tail, the long C-terminal extension, which is the subject of the splicing mechanism. The majority of cells tested for the presence of the short (BRII-SF) or the long form with the tail (BRII-LF) expresses the long BMP receptor variant (Foletta et al., 2003). Still, the impact of this extension is not fully understood. During this study the function of the BRII-tail, the association of different proteins and its impact on signal transduction should be examined in more detail.
As demonstrated, the BMP-mediated signal transduction differs, depending on the type I receptors involved (Chen et al., 1998), (Zou et al., 1997). BRIa is inducing adipogenesis in the mesenchymal precursor cell line 2T3, whereas BRIb is pushing the cells towards osteogenesis (Chen et al., 1998). On the other hand, stimulation of different receptors in the same cell type can evoke identical outcome, e.g. production of ALP (Fujii et al., 1999). Besides, some BMPs do prefer binding of a specific receptor subset and they exhibit different affinities to various receptors (Aoki et al., 2001). Another level of regulation of BMP signal transduction is, that cells do express different receptor combinations, due to developmental stages, with the consequences that only some factors can induce signalling (Nakamura et al., 2003).

1.3.5 Receptor binding and receptor oligomerization

Two distinct modes of the ligand-receptor interaction exist. One is represented by members of the BMP subfamily and the others by TGF-βs and Activins (Sebald and Mueller, 2003). BMP ligands (BMP2/4; GDF) bind type I receptors with high affinity and the respective type II receptors with a much lower affinity. Their binding affinity for the complex formed by type I and type II is stronger than the binding to one of the receptor chains, the so called cooperative binding mode (Sebald and Mueller, 2003). TGF-βs and Activins exhibit a high affinity for the type II receptors and a low affinity for type I receptors. They do not interact with the isolated type I receptors (Massague, 1998). Here, the ligand binds first strongly to the type II receptor and this binding allows the subsequent recruitment of the type I receptor, thus forming a large signalling complex (sequential binding mode).
Receptor endocytosis is an important regulatory event in signal transduction. TGF-β receptors signal to the Smad pathway through heteromeric Ser-Thr kinase receptors that are rapidly internalized and then downregulated in an ubiquitin-dependent manner.

Internalization occurs either via caveolae or via early endosomes. Moreover, the TGF-β receptors can be found in lipid raft and non-raft membrane domains. Clathrin-dependent internalization into the early endosome (that is EEA1-positive), where SARA is found, promotes TGF-β signalling. In contrast, the pathway, where internalization occurs via lipid rafts and caveolae, contains TGF-β receptors bound to Smad7-Smurf2 and is required for rapid receptor turnover. Thus, segregation of TGF-β receptors into distinct endocytic compartments regulates Smad activation and receptor turnover (Di Guglielmo et al., 2003). Still the question remains whether similar processes are involved in BMP receptor mediated signalling and whether receptor oligomerization is influencing receptor localization. These important questions have to be addressed in the future.

BMP receptor oligomerization

Homomeric receptor interactions are involved in receptor regulation and signalling outcome. As demonstrated, the TGF-β receptors form ligand-independent homodimers on the cell surface (Gilboa et al., 1998), (Henis et al., 1994). In contrast to the TGF-β receptors, the oligomerization pattern of the BMP receptors exhibits a higher flexibility. Prior to ligand binding, about 30 percent of the BMP receptors are found as preformed homo- and heterooligomers on the cell surface. Moreover, the percentage of homo- as well as hetero-oligomers increases upon ligand addition, except for BRII homodimers, who exhibit only low ligand-affinity (Gilboa et al., 2000), (Nohe et al., 2002). This more flexible pattern results in a greater variability of receptor combinations and signal transduction. Besides there is evidence that the oligomerization mode is determining signalling and cellular outcome (Nohe et al., 2002).

1.4 Signal transduction pathways

After binding to the receptors BMP2 exerts at least two different pathways. On the one hand, pathway restricted Smad proteins (R-Smads) are recruited to and phosphorylated by the receptors (Smad-pathway), and on the other hand p38-MAPK gets activated upon receptor stimulation (MAPK pathway), moreover Ras and ERK pathways.

1.4.1 Smad-dependent pathways

After ligand-receptor binding and subsequent receptor activation the activated type I receptor is phosphorylating intracellular signalling mediators, the Smads; in case of BMP signalling the so called “BMP-Smads”, Smad1/5/8 get activated. The TGF-β type I receptor Alk5 is phosphorylating Smad2/3. Upon phosphorylation, the Smads interact with the common-mediator (Co-Smad) Smad4, form a complex, translocate to the nucleus and regulate transcription.

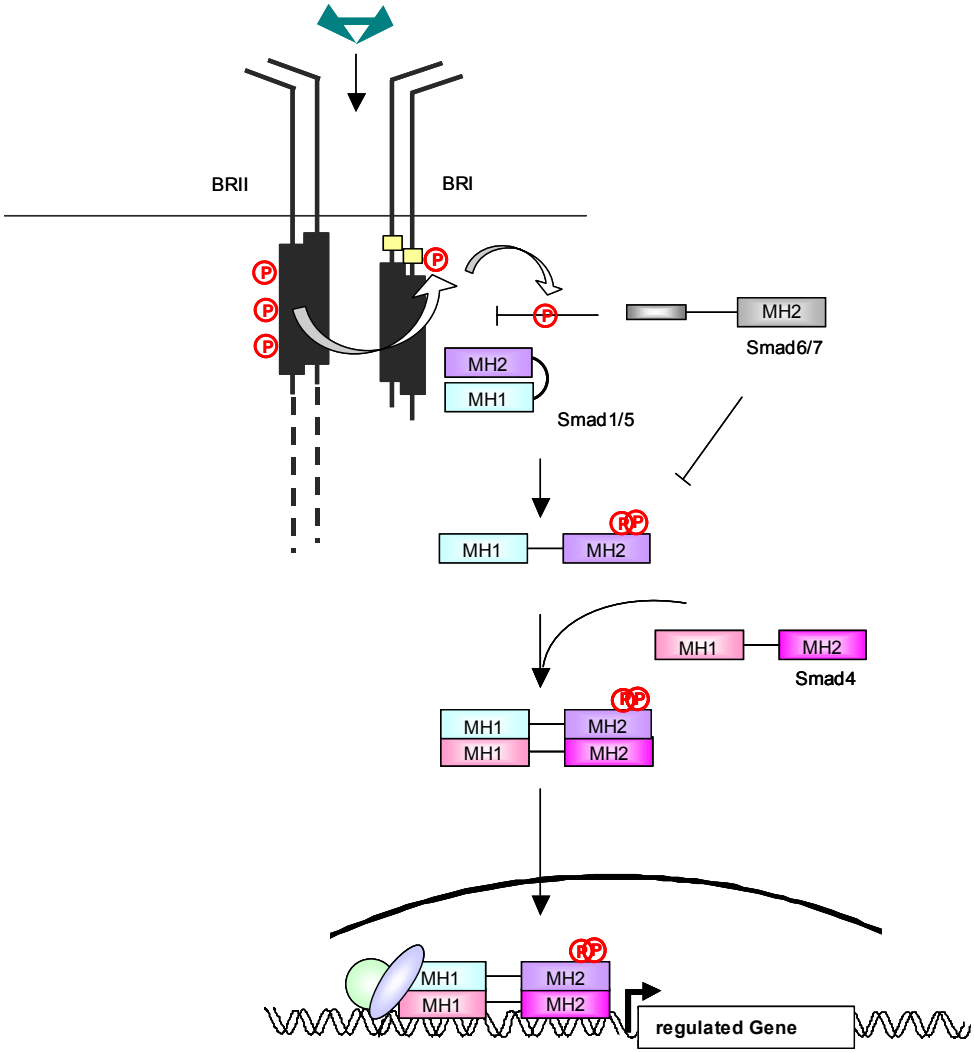


Figure 1.3 BMP2-induced Smad-pathway
BMP2 signals via a complex of type I and type II receptors. Induction of the Smad-pathway occurs via ligand binding to preformed hetero-oligomeric complexes (Nohe et al., 2002).

1.4.1.1 The Smads

Functionally, Smads can be divided into three subclasses: the receptor associated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5 and Smad8) which become phosphorylated by the type I receptors, the common-mediator Smad4 (Co-Smad), which oligomerizes with activated R-Smads and the inhibitory Smads (I-Smads, Smad6 and Smad7), which get activated upon ligand stimulation. Smad6 and Smad7 exert a negative feedback effect by marking the receptors for degradation and by competing with R-Smads for receptor interaction.

Smad proteins have conserved N- and C-terminal regions, named the MH1 and MH2 (MAD homology) domains, respectively. Both domains are bridged by a proline-rich linker region. The MH2 domains are well conserved among Smad proteins, whereas the MH1 domains are conserved in R-Smads and Co-Smads, and the N-terminal regions of I-Smads are highly divergent. The MH1 domain of R- and Co-Smads is binding to specific DNA sequences and

regulates nuclear import, whereas the MH2 domains are responsible for homo- and heteromeric complex formation. Upon receptor activation, R-Smads become phosphorylated at their C-terminal SSXS motif, form oligomers with Smad4 and translocate into the nucleus (Zhang et al., 1996). Here, they bind to DNA alone or in combination with transcription factors and other regulatory molecules. Although the structure of the Smad heterocomplex is not determined yet, a heterotrimer of two R-Smads and one Co-Smad or a heterodimer composed of one R-Smad and one Co-Smad molecule are proposed (Qin et al., 2001), (Wu et al., 2001), (Inman and Hill, 2002).

1.4.1.2 The BMP R-Smads: Smad 1/5/8

As R-Smads for the BMP receptors Smad1, Smad5 and Smad8 (only in *Xenopus*) have been identified (Liu et al., 1996), (Yamamoto et al., 1997), (Nakayama et al., 1998). Whereas BRIa/Alk3 and BRIb/Alk6 activate all three R-Smads, Alk2 does not activate Smad8 (Heldin et al., 1997), (Aoki et al., 2001). Inactive, cytoplasmic Smads are auto-inhibited through an intramolecular interaction between MH1 and MH2 domains (Hata et al., 1997). All R-Smads reside in the cytoplasm, but the MH1 domains of all R-Smads contain a lysine-rich potential nuclear localization sequence. Moreover,,Smad1 has, along with other Smads, constitutive nuclear export activities due to an NES (nuclear export sequence) in the MH2 domain (Xiao et al., 2003a; Xiao et al., 2001). There is evidence, that BMP dependent pathways are regulated via ubiquitin-dependent mechanisms. The HECT ubiquitin E3 ligase Smurf1 is interacting with Smad1 and Smad5 and thus mediating degradation of these proteins (Murakami et al., 2003). Besides, the osteoblast transcription factor Cbfa1 is interacting with and degraded by Smurf1 (Zhao et al., 2004), presenting an efficient mechanism to inhibit BMP mediated signalling. Another way of Smad1 degradation and inhibition of the transcriptional activities of the Smad1/Smad4 complex is mediated by CHIP. CHIP is an U-box-dependent E3 ubiquitin ligase, previously identified as a co-chaperone protein, that mediates Smad1 degradation in an ubiquitin-dependent manner (Li et al., 2004). Besides degradation there are other posttranslational mechanisms to regulate Smad1 activity. Smad1 phosphorylation via ERK, that is induced by HGF and EGF, inhibits Smad1 nuclear accumulation and thus blocking transcriptional activation (Kretzschmar et al., 1997), (Pera et al., 2003). Smad2 activity is negatively regulated by Calmodulin (Xu et al., 1999). In contrast, Smad1 activity seems to be enhanced via that mechanism. Moreover, Calmodulin binding to Smad1 inhibits RTK driven Erk-dependent phosphorylation of Smad1 and vice versa, generating a mechanism to regulate Smad1 activity (Scherer and Graff, 2000). The nuclear envelope protein XMAN1 acts as a Smad-binding protein to antagonize BMP signalling during *Xenopus* embryogenesis (Osada et al., 2003). SANE (Smad1 Antagonistic Effector) shows significant sequence similarity to nuclear envelope proteins, such as MAN1 and

inhibits Smad1 phosphorylation and thus blocking the nuclear localization of Smad1 (Raju et al., 2003).

1.4.1.3 Smad4

Mammalian Smad4 displays constant nucleocytoplasmic shuttling and is capable of autonomous nuclear import and export (Xiao et al., 2003b). Phosphorylated R-Smads quickly form complexes with Smad4, possibly prior to nuclear localization (Lagna et al., 1996). Smad4 contains an unique loop in its MH2 domain to prevent oligomerization in the absence of signalling (Tada et al., 1999). R-Smads can enter the nucleus without Smad4. Smad4, indeed needs R-Smads for nuclear import (Hoodless et al., 1999). In the nucleus, Smad4 plays a crucial role in regulating the efficiency of transactivation. It supports the activation of transcription by inducing large-scale chromatin unfolding (Yan et al., 2004). C-Ski is a transcriptional corepressor that interacts strongly with Smad2, Smad3, and Smad4 but only weakly with Smad1 and Smad5. Through binding to Smad proteins, c-Ski suppresses signalling of transforming growth factor-beta (TGF-β) as well as bone morphogenetic proteins (BMPs). (Takeda et al., 2004). Recent studies have shown that Smad4 is SUMOylated at two specific lysines in the MH1 domain (Lin et al., 2003a). Attachement of SUMO (small ubiquitin-related modifier)-1 and Ubc9, the ubiquitin carrier protein (E2) conjugating enzyme, is regulating its metabolic stability and subcellular localization, but influencing the intrinsic transcriptional activity of Smad4, thus having either positive or negative effects on Smad4 mediated transcription, depending on the target gene (Liang et al., 2004; Long et al., 2004).

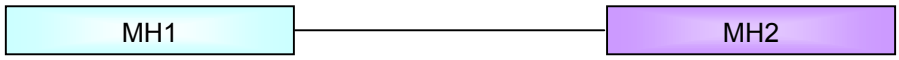
1.4.1.4 Smad6/7

The I-Smads downregulate ligand-mediating signalling by interacting with activated receptor complexes, thereby preventing the access of R-Smads. Both I-Smads only exhibit a MH2 domain and a linker region, but lack the MH1 domain. Smad6 is downregulating BMP dependent pathways (Hata et al., 1998), whereas Smad7 blocks both, BMP and TGF-β mediated signalling (Casellas and Brivanlou, 1998). In the absence of ligand, the I-Smads are located in the nucleus, upon ligand stimulation they are exported in the cytoplasm, followed by receptor interaction. As demonstrated, Smad6 can compete with Smad4 in complex formation with Smad1 (Itoh et al., 2001). Smad6 receptor interaction is enhanced by binding of Tob. Tob and Tob2 cooperate with Smad6 to inhibit BMP signalling (Yoshida et al., 2003). Besides its cytoplasmic functions, Smad6 exerts nuclear funtions as well. Lin et al. have demonstrated that Smad6 represses BMP-induced Id1 transcription through recruiting CtBP. (Lin et al., 2003b). Moreover, Smad7 interacts with HECT ubiquitin ligases via its Prolin-Tyr motiv, that binds the HECT domain and WW domains of Smurf1 and Smurf2, inducing degradation of Smads and associated receptor complexes.

1.4.2 Smads and other transcription factors in the nucleus

Upon phosphorylation of R-Smads at the last two serines at the C-terminal SSXS motif, Smads oligomerize with Smad4, the heteromeric complexes are translocated to the nucleus where they regulate, in co-operation with other transcription factors, coactivators and corepressors, the transcription of target genes. Although Smad4 and the BMP R-Smads possess DNA binding ability, the affinity can be enhanced by these factors.

Moustakas et al. present a table of Smad-interactors and their regulation in TGF-β signal transduction (Moustakas et al., 2001).



Function	• nuclear import • cytoplasmic anchoring • DNA binding • interaction with transcription factors • autoinhibition	• ubiquitination	• receptor association • oligomerization • cytoplasmic anchoring • interaction with transcription factors • nuclear export
Regulatory phosphorylation		ERK (S1-3)	type I receptors (S1-2, S5, S8)
Receptors			Alk 1, 2, 3, 6
Oligomerization			R-Smads, Co-Smad
Cytoplasmic adaptors, affectors	Calmodulin (S1-4) Filamin (S1-6)	Filamin (S1-6) TAK1 (S6)	STRAP (S2, 3, S6, S7)
Ubiquitination adaptors, substrates		Smurf1 (S1, S5, S7) Smurf2 (S2, S3, S7)	
Transcriptional coactivators	pX HBV (S4)	Swift (S1, S2) SMIF (S4) ZEB-1/ deltaEF1	MSG1 (S4) p300/CBP (S1-4) p/CAF (S1-4)
Transcriptional (co-) repressors	Hoxc-8 (S1, S6) SNIP1 (S1, S2, S4)	Hoxc-8 (S1, S6) mZnf8 (S1) CtBP (S1-S6) CIZ (S1, 5) Nkx3.2 (S1, S4) XMan (S1)	ZEB-2/SIP1 (S1-3, S5) Ski (S2-4) SnoN (S2-4) Tob (S1, S4, S5, S8) SANE (S1)
Transcription factors	YY1 (S1, S3, S4)	Gli3 ΔC-ter (S1-4)	OAZ (S1, S4) EHZF (S1, S4) Runx/Cbaf1/AML (S1-4)

Tab 1.2 BMP Smad interacting proteins
Here, only proteins involved in BMP mediated signal transduction are shown. This is an (incomplete) list of Smad interacting proteins, shown along with the interaction site in the Smad protein and with the function of the interacting proteins. The specific Smad members that are known to fulfill the indicated function are shown in parenthesis and are abbreviated with S1-S8 for Smad1 to Smad8, respectively. Proteins, for which the interaction site is not yet determined are highlighted with yellow colour.

1.4.2.1 Transcription factors

Only few transcription factors are found to directly interact with BMP-Smads or to synergize with them.

OAZ (Olf-1/EBF-associated zinc finger), a 30-zinc finger DNA-binding factor, has been identified to associate with Smads in response to BMP2 (Hata et al., 2000). Early haematopoietic zinc finger (EHZF), with high homology to mouse Evi3 and OAZ, complexes Smad1 and Smad4, binds to and enhances the transcriptional activity of a BMP2/4 responsive element (Bond et al., 2004).

Hoxc-8, a homeodomain transcription factor interacts with Smad1. This binding dislodges Hoxc-8 from DNA and can induce transcription of specific genes (Shi et al., 1999). Smad6 can bind to Hoxc-8 or Hoxc-9, form a heterodimer and subsequently block Smad1/Hox mediated gene induction, acting as a transcriptional corepressor (Bai et al., 2000).

Some BMP-responsive genes contain AP-1 binding sites, which are activated by the homomeric c-Jun/c-Fos complex. Smads, together with MAPK are binding to promoters with these AP-1 or CREB/ATF binding sites (Akhurst and Derynck, 2001). Moreover, Smad4 is binding to bZIP transcripton factors like c-Jun, ATF-2 (together with Smad1) and CREB (cAMP response-element binding protein) (Monzen et al., 2001).

Smad1 and Cbfa1/Runx2, an indirect transcriptional target of BMP2 itself, were found to act cooperatively at the chicken type X collagen promoter (Drissi et al., 2003).

YY1 represses the induction of immediate-early genes to TGF-β and BMP, as PAI-1 and Id1, by binding to the MH1 domains of Smad4 and weaker to Smad1 and Smad2 and thus inhibiting DNA binding (Kurisaki et al., 2003).

1.4.2.2 Coactivators and Corepressors

Coactivators:

Transcriptional coactivators, such as p300, CBP (CREB-binding protein) and p/CAF contain Histone acetyl transfease (HAT) activity domains. Through acetylation of histones these coactivators help Smads to activate the transcription of target genes.

It has been shown that BMP-Smads bind to the master transcriptional co-activator p300/CBP in the nucleus and that this binding is essential for transcriptional activation and the function of Smads (Pearson et al., 1999). Moreover, pCAF (p300/CBP-associated factor) is recruited via the MH2 domain of Smad in a ligand dependent manner (Akhurst and Derynck, 2001).

P300 is bridging the nuclear protein MSG1 and Smad1. MSG1 nuclear protein has a strong transcriptional activating activity but does not bind directly to DNA. It enhances transcription mediated by Smads in a ligand-dependent manner (Yahata et al., 2000).

SMIF is a Smad4-interacting transcriptional co-activator, that forms complexes with Smad4 upon TGF-β or BMP4 stimulation, translocates into the nucleus and induces transcription, depending on p300/CBP and in a ligand-dependent manner (Bai et al., 2002).
ZEB-1/deltaEF1, a member of the family of zinc finger factors, is positively regulating BMP mediated transcription via interaction with p300/CBP (Postigo, 2003; Postigo et al., 2003).

Corepressors:

The other member of the zinc factor family, ZEB-2/SIP1, represses Smad-mediated transcriptional activation by recruiting the transcriptional repressor CtBP (Postigo, 2003; Postigo et al., 2003), (van Grunsven et al., 2003). SIP1, identical with ZEB-2 is belonging to the two-handed zinc finger proteins and was identified binding to E2-box sequences present in the promoter region of liver/bone/kidney ALP. Binding to the promoter represses transcription. Potential interaction with Smad1 could dislodge SIP1 from DNA. SIP1 shows high homology to δ-crystallin enhancer binding factor (deltaEF1) (Tylzanowski et al., 2001), (van Grunsven et al., 2001).
CtBP belongs to a family of proteins, that recognize PXDLS motifs in DNA-binding proteins and function as transcriptional co-repressors in *Drosophila*, *Xenopus* and mammals. The active protein is a dimer, that can contact Histone deacetylases (Lin et al., 2003b), (Subramanian and Chinnadurai, 2003).
SNIP1 is a recently discovered novel repressor of p300/CBP. It interacts via its N-terminus with Smad4 and p300/CBP. The C-terminus links Smad1 or Smad2 to SNIP1. SNIP1 degradation is mediated via Smad1, that forms a complex with the 26S proteasome beta subunit HsN3 and the ornithine decarboxylase antizyme. After BMP stimulation, the complex is translocated into the nucleus and SNIP1 is associated and subsequently degraded (Lin et al., 2002) (Kim et al., 2000).
Tob and Tob2 have been identified to suppress BMP-mediated signalling e.g. by increasing the number of osteoblasts and subsequent the bone mass in Tob deficient mice. Tob interacts with Smad1/5/8 and inhibits transcription, its interaction with Smad6 and Smad7 enhances Smad6-receptor binding, thus facilitating inhibitory Smad functions (Yoshida et al., 2003), (Yoshida et al., 2000).
The transcriptional corepressor c-Ski suppresses signalling of TGF-β as well as BMP through binding to Smad proteins. Whereas the binding of Smad2 or Smad3 is sufficient for TGF-β inhibition, the binding to Smad4 is required for BMP inhibition. This is due to the fact of weak interaction between Smad1/5 and c-Ski (Takeda et al., 2004) (Luo, 2003).
Mouse Kruppel-type zinc finger protein, mZnf8, and Smad1 interact in the nucleus, suppressing BMP induced gene expression (Jiao et al., 2002). CIZ (Cas-interacting zinc

finger protein) and Nkx3.2 are novel inhibitors of BMP mediated signalling (Shen et al., 2002). Nkx3.2 forms a complex with histone deacetylase 1 and Smad1 and Smad4 in a BMP-dependent manner to act as a transcriptional repressor (Kim et al., 2003). The mechanism of action of CIZ is not discovered yet.

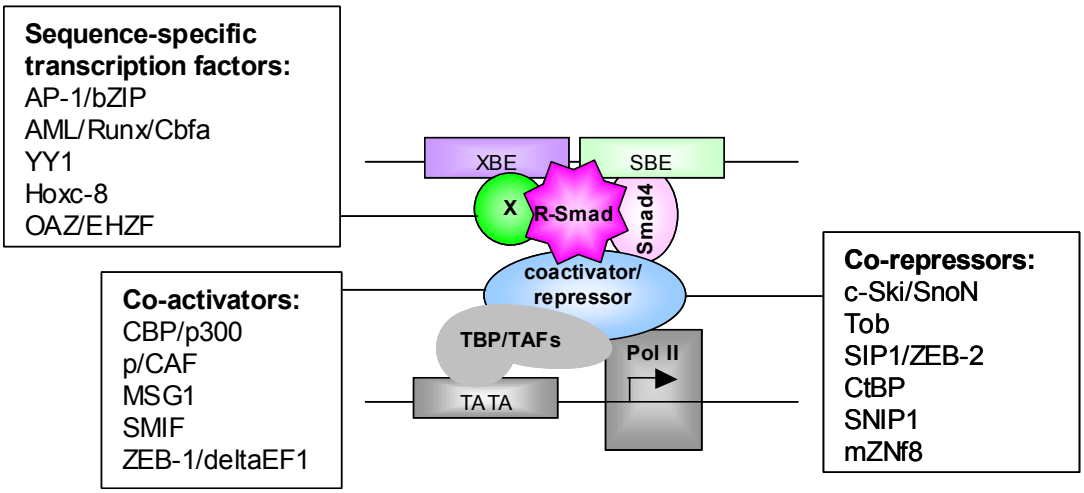


Figure 1.4 Schematic representation of Smad and transcription factor complexes on DNA
Potential architecture of Smad-DNA binding complexes. The BMP R-Smad is bound to DNA in complex with Smad4 and a sequence-specific transcription factor that is either enhancing the affinity for DNA or mediating specificity.Smad1/5 specific co-factors might be involved as well, that mediate interaction with the transcription machinery.

BMP target genes

Nuclear cofactors have been identified that cooperate with the Smads in regulating specific target genes depending on the cellular context. Some of the identified BMP target genes are described in the following.

Among BMP and TGF-β target genes Id1, Id2 and Id3 can be found. Id proteins (inhibitors of differentiation) were first shown to act as dominant negative antagonists of the basic helix-loop-helix family of transcription factors, which positively regulate differentiation in many cell lineages. Moreover, Id proteins interact with transcription factors and the Rb family of tumor suppressor proteins (Hollnagel et al., 1999). Other BMP targets are the transcription factors Msx1 and Msx2, *Xenopus* Vent-2 and JunB, which in turn induces Cbfa1, an indirect BMP target (Hollnagel et al., 1999). BMP2 stimulates the upregulation of the osteogenic master transcription factors: Cbfa1/Runx2, Dlx5, and Osterix (Osx). The upregulation of Osx requires the synthesis of new protein, which is mediated by Dlx5 (Lee et al., 2003a).

During cellular differentiation processes, induced by BMP ECM proteins as osteocalcin, collagen, bone sialoprotein and decorin are upregulated. Along with that, indirectly upregulated genes are osteopontin, ALP and osteocalcin and the cartilage markers collagen X and fibronectin (Lee et al., 2000). Downregulated by BMP are the muscle differentiation genes MyoD and myogenin (ten Dijke et al., 2003).

During chondrocytic differentiation induced by BMP2 first type II collagen is expressed, the second step is upregulation of Indian hedgehog, parathyroid hormone (PTH)/parathyroid hormone-related peptide receptor (PTHrP), type X collagen, and ALP and last osteocalcin (Valcourt et al., 2002).

1.4.3 Smad-independent pathways

Upon BMP stimulation several cellular phosphorylation events can be observed. The best characterized phosphorylation targets are the Smad-proteins, but besides the onset of various other pathways can be observed. It has been shown that BMP induces p38-phosphorylation, ERK1/2 phosphorylation and PI3K activation, leading to Akt phosphorylation. Upon PKD activation JNK and p38 can be phosphorylated and BMP-induced apoptosis is mediated by PKC phosphorylation events.

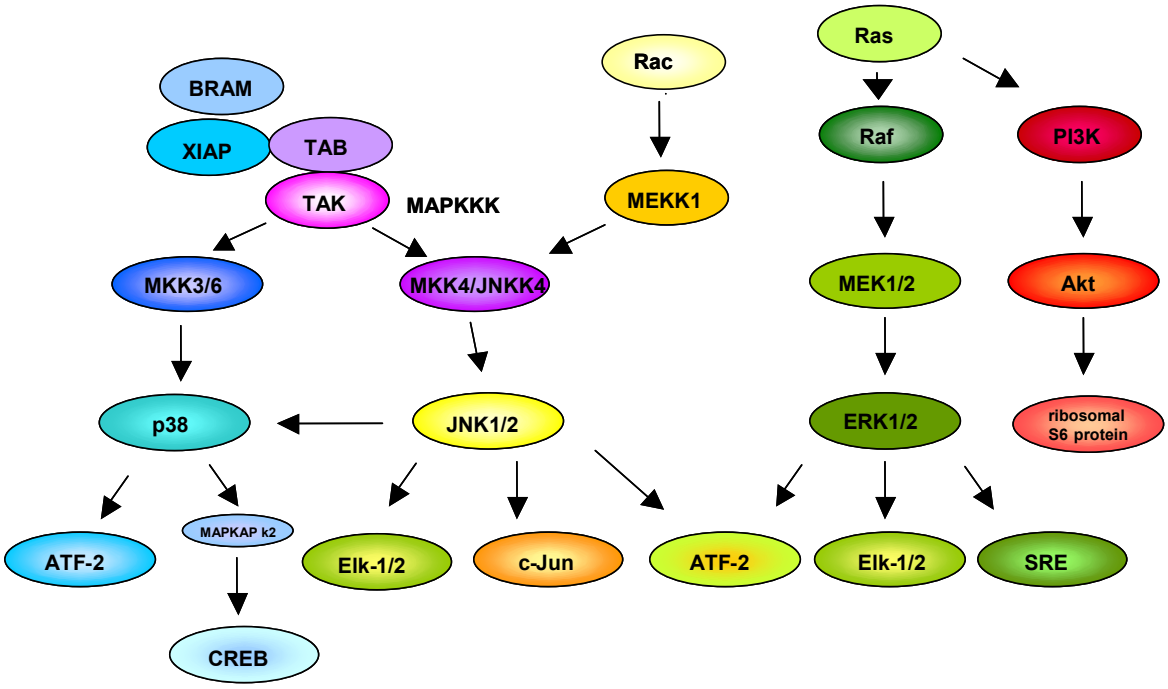


Figure 1.5 Network of non-Smad pathways induced by BMP
BMP stimulation induces a variety of different pathways, as depicted above. The activation of PKC and PKD is not shown. Via BRAM and XIAP the p38 or JNK1/2 pathway could be activated. For some other pathways the link to the receptor is still unknown.

The p38-pathway

One of the major target proteins upon BMP2 stimulation is p38 (Iwasaki et al., 1999). Interestingly several ways seem to induce phosphorylation of this MAPK.

One of the few BRI interacting proteins is XIAP (X-chromosome linked inhibitor of apoptosis), whose interaction is mediated by BRAM. Via XIAP TAB (TAK binding protein) and TAK1 (TGF-β activated kinase) are linked to the receptor. Whereas TAB is stimulating TAK1,

TAK1, furthermore, activates stress-activated protein kinases (SAPKs), p38 through MKK3 or MKK6 and c-Jun N-terminal kinases (JNKs) via MKK4 (Shirakabe et al., 1997). Upon BMP2 stimulation, p38-phosphorylation can be observed (Gallea et al., 2001), which in turn can activate the transcription factors ATF-2 and CREB (Sano et al., 1999), (Nakamura et al., 1999). This pathway can be downregulated by the I-Smads, Smad6 and Smad7 (Yanagisawa et al., 2001). Smad6 can interact with TAK1, block TAK1 activity and thus inhibit BMP2 induced p38 phosphorylation. Kimura et al. speculate that TAK1/p38 may be the mediator of BMP2/4 induced apoptosis (Kimura et al., 2000). Experiments performed in TGF-β responsive prostate cancer cells revealed a connection between Smad7, p38, TAK1 and MKK3. Smad7 was shown to form a complex with MKK3 and p38, indicating its function as a scaffolding protein to facilitate TAK1 and MKK3 mediated activation of p38 (Edlund et al., 2003).

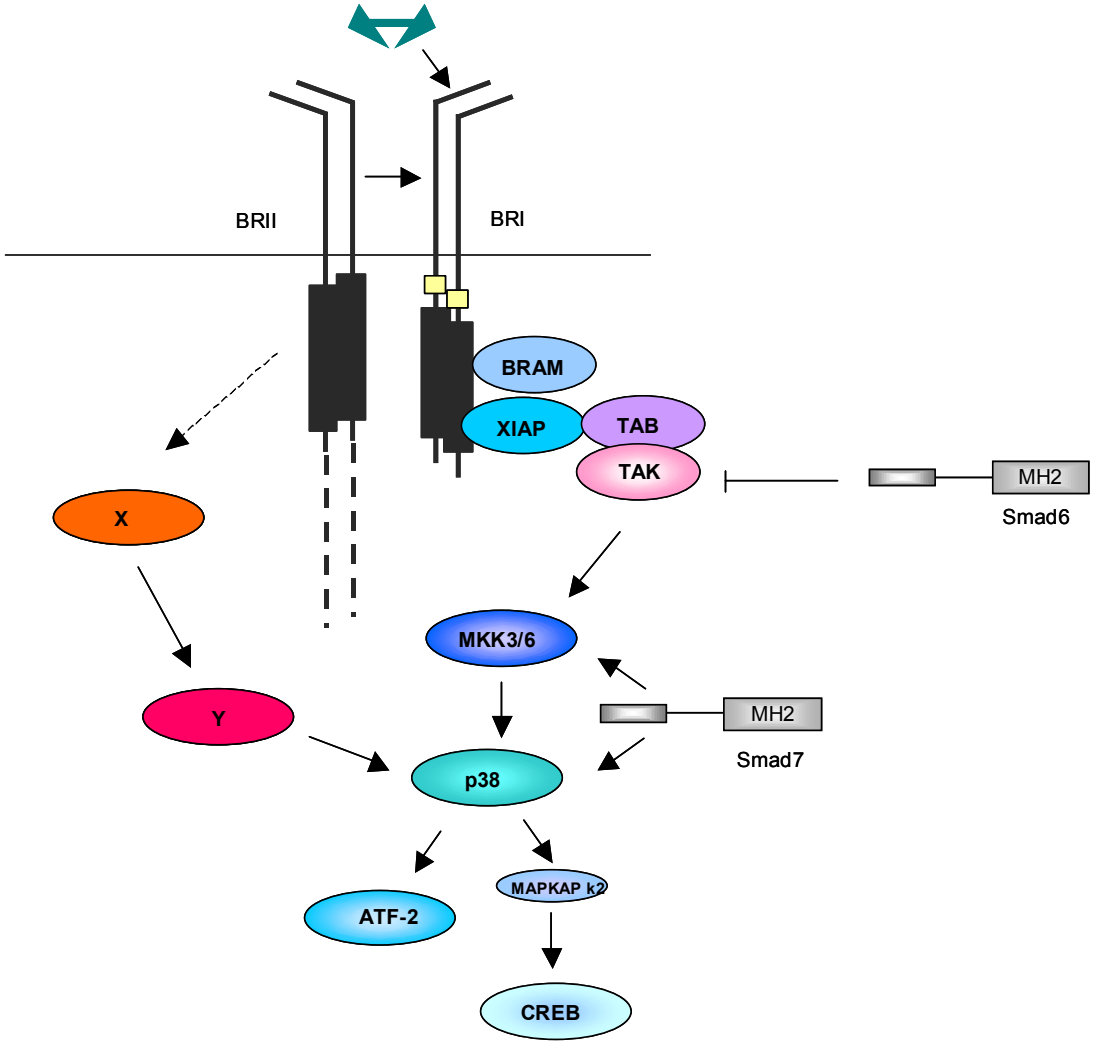


Figure 1.6 p38 pathway induced by BMP2
There are different ways how BMP2 can induce the p38-pathway. We hypothesize the p38 phosphorylation being mediated by BRII and involving one or two proteins, here X and Y. On the other hand BRAM and XIAP link the BMP receptors to the TAB-TAK-MAPK pathway which leads to subsequent p38 phosphorylation.

The second pathway leading to phosphorylation of p38 is dependent on new protein synthesis and thus showing delayed p38 activation after ligand stimulation. As demonstrated, Gadd45β (growth arrest DNA-damage) is an immediate early response gene for TGF-β. Its promoter is activated by TGF-β through Smad2, Smad3, and Smad4. Upon expression of GADD45β, it binds to MEKK4, which is an upstream activator of p38, p38 is activated and triggers apoptotic cell death (Takekawa et al., 2002), (Yoo et al., 2003). Gadd45β involvement has not been shown for BMP pathways so far.

We could show that the activation of either the Smad1/5 or the p38 pathway is triggered by the formation of different complexes and their respective recognition by ligand. Whereas preformed complexes (PFCs) signal via Smad1/5, ligand-induced complexes transmit their signals via p38. By inhibiting ligand-independent complex formation with a truncated BMP type II receptor and working with kinase deficient mutants we could demonstrate that p38 activation is dependent on BRII kinase activity and on the respective complex . It can not be excluded that the BRII is representing an anchor for yet undiscovered p38 activating proteins (Nohe et al., 2002).

Despite the fact that Kozawa and co-workers could not see JNK activation upon BMP stimulation (Kozawa et al., 2002), it has been proven that PKD activates JNK and p38 in a PKC independent mechanism and is subsequently influencing ALP production (Lemonnier et al., 2004). In addition, during osteoblastic cell differentiation, a JNK inhibitor was shown to influence osteocalcin expression (Guicheux et al., 2003).

The ERK-pathway

It has been shown, that p38 is essential in BMP2 induced upregulation of type I collagen, fibronectin, osteopontin, osteocalcin, and alkaline phosphatase activity, whereas ERK mediated BMP2 stimulation of fibronectin and osteopontin. This is indicating that ERK and p38 differentially mediate BMP2 functions in osteoblasts (Lai and Cheng, 2002).

In C2C12 cells, BMP2 is inducing ERK1/2 phosphorylation (Gallea et al., 2001). ERK1/2 activation occurs via the Ras-MEK1-ERK1/2 pathway (Palcy and Goltzman, 1999), (Lou et al., 2000). In ROS cells, BMP2 induced ERK phosphorylation leads to upregulation of Collagen Iα (Palcy and Goltzman, 1999). Moreover, the RAS-ERK pathway is supporting Smad1 mediated transcription (Suzawa et al., 2002). From in vivo experiments in *Xenpous*, data were obtained that hint towards Ras mediating BMP4 signalling in dorsal-ventral patterning (Xu et al., 1996).

Other pathways

So far, there are two examples for PKC and PKD involvement in BMP2 mediated signalling.

Hay et al. have presented, that in human osteoblasts, BMP2 uses a Smad-independent, PKC-dependent pathway to promote apoptosis via Bax/Bcl-2. Hereby a cytochrome c,

caspase-9, caspase-3, caspase-6, and caspase-7 cascade is involved (Hay et al., 2001). During osteoblastic differentiation of MC3T3-E1 cells an implication of PKD in activation of JNK and p38 induced by BMP2 could be demonstrated (Lemonnier et al., 2004).
Moreover, BMP2 induces the PI3K/p70 S6K in addition to the already described p38 pathway in C2C12 cells. Abrogation of both pathways was demonstrated to lead to increase of ALP activity (Vinals et al., 2002). During the osteoblastic differentiation of 2T3 cells expression of a dominant negative PI3K abolished induction of ALP. The action of this lipid kinase is mediated by phosphorylation of Akt, which in turn has been shown to regulate nuclear translocation of Smad1 and Smad5 (Ghosh-Choudhury et al., 2002).

1.4.4 Cross talk between BMPs and other factors

BMP as one central cytokine during development is embedded in a network of interaction and synergy with other signal transducing molecules. Only some of these pathways should be mentioned here briefly.
FGF-2, EGF and HGF signal via receptor tyrosine kinases (RTKs) and transmit their signals via ERK. It has been shown that ERK-induced phosphorylation in the linker of Smad1 inhibits the nuclear accumulation of Smad1 and Smad1 mediated signalling (Kretzschmar et al., 1997). Althini and colleagues obtained contradictory results, showing that BMPs potentiate NGF-induced neuronal differentiation in PC12 phaeochromocytoma cells (Althini et al., 2003). Now, it has been shown that the MAPK-Smad1 link may be critical for neuralization and dorsal-ventral differentiation during *Xenopus* gastrulation (Pera et al., 2003).
Direct crosstalk between Wnts and BMP2 is indicated by the up-regulated interaction between β-catenin and Smad4 in response to BMP2. This leads to enhancement of chondrogenesis, induced by BMP2 and promoted by a member of the Wnt family. Here, Wnt-3A acts in a manner opposite to that of other Wnts, such as Wnt-7A, which were previously identified as inhibitory to chondrogenesis (Fischer et al., 2002).
In endothelial cells (EC) the Notch target gene, Herp2, is synergistically induced upon activation of Notch and BMP receptor signalling. This occurs by binding of Smad1 and Notch intracellular domain (NIC) to RBP-Jκ/CBF-1 and GC-rich palindromic sites in the Herp2 promoter and by interaction of NIC with Smad1 in the presence of p-CAF and p300. This abrogates BMP induced EC migration, induced by the BMP/Id1-pathway, demonstrating a switch to balance both pathways (Itoh et al., 2004).
It has been shown that LIF and BMP2 act synergistically on the differentiation of fetal neural progenitor cells into astrocytes. LIF is a member of the IL-6 family, sharing gp130 as a common receptor. Ligand binding dimerizes the receptors and induces the JAK/Stat-pathway. The activated Stat3 (by LIF) and the phosphorylated Smad1 (by BMP2) form a

nuclear complex, bridged by p300/CBP, thus exerting synergy between these ligands (Nakashima et al., 1999).

1.5 Receptor interacting proteins

1.5.1 TGF-β receptor interactors

Many different proteins have been identified to interact with TGF-β receptors (see Fig 1.7).
All these proteins are somehow involved in the modulation of receptor mediated signalling. The immunophilin FKBP12 functions as a "guardian" for the type I receptor to prevent it from leaky signalling under sub-optimal ligand concentrations. Although TGF-β promotes the association of TβRI with TβRII, these receptor components have affinity for each other which can lead to their ligand-independent activation (Wang et al., 1994), (Chen et al., 1997).
FKBP12 is constitutively associated with the juxtamembrane domain of TβRI, from which it is released upon a ligand-induced, type II receptor mediated phosphorylation of the type I receptor. This leads to conformational changes and subsequent activation of TβRI (Huse et al., 1999), (Wang and Donahoe, 2004). Kurozumi et al. report an interaction of FKBP12 with BRIa as well, but this has not been proven so far (Kurozumi et al., 1998).
SARA (Smad anchor for receptor activation) is a FYVE protein that is important for cellular localization of the TGF-β Smads (Tsukazaki et al., 1998). Via its FYVE domain SARA is anchored to the cell membrane. It directly interacts with Smad2 and Smad3 and recruits Smad2 to the activated TGF-β receptors by controlling the subcellular localization of Smad2 and by interacting with the TβRI via its C-terminus (Wu et al., 2000). Phosphorylation of Smad2 by TβRI releases Smad2 from SARA (Tsukazaki et al., 1998). Smad2 has intrinsic nuclear import activity mediated by its MH2 domain. SARA is retaining unphosphorylated Smad2 in the cytoplasm by masking the nuclear localization signal (Xu et al., 2000a).
Disabled-2 (Dab2) has been shown to directly interact with TβRI and TβRII in vivo. Besides that, it is binding to Smad2 and 3 (Hocevar et al., 2001). Dab2 contains a N-terminal phosphotyrosine binding site (PTB), that specifically interacts with Smad2 and Smad3 and a C-terminal proline-rich domain (PRD), showing its adapter functions. Connecting both TGF-β receptors with the Smad proteins Dab2 seems to function as a adapter molecule in a multiprotein complex, serving to bridge the TGF-β receptors to the Smad pathway. Besides that, Dab2, is implicated in clathrin-coat assembly, linking the TGF-β receptors to clathrin-mediated endocytosis (Kowanetz et al., 2003).
TRAP1 (TGF-β receptor associated protein) has shown to be a molecular chaperone for Smad4, which facilitates its transfer to the R-Smads. TRAP1 binds strongly via multiple domains to inactive TGF-β and Activin receptors (TβII, ActRIIB). When the receptor is activated, TRAP1 dissociates, interacts with Smad4 and facilitates its interaction with R-

Smads (Wurthner et al., 2001). In contrast to TRAP1, TLP (TRAP1 like protein) interacts with both, active and inactive TGF-β and Activin receptors. When overexpressed it enhances Smad2 mediated signalling whereas it suppresses Smad3 mediated signalling. This suggests TLP regulating the balance of Smad2 and Smad3 signalling by localizing Smad4 intracellularly (Felici et al., 2003).

Smad7 (Hayashi et al., 1997), (Nakao et al., 1997), STRAP (Datta et al., 1998; Datta and Moses, 2000), Smurf 1 (Zhu et al., 1999) and Smurf 2 (Zhang et al., 2004), (Kavsak et al., 2000) downregulate TGF-β pathways by direct receptor interaction.

The inhibitory Smad, Smad7 can specifically interact with TβRI via its N-terminus and therefore block TGF-β mediated signalling by preventing R-Smad association and phosphorylation (Hayashi et al., 1997), (Nakao et al., 1997). Now, Smad7 has been identified as an adaptor in an E3 ubiquitin ligase complex that targets TGF-β receptors for degradation (Kavsak et al., 2000). STRAP (serine-threonine kinase receptor-associated protein) has been shown to interact with both, type I and type II TGF-β receptors and to be phosphorylated on its N-terminus. It is a WD domain-containing protein, recruiting Smad7 to the receptor complex and thus preventing the interaction and phosphorylation of Smad2/3 (Datta et al., 1998; Datta and Moses, 2000). The two HECT E3 ubiquitin ligases Smurf1 and Smurf2 are responsible for TGF-β receptor ubiquitination and degradation. Smurf1, originally identified degrading BMP receptors, also interacts with Smad7 and induces Smad7 ubiquitination and translocation into the cytoplasm. Besides, Smurf1 associates with TβRI via Smad7, leading to enhanced turnover of TβRI and Smad7 (Ebisawa et al., 2001). Smurf2 is also linked to the TβRI via Smad7 (Kavsak et al., 2000). Both Smurfs bind to I-Smads and R-Smads via their WW domains, recruit the TGF-β receptors into the complex and lead to subsequent proteasomal and lysosomal degradation of the whole complex (Ebisawa et al., 2001). TAB1 and 2 (TAK1 binding protein) may function as an activator of the TAK1 MAPKKK in TGF-β signal transduction (Shibuya et al., 1998), (Shibuya et al., 1996). TAK1 (TGF-β activated kinase1) a member of the MAPKKK family has been identified to be activated upon TGF-β stimulation and to trigger the p38 as well as the JNK pathway (Yamaguchi et al., 1995), (Hanafusa et al., 1999). A definite interaction of TAB or TAK1 with one of the TGF-β receptors, however has not been shown so far. Moreover, TAB1 binds to XIAP (X-chromosome-linked inhibitor of apoptosis), a protein containing a C-terminal RING and three N-terminal baculovirus IAP repeats (BIR) (Yamaguchi et al., 1999), (Lewis et al., 2004). XIAP is shown to associate with several type I receptors of the TGF-β superfamily. It is not clear yet how TGF-β activation can induce pathways mediated by XIAP. But, XIAP mediated JNK and NFκB activation were shown to be Smad4 dependent, whereas XIAP induced apoptosis was Smad independent (Birkey Reffey et al., 2001). Lewis et al. have mapped the signalling

domains in XIAP. The E3 ubiquitin ligase activity of the RING domain is responsible for NFκB activation and the BIR and loop regions of XIAP regulate Smad-pathways (Lewis et al., 2004).

Several other molecules have been identified to be interacting with the TGF-β receptors, but their positioning in the TGF-β dependent signalling network is still unclear. The protein phosphatase PP2A, subunit Bα and the FT-α belong both to the group of WD domain-containing proteins and both interact with and are phosphorylated by TβRI (Griswold-Prenner et al., 1998), (Wang et al., 1996a). The association of PP2A Bα depends on the kinase activity of the type I receptor, is increased by coexpression of the type II receptor and potentiates antiproliferative effects of TGF-β (Griswold-Prenner et al., 1998). TRIP-1, another WD-domain protein, specifically associates with TβRII in a kinase dependent way and serves as a target for phosphorylation (Chen et al., 1995). TRIP-1 acts as a modulator of TGF-β gene response by specifically inhibiting gene expression driven by the PAI-1 promoter but not repressing cyclin A inhibition and growth inhibition (Choy and Derynck, 1998).

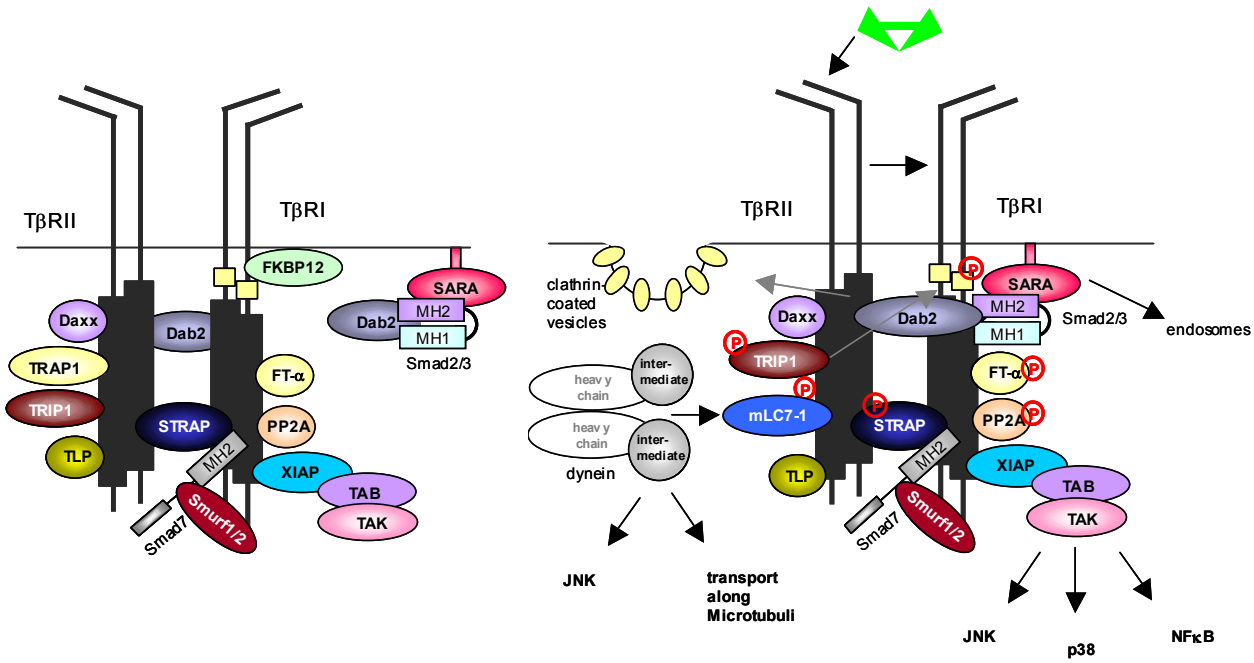


Figure 1.7 TGF-β receptor associated proteins before and after ligand stimulation
In the absence of ligand many different proteins are associated with the TGF-β receptors. Their functions are described in the text. Stimulation with ligand induces processes as recruitment of SARA and Smads, dissociation of TRAP1 and others.

14-3-3ε was originally found in *Schistosoma mansoni,* here interacting with SmRK1, a TβRI homologue. It has been shown to interact with hTβRI in a phosphorylation dependent manner and to act as a positive regulator of TGF-β mediated growth arrest (McGonigle et al., 2001). Sorting nexin 6 (SNX6) are involved in receptor trafficking. Most of the Sorting nexins

bind to tyrosine kinase receptors with high affinity. SNX6 and 1-4 have been found associated with ActRIIB and the kinase dead TGF-β receptors, linking them to tyrosine kinase receptors (Parks et al., 2001). In a screen for TβRII interactors Daxx, a protein associated with the Fas receptor, was isolated (Perlman et al., 2001). Daxx mediates activation of JNK and apoptosis induced by Fas. It binds to constitutively active TβRII, thus being a link between TGF-β and Fas and the apoptotic machinery.

1.5.2 BMP receptor interactors

So far very little is known about BMP receptor interacting proteins. For the BRI only two direct interactors are described: BRAM1 and XIAP. BRAM1 (BMP receptor associated molecule1), was fished in a yeast two-hybrid screen with BRIa as a bait. BRAM1 is a alternative splice variant of BS69, a factor previously identified as an adenovirus E1A-associated protein. Only the C-terminus is sufficient for BRI interaction, but the physiological relevance of this interaction has not been investigated so far (Kurozumi et al., 1998). XIAP, (X-chromosome-linked inhibitor of apoptosis), has been shown to interact with many type I receptors. Its interaction with the BRIa has been proven in the mammalian and in the *Xenopus* system (Yamaguchi et al., 1999). XIAP associates not only with TAB1 but also with the BMP receptors in vivo. Direct receptor binding of XIAP recruits the associated proteins TAB1/2 and TAK to the BMP receptor complex. TAB is the upstream signalling molecule of TAK (MAPKKK), which triggers itself multiple signal transduction pathways (Yamaguchi et al., 1995, Shibuya, 1996 #219). Activated by BMP4, XIAP, TAB and TAK1 act downstream of BRIa (Shibuya et al., 1998).

LIMK1 (Foletta et al., 2003), a serine-only protein kinase that contains LIM and PDZ protein-protein interaction domains is a key regulator of actin dynamics. It can regulate cell shape and motility through phosphorylation of cofilin, an actin-depolymerizing factor of actin filaments, which is then unable to bind and depolymerize F-actin (Sumi et al., 2002). Interaction between LIMK1 and BMPR-II inhibited the ability of LIMK1 to phosphorylate cofilin, whereas addition of BMP4 reduced this effect (Foletta et al., 2003). A truncated PPH mutant of BRII was unable to bind and inhibit LIMK1, suggesting that deregulation of actin dynamics may contribute to the etiology of PPH (Foletta et al., 2003).

The function and mechanism of Tctex-1, the second BRII-tail interaction partner, are not fully understood (Machado et al., 2003). Tctex-1, a light chain of the motor complex dynein, interacts with the cytoplasmic domain of BRII and is phosphorylated by the kinase of BRII. PPH mutant proteins, which carry mutations within the tail are still able to interact with Tctex-1 but are not able to phosphorylate it (Machado et al., 2003).

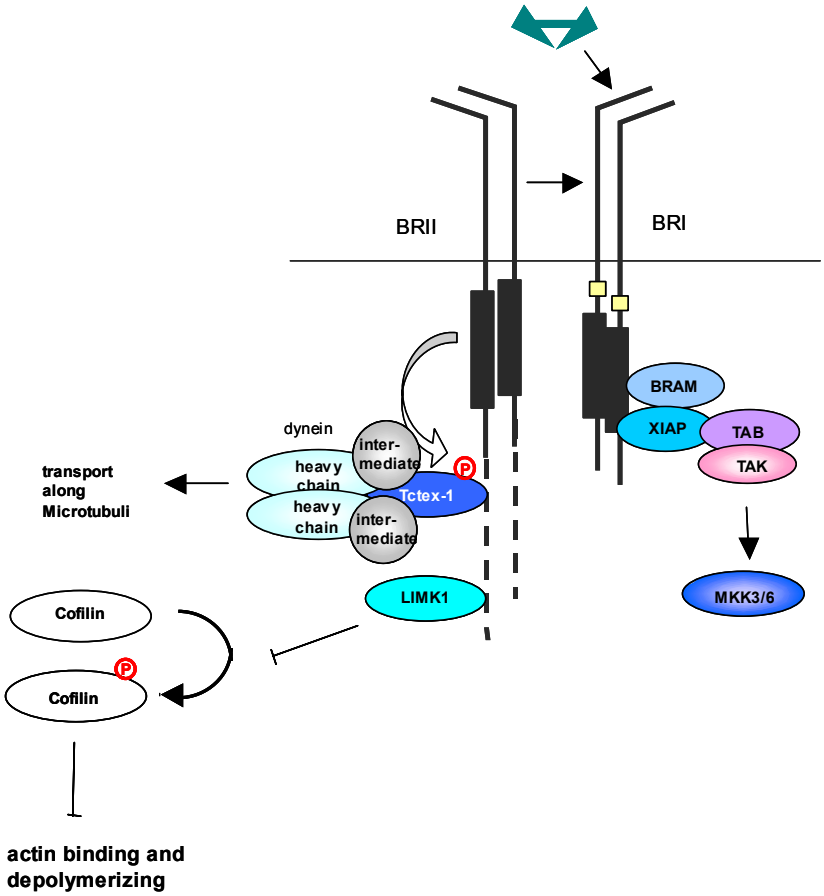


Figure 1.8 BMP receptor associated proteins
As only few BRII interactors are described yet the scheme of BRII associated proteins is rather simple. With BRI only BRAM and XIAP are linked. And BRII is interacting with Tctex-1 and LIMK1 via its C-terminal tail. Addition of BMP2 does not induce a change in the association pattern.

1.6 Phosphorylation of BMP and TGF-β receptors

There is biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor beta receptor kinases. Overexpressed TβRII is a constitutively active kinase which phosphorylates the ligand recruited TβRI on serine and threonine residues, even as substrate in an in vitro kinase assay. The phosphorylation occurs mainly in the juxtamembrane region, the GS box (Wrana et al., 1994). It can be observed that, in contrast to the in vivo situation, overexpressed type I and type II receptors show ligand-independent heteromeric complex formation (Ventura et al., 1994), (Chen and Weinberg, 1995), (Souchelnytskyi et al., 1996).

TβRI that is overexpressed in COS1 cells exhibits different phosphorylation patterns. As mentioned, overexpressed TGF-β receptors have the tendency to form heterooligomers (Chen and Weinberg, 1995). Non complexed TβRI is barely phosphorylated, complex formation with TβRII enhances the phosphorylation status and ligand addition increases phosphorylation even more. Complexed, stimulated TβRI exhibits 16 phospho-spots, among

them 5 are TGF-β dependent (Souchelnytskyi et al., 1996). Three major hot spots of ligand-dependent phosphorylation can be observed: the GS-domain with the phosphorylated sites: T185, T186, S187, S189, S191 (all phosphorylated or only T186, S189 and S191 together) and two regions more N-terminal with S165 and S172 plus T176 (Souchelnytskyi et al., 1996), (Saitoh et al., 1996). Phosphorylation of residues in the GS-box is activating the TβRI, leading to C-terminal Smad2/3 phosphorylation (Wrana et al., 1994). The phosphorylation of S165 is a receptor modulating phosphorylation. Exchanging of that serine to alanine promotes TGF-β dependent growth inhibition and extracellular matrix production, but does not influence TGF-β mediated apoptosis (Souchelnytskyi et al., 1996). Phosphorylation of S172 or T176 changes receptor behaviour. Receptors with mutations that change serine 172 to alanine (S172A) or threonine 176 to valine (T176V) were similar to wild-type TβRI. However, these mutant receptors were impaired to mediate a growth inhibitory signal from TGF-β. These observations indicate that serine 172 and threonine 176 of TβRI are dispensable for extracellular matrix protein production but essential to the growth inhibition by TGF-β (Saitoh et al., 1996). GADD34, which is a regulatory subunit of protein phosphatase PP1c, that recruits the catalytic subunit, is linked to TβRI via Smad7. After forming the holoenzyme PP1c is dephosphorylating TβRI and thus downregulating TGF-β mediated signalling. SARA enhances the recruitment of PP1c by controlling its subcellular localization (Shi et al., 2004). Now, four ways how Smad7 blocks TGF-β signalling are described: by mechanical blockage of R-Smad phosphorylation, by proteasomal degradation of the receptor complex, by de-phosphorylation of TβRI and by interacting with TAK1 and thus blocking the p38-pathway.

The TβRII exhibits strong auto-phosphorylation in the absence of ligand (Chen and Weinberg, 1995). When overexpressed in COS1 cells, alone or together with TβRI, no ligand induced phosphorylation can be observed. It has been shown that TβRII is phosphorylated at 17 spots in vivo (Souchelnytskyi et al., 1996). When complexed with TβRI the TβRII exhibits phosphorylation on S223,(S226), S227, S549, and S551 (Souchelnytskyi et al., 1996).

Experiments using bacterially overexpressed TβRII and subsequent in vitro kinase assays revealed that the mutant K277R is not phosphorylated. The phosphorylation observed on wildtype TβRII results from auto-phosphorylation or other kinases activated by the TβRII kinase (Luo and Lodish, 1997). Phosphorylation on serines, threonines and tyrosines was determined. Threonine phosphorylation was detected via phosphoamino acid analysis, but the target amino acids were not specified (Lin et al., 1992). Luo et al. discovered phosphorylated serine residues, S213 in the membrane proximal region, S409 and S416 in the substrate-binding site of the kinase (T-loop region) (Luo and Lodish, 1997). The phosphorylation event for S213 is intramolecular, S409 and S416 are phosphorylated by an

intermolecular mechanism, that is enhanced by dimerization (Luo and Lodish, 1997). As demonstrated, this is facilitated by the high affinity of the TβRII monomers for each other, leading to ligand-independent dimer-formation (Henis et al., 1994) (Chen and Derynck, 1994). Whereas S409 phosphorylation is shown to be a receptor activating one, the phosphorylation of S416 is inhibiting receptor activity. Thus never both serines are phosphorylated on the same receptor chain (Luo and Lodish, 1997). Lawler demonstrated receptor-activating tyrosine phosphorylation on Y259, Y336, Y424 (Lawler et al., 1997). Therefore active TβRII is phosphorylated on S213, S409, Y259 or on S213, S409, Y259, Y336, Y424 at the same time (Lawler et al., 1997).

Nothing is known so far about BMP receptor phosphorylation. In this thesis BRII phosphorylation will be examined and compared with the TGF-β receptors.

1.7 BMP2 and its receptors – diseases and functions in the body

BMP and other proteins of that tightly regulated signal transduction network play important roles in the body. Mutations in proteins involved in this network identified so far lead to severe diseases.

1.7.1 Primary pulmonary hypertension

Primary pulmonary hypertension (PPH) is an autosomal dominant disorder with decreased penetrance and female predominance, characterized by the obliteration of small pulmonary arteries and arterioles (Loyd et al., 1984). This leads to the persistant elevation of pulmonary vascular resistance, pulmonary hypertension and right heart failure (Palevsky et al., 1989). The disease can occur at any age, but most patients develop symptoms in their third or fourth decade (Thomson et al., 2000). The majority of patients has no known family history of the disease, but some familial occurrence can be observed (Machado et al., 2001). Some persons carrying PPH mutations do not exhibit any symptoms, suggesting that additional or genetic factors may be necessary to develop symptoms (Thomson et al., 2000). Mapping of the putative susceptibility site in the human genome locus pointed towards 2q33 (Nichols et al., 1997), and a candidate-gene approach from the human genome project (HGP) showed that germline loss-of function mutations in the bone morphogenetic protein receptor 2 (*BMPR2*) gene causes familiar PPH (Lane et al., 2000), (Deng et al., 2000), (Thomson et al., 2000). This suggests that BMPs may play important roles in the homeostasis of the pulmonary vascular system. So far, 41 unique mutations have been identified (Waite and Eng, 2003). 75 percent of the mutations lie in the ligand-binding and kinase domains, even if these domains represent only about 50% of the receptor.

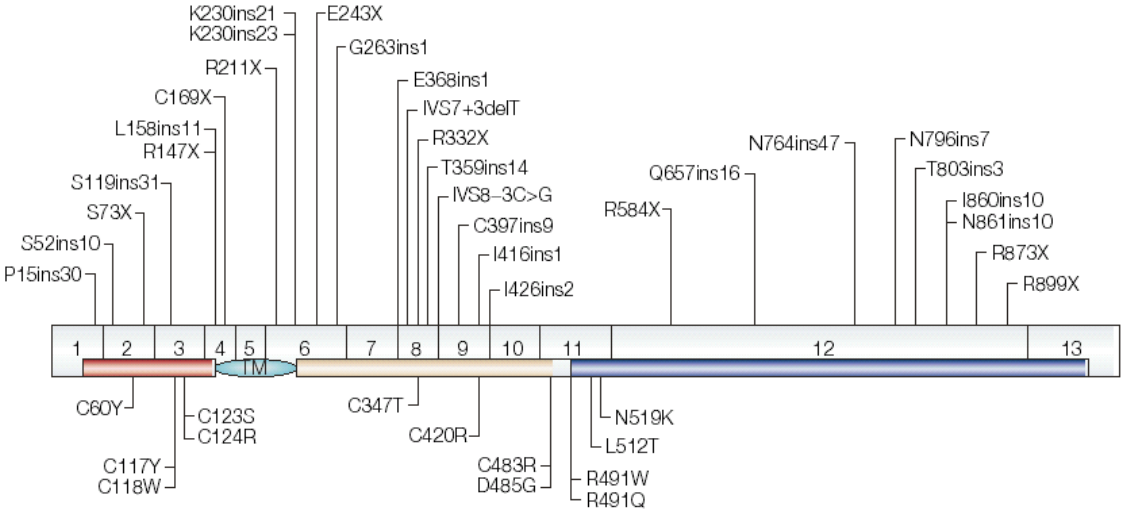


Figure 1.9 PPH mutations identified in germline *BMPR2* gene
BMPR2 is a gene with 13 exons that encodes an extracellular domain (red), a transmembrane domain (blue), a kinase domain (yellow) and a long C-terminal tail (blue). Truncating mutations are indicated above and missense mutations below. “ins” is depicting an insertion.
Figure from Waite et al. (Waite and Eng, 2003), combining experimental data from (Thomson et al., 2000), (Machado et al., 2001) and (De Caestecker and Meyrick, 2001).

It is remarkable, that a relatively high percentage of the mutations affect cysteines (Rudarakanchana et al., 2002). Substitution of these cysteine residues in the ligand binding or kinase domain affects trafficking of the BRII to the cell surface and results in reduced ligand binding, as observed with ^{125}I-BMP4 (Rudarakanchana et al., 2002). In addition, these mutants have a dominant-negative effect on the Smad-pathway, measured on pGCCG-lux. Non-cysteine mutations in the extracellular or kinase domain are inserted in the cell membrane but show the Smad-inhibition as well, whereas mutations in the tail domain exhibit a normal Smad-signalling. All mutants investigated show a upregulation of the p38 pathway leading to increased proliferation, though (Rudarakanchana et al., 2002).

To investigate the speciality of the BRII-tail, Machado et al. were looking for BRII-tail associated proteins. Tctex-1, a light chain of the motor complex dynein of 14kDa, interacts with the cytoplasmic domain of BRII and is phosphorylated by the BRII. This event is disrupted in PPH mutations within exon12, the tail (Machado et al., 2003). Dynein, a molecular motor protein, consists of two heavy, two intermediate and a variable number of light chains. The light chain is the specific adaptor for cargo binding (Karki and Holzbaur, 1995).

Tctex-1 interacts with BRII via its C-terminal 37 amino acids, a region that contains consensus sequences for CKII and PKC. The interaction site of BRII extends from amino acids 509-764 (Machado et al., 2003). PPH mutations were able to bind Tctex-1, but they were unable to trans-phosphorylate it, suggesting that this interaction is important for BRII

mediated signal transduction (Machado et al., 2003). Interestingly, the activated TGF-β receptor complex has also been shown to interact with dynein by phosphorylating the light chain of mLC7. Overexpression of mLC7-1 induces specific TGF-β responses, including activation of Jun N-terminal kinase (JNK), a phosphorylation of c-Jun, and an inhibition of cell growth (Tang et al., 2002).

Different mutations in the *BMPR2* gene have been shown to lead to PPH. Nishihara et al. tested three different types of mutations (extracellular, kinase domain and tail domain) for their influence on signalling. Whereas BRII containing cysteine mutations in the extracellular or kinase domain were detected to be stuck in the cytoplasm and showed impaired Smad signalling, the tail mutations retained the ability to transduce signals. These observations suggest that additional genetic or environmental factors may play critical roles in the pathogenesis of PPH (Nishihara et al., 2002).

Pulmonary vascular medial hypertrophy in this disease is mainly caused by increased proliferation and decreased apoptosis in pulmonary-artery smooth muscle cells (PASMCs) and endothelial cells (HPAECs). As demonstrated, BMP signals are involved in the regulation of proliferation of human pulmonary smooth muscle cells (Nakaoka et al., 1997).

Zhang et al. could show that BMPs have antiproliferative effects on wildtype PASMCs, increasing the percentage of cells undergoing apoptosis. The BMP2-mediated apoptosis in normal PASMCs was associated with a transient activation or phosphorylation of Smad1. In PASMCs from PPH patients, BMP-induced apoptosis was significantly inhibited. These results suggest that the antiproliferative effect of BMPs is partially due to induction of PASMC apoptosis, which serves as a critical mechanism to maintain normal cell number in the pulmonary vasculature. Inhibition of BMP-induced PASMC apoptosis in PPH patients may play an important role in the development of pulmonary vascular medial hypertrophy in these patients (Zhang et al., 2003b).

1.7.2 Hereditary Hemorrhagic Telangiectasia

A disease, that is connected to mutations, that are more related to the TGF-β mediated signal transduction is HHT (Hereditary Hemorrhagic Telangiectasia) or Osler-Weber-Rendu Syndrome. HHT is an autosomal dominant disorder that is characterized by multi-system vascular dysplasia. HHT primarily affects 4 organ systems; the lungs, brain, nose and gastrointestinal (stomach, intestines or bowel) system. The affected arteries either have an abnormal structure causing increased thinness or an abnormal direct connection with veins (arteriovenous malformation). Patients can have a wide range of clinical features, because many different organs can be affected having vascular lesions. Bleeding from affected vessels progresses with age and the 10% mortality that is associated with that disease is due to haemorrhagic stroke and/or brain abscesses.

Mutations connected to that disease are found in two components of the TGF-β/BMP pathway, in Endoglin (*ENG*) for HHT1 and ALK1 (*ACVRKLK1*) for HHT2 (McAllister et al., 1994), (Johnson et al., 1996a). Endoglin increases the activation of TGF-β receptors by a yet undefined mechanism. This can be connected to the TβRII, which is important for ligand recognition and therefore upstream of ALK1 or ALK5 who are transmitting the signal. Besides TGF-β1 and TGF-β3 endoglin is capable to bind BMP2 and BMP7 as well as Activin A, but requires coexpression of the respective ligand binding kinase receptor for this association (Barbara et al., 1999).
The exact mechanism how endoglin or ALK1 mutations trigger HHT is still unknown, but in general the phenotype caused by mutations in endoglin is distinct from, and more severe than the one caused by mutations in ALK1 (Berg et al., 2003).

1.7.3 Juvenile polyposis

Juvenile polyposis (JPS) is one of the autosomal dominantly inherited hamartoma polyposis syndromes, with an elevated risk for gastrointestinal cancers and juvenile polyps. Up to 50 percent of affected individuals carry germline mutations of either *BMPRIa* or *MADH4*. Mostly, these mutations are truncating mutations and lead to shorter and inactive proteins. In case of point mutations, they are in highly conserved regions and result in loss of function (Zhou et al., 2001).

1.7.4 Fibrodysplasia ossificans progressiva

FOP (Fibrodysplasia ossificans progressiva) is a rare autosomal dominant disorder with overexpression of BMP4 in lymphoblastoid cells (Kaplan and Shore, 1998). This is accompanied by deficiency of the BMP antagonist Noggin (Xu et al., 2000b). Patients show sporadic episodes of explosive growth of mesenchymal cells in skeletal muscle followed by cellular differentiation to heterotopic bone through an endochondral process. The FOP gene has recently been mapped to human chromosome 4q 27-31 (Mahboubi et al., 2001). A newly developed therapy is systemic or local application of an engineered Noggin mutein hNOGDeltaB2, that was shown to circulate systemically, preventing the BMP4-induced heterotopic ossification (Glaser et al., 2003).

1.7.5 Persistent Mullerian duct syndrome

Persistent Mullerian duct syndrome (PMDS) is a rare form of male pseudohermaphroditism. It is characterized by the lack of regression of Mullerian derivatives, uterus and tubes, in otherwise normally virilized males. Mutations of the Mullerian inhibiting substance (*MIS*) gene or the MIS type II receptor (*MISRII*) gene have been identified in PMDS patients with

autosomal recessive transmission (Hoshiya et al., 2003). The most common mutation is a 27bp deletion in exon 10 of the *AMHR* gene (Imbeaud et al., 1996).

1.7.6 BMP2 and radiation

Heterotopic bone formation (HBF) in the soft tissues surrounding the hip joint is a frequent complication of hip surgery. To avoid this complication, anti-inflammatory drugs are given daily for some weeks after the surgery or a single dose of radiation is given the day before or after the operation (Koelbl et al., 2001), (Pohl et al., 2003).

1.8 c-kit

The c-kit proto-oncogene is a receptor protein-tyrosine kinase (RTK) associated with several highly malignant human cancers. Upon binding its ligand, stem cell factor (SCF), c-kit forms an active dimer that autophosphorylates itself and activates a signalling cascade that induces cell growth. Disease-causing human mutations that activate SCF-independent constitutive expression of c-kit are found in acute myelogenous leukemia, human mast cell disease, and gastrointestinal stromal tumors. Originally, c-kit is important for survival and development of haematopioetic progentiors, mast cells, germ cells (gametogenesis), melanocytes and erythrocytes.

1.8.1 Defects of c-kit

Malignant gastrointestinal tumours bear a constitutively active c-kit receptor. Mutations in c-kit result in ligand-independent tyrosine kinase activity, autophosphorylation of c-kit, uncontrolled cell proliferation, and stimulation of downstream signalling pathways (Hirota et al., 1998). The mutation D816V is shown to occur in sporadic adult mastocytosis and other tumours (Longley et al., 1999), (Longley et al., 1996). Here, D816V plays a major role, leading to uncontrolled cell growth, V560G, another mutation identified in mast cell leukemia, has only minor impact (Piao et al., 1996). But V560G and other mutations in the juxtamembrane region are common in gastrointestinal stromal tumours (Frost et al., 2002). Besides that, mutant c-kit has shown to be involved in development of acute myeloid leukemia and germ cell tumours. Taken together, mutations in the juxtamembrane region seem to induce human gastrointestinal stromal tumours and kinase mutations are found in mast cell and myeloid leukemias and in human germ cell tumours (Hirota et al., 1998), (Nishida et al., 1998), (Tian et al., 1999).

Joensuu and coworkers demonstrated that STI571, an inhibitor of tyrosine kinase activity in BCR/Abl-positive leukemia, was effective in treating gastrointestinal stromal tumours (Joensuu et al., 2001). STI571, known as Imatinib and by the trade name Gleevec, was approved by the Food and Drug Administration in 2002 for the treatment of gastrointestinal stromal tumours [Savage, 2002 #707].

Besides its involvement in cancer, c-kit is a candidate gene for the degree of spotting in cattle and for white skin colour in pig and mice, so called dominant white spotting (W) (Chabot et al., 1988). In humans, Piebaldism, that involves distinct patches of skin and hair that contain no pigment, an autosomal dominant inherited disease, is due to mutation in the c-kit gene (Giebel and Spritz, 1991).

1.8.2 The c-kit receptor

All members of the RTK family have an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic kinase domain with tyrosine kinase activity. C-kit is the non-oncogenic homologue of v-kit, which was identified as the transforming oncogene in the Hardy-Zuckerman 4 feline sarcoma virus (Besmer et al., 1986). V-kit shows no extracellular domain and is membrane-anchored via myristylation. C-kit is belonging to the type III RTK family, including the PDGFRα and β, the CSF-1R and the FMS-related receptor FLT-3 (Yarden et al., 1987), (Rosnet et al., 1993), (Mol et al., 2003). Structurally, c-kit contains extracellularly five immunoglobulin-like (Ig-like) domains and a catalytic domain divided into two regions by a 77 amino acid insert intracellularly, a so called split-kinase. The three N-terminal Ig-like domains are responsible for ligand binding, thus leading to receptor dimerization. The fourth one is involved in stabilization of the receptor dimer and the membrane proximal Ig-like domain contains a proteolytic cleavage site. Cleavage here results in a soluble, ligand binding domain, that functions as an extracellular inhibitor of SCF and SCF-mediated signalling (Broudy et al., 2001), (Dahlen et al., 2001), (Wypych et al., 1995).

Recently, the crystal structure of c-kit kinase with c-kit being in a fully active form, with ordered kinase activation and phosphate-binding loops, has been identified. Comparing MS/MS analysis and in structural data in parallel, Y568 and Y570 were indentified as in trans auto-phosphorylated sites (Mol et al., 2003).

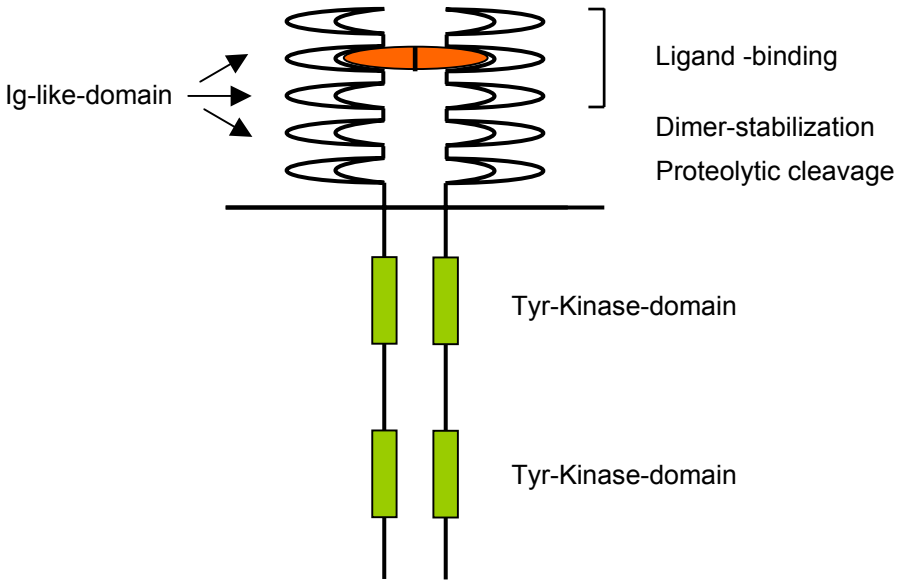


Figure 1.10 Schematic representation of the domain structure of c-kit
C-kit belongs to the receptor tyrosine kinase family and carries extracellularly five Ig-like domains, that are responsible for ligand-binding and dimer stabilization. The fifth domain represents a cleavage site for proteases. The cytoplasmic part exhibits a split kinase domain with tyrosine kinase activity.

1.8.3 Signal transduction induced by c-kit

Ligand binding results in dimerization, followed by receptor kinase activation and subsequent auto- and transphosphorylation. Direct receptor-receptor interaction via the Ig-like domain four is supporting that process (Blechman et al., 1995).

A variety of different pathways is induced by c-kit activation. Members of these pathways interact with each other via different protein domains, as SH2, SH3 (Src homology) and PH (Pleckstrin homoloyg). It has been shown, that phosphorylated tyrosines display the interaction sites of the associated proteins. Many sites and their interaction partners have been identified so far, indicating the downstream pathways of c-kit (see Fig. 1.11). Moreover c-kit undergoes phosphorylation on serine residues following ligand stimulation; this phosphorylation is dependent mainly on the activity of protein kinase C (PKC) (Blume-Jensen et al., 1994).

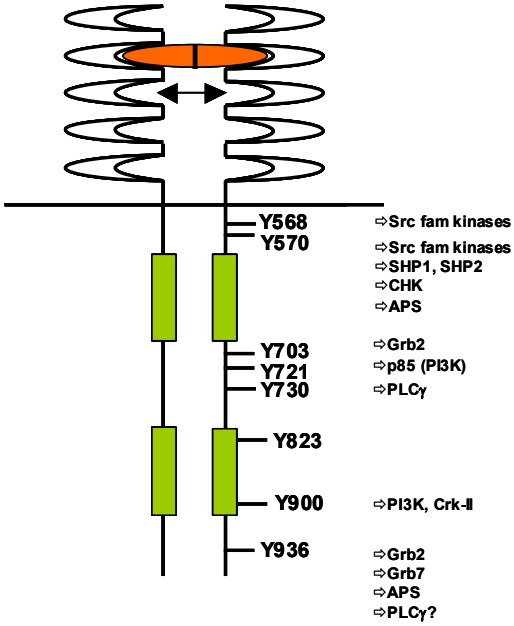


Figure 1.11 Tyrosines as adapters for signalling molecules
C-kit is signalling via a variety of different pathways. Phosphorylated tyrosines represent links towards these pathways. Here a scheme of the phosphorylation sites and the identified interactors is depicted.

Among the pathways induced by SCF the Ras-Raf-ERK pathway is playing an important role. It is either activated via the adaptor protein Grb2 or via Src family kinases that lead to phosphorylation of the adaptor Shc (Lennartsson et al., 1999; Thommes et al., 1999). In haematopoietic cells the described ways can be bypassed by the activation of the GEF (guanine nucleotide exchange factor) Vav and subsequent Raf activation. Here, Vav is linked to c-kit via Grb2 (Gulbins et al., 1994), (Ye and Baltimore, 1994). Moreover, Stats (signal transducers and activators of transcription) play important roles in c-kit mediated signal transduction. It has been shown that JAK2 as well as Stat1 can associate with c-kit and mediate its signal transduction (Deberry et al., 1997; Linnekin et al., 1997). Besides Stat1

and Stat5, that are phosphorylated on tryosines through c-kit, Stat3 is phosphorylated on serines upon ligand stimulation (Ryan et al., 1997), (Gotoh et al., 1996). PI3K is activated in response to SCF. Its activation includes its association with p-Tyr on the c-kit receptor and subsequent phosphorylation of the regulatory subunit p85 via the receptor or Src family kinases (Pleiman et al., 1994), (Cuevas et al., 2001). Now Ca^{2+}-independent PKC or Akt/PKB can be activated. There are two ways to activate PKC. The most prominent is via PLCγ (phospholipase C), which is in turn activating DAG (Diacylglycerol) and Ca^{2+} release. This is leading to PKC activation and subsequent cellular response. The pathway, which seems to be the most prominent, is not the main way to induce PKC dependent on SCF. Upon stimulation, PI3K gets activated which in turn induces PLD (phospholipase D), which can induce the production of DAG and thus activate PKC (Kozawa et al., 1997). An overview of c-kit induced signalling can be found in the review of Linnekin (Linnekin, 1999).

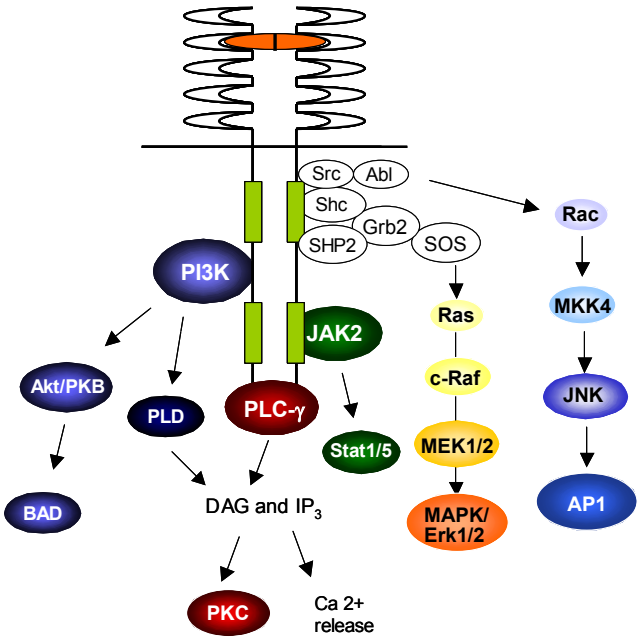


Figure 1.12 Signal transduction pathways originating from c-kit
C-kit is signalling via a variety of different pathways. Here a scheme of the characterized pathways is depicted.

RTKs are negatively regulated by protein tyrosine phosphatases (PTPs). The phosphatase SHP1 has been shown to downregulate c-kit mediated signalling. A c-kit mutant defective in SHP1 binding (Y570F) exhibits enhanced SCF-induced proliferation (Kozlowski et al., 1998). The Src family kinase Lyn is shown to be important for normal, ligand-dependent internalization of c-kit, leading to lysosomal degradation (Broudy et al., 1999). Upon ligand stimulation, c-kit is rapidly internalized and it has been demonstrated that the kinase activity is important for that process (Yee et al., 1994c). The internalization process is mediated via clathrin-coated pits, pinching off the vesicles might be supported by PI3K and subsequently

dynamin (Gommerman et al., 1997). One sign for degradation is poly-ubiquitination. RTKs get poly-ubiquitinated via c-Cbl, a RING finger ubiquitin ligase. After attachment of ubiquitin, the receptor gets rapidly degraded via the lysosomal pathway.

1.8.4 Possible links between c-kit/ SCF and BRII/BMP2

We could show a link between the BRII/BMP- and the c-kit/SCF pathway. To examine potential targets and the physiological relevance of this crosstalk, possible connections between the BMP and the c-kit pathway should be elucidated. Here, the focus is more on the whole organism and the whole cell than on signalling, even though signalling can not be neglected in that context.

Stem cells and the stem cell niche

So far very little is known about the factors that regulate adult murine and human blood stem cells. It has been shown that certain mesodermal cells differentiate into haematopoietic and endothelial cell lineages and the involvement of different cytokines during these processes has been investigated (Zon, 1995), (Choi et al., 1998). Very little attention has been paid on other factors. BMP was shown to regulate proliferation and differentiation of highly purified primitive human haematopoietic stem cells (HHSC) (Bhatia et al., 1999). Besides their reaction upon BMP2/4 or BMP7 stimulation, these cells express BRIa and BRIb as well as the BMP-Smads. Stimulation of $CD34^{+}CD38^{-}Lin^{-}$ cells with BMP2/7 or TGF-β resulted in inhibition of proliferation and maintenance of the primitive phenotype. Low doses of BMP4 induced proliferation and differentiation, whereas higher doses delayed differentiation and kept the immature state. Although the differences between BMP2 and BMP4 in that system can not be explained, the effect of BMPs on immature stem cells is undisputed and represents a new way of regulation (Bhatia et al., 1999). Nakayama and colleagues demonstrated the participation of BMP4 in differentiation of ES cells into lymphoid or erythro-myeloid progenitors. This process is synergistically enhanced by VEGF (Nakayama et al., 2000). Moreover BMP2/4 have been shown to be essential for haematopoietic cell genesis in *Xenopus* and *Zebrafish* (Graff et al., 1994). During the formation of embryonic bodies originating from ES cells, the BMP pathway is shown to direct the expression of the erythroid transcription factors EKLF and GATA1 (Adelman et al., 2002).

Mature cell populations are replenished by adult stem cells that are located at certain regions in the body. The birth and maturation of these cells is regulated by the surrounding micro-environment, the so called niches. Although the niches for gut and some skin stem cells haven been identified, the niche for human haematopoietic stem cells has been undiscovered. Recently it has been confirmed that osteoblasts play important roles in stem-cell regulation. HHSCs colocalize with spindle-shaped osteoblasts that are lining the inner bone surface. Both cells might be connected via β-catenin and N-cadherin, that helps the

cells to adhere. The activity of BMP has been shown to be essential for the development of blood-forming tissues. Manipulation in the BMP-mediated signalling in the spindle-shaped osteoblasts, either by knocking-down of BRIa or by overexpression of PTH receptors, the number of spindle-shaped osteoblasts increased and, along with it, the number of long-term HHSCs (Lemischka and Moore, 2003), (Calvi et al., 2003), (Zhang et al., 2003a).

BMPs have been shown to be implicated in the maintenance of the immature state of embryonic stem cells (ES). The blockade of lineage-specific transcription factors by BMP-induced Id proteins enables the self-renewal response to LIF/STAT3. Thus, the BMP-mediated induction of Id proteins suppresses the differentiation and sustains the embryonic stem cell self-renewal potential in collaboration with Stat3 (Ying et al., 2003), (Rajan et al., 2003).

Granulosa cells

In the rat, a so far undescribed system of feedback mechanism between granulosa cells and oocytes has been demonstrated. In a co-culture system of oocytes and granulosa cells BMP15 stimulated SCF expression in granulosa cells, whereas the produced SCF inhibits BMP15 expression in oocytes, thus forming a negative feedback loop. This interplay between SCF, BMP15 and the respective receptors might play important roles in early folliculogenesis (Otsuka and Shimasaki, 2002).

1.9 Aim of the project

BMPs regulate a plethora of cellular processes, such as proliferation, differentiation, migration and apoptosis and thus play central roles during developmental processes and tissue homeostasis. In this thesis BMP2 mediated signal transduction, BRII associated proteins and BRII phosphorylation should be examined.

We could show that the oligomerization pattern of BMP receptors exhibits a higher degree of flexibility compared to other receptors of that superfamily (Gilboa et al., 2000). One aim of this thesis was to identify the impact of receptor oligomerization on BMP2-mediated signal transduction. The initial trigger for BMP-dependent signalling is the phosphorylation of BRI by BRII. As the BRII, having a long C-terminal extension in the BRII-LF splice variant, plays central roles in transmitting signals and proteins inducing Smad-independent pathways might be associated with the BRII or the BRII-tail, one goal was to identify BRII-associated proteins. One of the interactors should be further characterized. As only few BRII interactors are described so far and the interaction of serine/threonine kinase receptors with tyrosine kinase receptors is opening new perspectives in signalling, the interactor chosen was c-kit.

In the present work, the impact of c-kit and SCF-mediated signalling on the BRII and various BMP-dependent pathways should be investigated. As c-kit is an active tyrosine kinase, analysis of the BRII phosphorylation in different states should show the different activation levels of BRII and the influences of c-kit on the latter.

2. Material and solutions

Due to the fact that one part of the experiments described has been performed in the department of Physiological Chemistry II, University of Würzburg and one part in the Ludwig Institute for Cancer research, Uppsala, Sweden, I will only focus on important equipment.

2.1 Chemicals

Basic chemicals of highest purity were purchased from Merck, Roth, Serva and Sigma. All solutions were prepared using deionized water of milipore quality.
Reagents for cell culture were from Biochrom, Bio Witthaker, Falcon, Gibco BRL, Greiner, Nunc, Merck and Sigma.
Substances for the preparation of bacterial growth media were from Roth, Pronadise and Difco.

Special chemicals:

Immunosorb A	EC Diagnostics AB, Uppsala, Sweden
Polyvinylpyrrolidone, K30	Aldrich, Steinheim, Germany
precoated thin layer cellulose plates for phosphopeptidmaps	Merck
membrane for phosphopeptidmaps	Hybond-C extra, Amersham

2.2 Technical devices

Phosphoimager	FujiX200, BAS-reader
CCD Camera	Fuji, LAS2000
Phosphoimager and CCD camera	AIDA program, Raytest, Straubenhardt, Germany
MALDI-TOF-MS	BRUKER autoflex® MALDI with AUTOFLEX program
Speedvac for drying of 32P samples	Savant SVS100
Hunter thin layer electrophoresis system	C.B.S Scientific Co, Del Mar, Canada

2.3 Radiochemicals

^{32}P γ-ATP in (in vitro kinase assay)	Amersham
^{35}S Methionine and cysteine	Dupont, Amersham
^{32}P orthophosphate in aequous solution (in vivo labelling)	Amersham

2.4 Enzymes

Restriction endonucleases	MBI and NEB

Trypsin for the digestion of proteins for mass spectrometry and 2D-phosphopeptide mapping was purchased from Promega (porcine, sequencing grade modified trypsin).

2.5 DNA-and RNA modifying enzymes

RNase	Roth/Sigma
Shrimp alkaline phosphatase (SAP)	Roche
T4-DNA Ligase	Promega

DNA Polymerases

DNA polymerase from Thermus aquaticus (Taq)	prepared by W. Hädelt (Würzburg)
DNA polymerase from Pyrococcus furiosus (Pfu)	Promega

2.6 Kits

DNA preparation, different formats	Qiagen
Dual Luciferase Assay kit	Promega
Gel extraction and PCR purification kit	Qiagen
RNA extraction	TriFast, peqLab Biotechnology

2.7 Oligonucleotides

Oligonucleotides were synthesized and purchased by the companies Interactiva/ Thermohybaid or Sigma in HPLC quality. Primers used for PCR mutagenesis, sequencing and PCR reactions are listed in the appendix (see appendix A.2).

2.8 Plasmids

2.8.1 Expression vectors

pcDNA1 (Invitrogen)	mammalian expression vector for high level constitutive expression driven from a CMV promoter; the plasmid encodes the tRNA suppressor F gene (supF) which demands transformation in specific bacterial strains that harbor the P3 episome *(i.e.* MC1061/P3); sensitivity to tetracycline and ampicillin is generated by suppression of the amber mutations

pcDNA3 (Invitrogen)	mammalian expression vector for high-level constitutive expression driven from a CMV enhancer promoter
pEGFP (clontech)	mammalian expression vector with pUC backbone provides a high-copy-number origin of replication and an ampicillin resistance gene for propagation and selection in *E. coli*. The EGFP fusion protein is expressed from the lac promoter in *E. coli*. The vector carries a red-shifted variant of wild-type green fluorescent protein (GFP) which has been optimized for brighter fluorescence and higher expression in mammalian cells and which is optimized to mammalian codon usage.
pHIT60/gag-pol	expression vector for the gag-pol protein of foamy virus (obtained from D. Lindemann, Würzburg)
pczVSV-G wt	expression vector for the VSV-G envelope protein of foamy virus (obtained from D. Lindemann, Würzburg) (Yee et al., 1994a)
pGEX-KG	vector for the expression of recombinant protein in bacteria. The protein of interest is expressed as a C-terminal fusion with glutathione S transferase (GST)

2.8.2 Retroviral vectors

pczCFG EGIRT iAhCD8 α	retroviral vector containing a pcDNA3.1 backbone; the vector expresses the green fluorescent protein (GFP), an intervening ribosomal entry site (IRES) and the reverse transactivator (rtTA) under the control of a modified MuLV long terminal repeat (LTR). The expression of the gene of interest is driven by a tetracycline-inducible minimal CMV promoter (the vector was obtained from D. Lindemann, Würzburg)

pczCFG5 IEGN	retroviral vector for constitutive protein expression; the vector expresses the green fluorescent protein (GFP), an intervening ribosomal entry site (IRES) and the protein of interest under the control of a modified MuLV long terminal repeat (LTR). (the vector was obtained from D. Lindemann, Würzburg) (Knodel et al., 1999)
pczCFG5 IEYZ	the same backbone as pczCF5 IEGN, but the vector expresses the yellow fluorescent protein (YFP) (Kuss et al., 1999)

2.9 Bacterial strains

E. coli DH5 α	deoR, *end*A1, *gyrA*96, *hsdR*17 (r_k^-, m_k^+), *recA*1, *relA*1, *sup*E44, *thi*-1, *Δ(lacZYA-argF)*U169, *ϕ80*d*lac*ZΔM15, F - (Hanahan, 1983)
E. coli C600	*la*cY1, *le*uB6, *mc*rB+, *sup*E44, *thi*-1, *thr*-1, *tonA21*, F - (Young and Davis, 1983)
E. coli MC1061/P3	*ar*aD139, *ga*lK, *ga*lU, *hs*dR2(rk - mk +), *rp*sL, *th*i-1, *(ara-le*u)7696, *la*cX74, F-[P*3kan* r amber *amp* r amber *tet* r] (Invitrogen)
E. coli XL1-Blue	*recA*1, *endA*1, *gyrA9*6, *thi*-1, *hsdR17*(r_k^-, m_k^+), *supE4*4, *relA*1, *lac [F'*, *proA*B, *lac1*^{q} *Z*ΔM15, T*n10*(Tet^r)] (Stratagene)
BL21(DE3)	F-, *ompT, hsd*$S_β$, ($r_β^-$, $m_β^-$), *dcm, gal,* λ(DE3) tonA (Studier and Moffatt, 1986)

2.10 Cell lines

293	human embryonic kidney cell line transformed with adenovirus 5 DNA (CRL-1573, ATCC)
293T	human embryonic kidney cell line transformed with the large T-antigen from the SV40 virus (DuBridge et al., 1987)
COS7	African green monkey kidney fibroblast-like cell line (CRL-1651, ATCC)

MC3T3 mouse embryo/fetus calvaria fibroblasts established from the calvaria of an embryo/fetus C57BL/6 mouse; described to differentiate to osteoblasts and to produce collagen

C2C12 murine muscle myoblast line, described to differentiate to myotubes under serum starvation and to osteoblasts under BMP treatment (CRL-1772, ATCC). The C2C12 cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins.

2.11 Growth factors

BMP2 recombinant BMP2 was a generous gift from Prof. Sebald, Würzburg

SCF recombinant SCF was purchased from Amgen (Lars Rönnstrand, Malmö, Sweden)

TGFβ2 recombinant TGFß2 was purchased from R&D

2.12 Antibodies

The antibodies used are listed below. The appropriate buffers for blocking and antibody solution are indicated. To all the primary antibody solutions 0,05% Azid in dH_2O was added to avoid contaminations.

Antibody	Dilution for WB	Blocking	Conc. for IP	Type, origin	Epitope, recognition
first antibody					
BRIa 14/15 P510 (Gilboa et al., 2000)	1:100 in PBS	3% BSA in $PBST_{0,1\%}$	10µl/ml	polyclonal antiserum, rabbit	recognizes the juxtamembrane domain of BRIa (LEQDEAFIPVGESLKDLC)
BRII 59/60, P507 (Gilboa et al., 2000)	1:100 in PBS	3% BSA in $PBST_{0,1\%}$	10µl/ml	polyclonal antiserum, rabbit	recognizes the juxtamembrane domain of BRII (SMNMMEAAASEPSLDLDN)
BRII-LF 61/62, P508 (Gilboa et al., 2000)	1:100 in PBS	3% BSA in $PBST_{0,1\%}$	10µl/ml	polyclonal antiserum, rabbit	recognizes the c-terminal domain of BRII (CEGGTATTMVSKDIGMN)
BRII SMN from S. Souchelnytskyi (Uppsala) (Rosenzweig et al., 1995)	-	-	20µl/ml	polyclonal antiserum, rabbit	recognizes the amino acids 185-202 of hBRII (SMNMMEAAASEPSLDLDN)

Antibody	Dilution for WB	Blocking	Conc. for IP	Type, origin	Epitope, recognition
first antibody					
BRII-LF NRR (from S. Souchelnytskyi (Uppsala) (Rosenzweig et al., 1995)	-	-	20µl/ml	polyclonal antiserum, rabbit	recognizes the amino acids 534-556 of hBRII (NRRVPKIGPYPDYSSSSYIEDSI)
BRII pab-10462 (orbigen)	-	-	1µg/IP	affinity purified rabbit IgG	raised against the human BRII (CAFKDPYQQDLGIGES)
BRII, G-17 sc-5682	1:200 in $TBST_{0,1\%}$, 3%milk	3% milk in $TBST_{0,1\%}$	1µg/IP	affinity-purified goat polyclonal antibody	raised against the intracellular part of human BRII, recognises SF and LF
BRII, T-18 sc-5683	1:200 in $TBST_{0,1\%}$, 3%milk	3% milk in $TBST_{0,1\%}$	1µg/IP	affinity-purified goat polyclonal antibody	raised against the C-terminal part of human BRII, recognises BRII-LF
ERK (EET) from L. Rönnstrand, (Malmö) (Leevers and Marshall, 1992)	1:250 in $PBST_{0,1\%}$	5% milk in $TBST_{0,1\%}$	-	polyclonal antiserum, rabbit, affinity purified	raised against human ERK-2, (EETARFQPGYRS)
pERK p44/42 MAPK (cell signalling)	1:1000 in $PBST_{0,1\%}$, 0,5% milk	3% BSA in $TBST_{0,1\%}$	-	polyclonal, rabbit, affinity purified	raised against the phosphorylated form of human ERK (Thr 202/Tyr204),
HA (home made)	1:100 in PBS	3% BSA in $TBST_{0,1\%}$	10µl/ml	mouse monoclonal, prepared by T.Lutz (Universität Würzburg) according to standard protocols	12CA5 from the hemagglutinin protein YPYDVPDYA, hybridoma cell line from BAbCO
HA (Roche)	1:1000 in $TBST_{0,1\%}$	5% BSA in $TBST_{0,1\%}$	1µg/IP	mouse monoclonal, $IgG_{2b}\kappa$	12CA5 from the hemagglutinin protein YPYDVPDYA
HA (sc-805)	1:1000 in $TBST_{0,1\%}$, 1,5% BSA	1,5% BSA in $TBST_{0,1\%}$	1µg/IP	rabbit polyclonal, Y-11	12CA5 from the hemagglutinin protein YPYDVPDYA
c-kit-C1 from L. Rönnstrand, (Malmö) (Blume-Jensen et al., 1993)	1µg/ml in $PBST_{0,1\%}$	$PBST_{0,5\%}$	1µg/IP	polyclonal antiserum, rabbit, affinity purified	recognizes the C-terminal tail of human c-kit; SVGSTASSSQPLLVHDDV
c-kit, C-19 (sc-168-G)	-	-	1µg/IP	goat affinity purified polyclonal antibody	raised against the C-terminal domain of human c-kit
myc (home made)	1:100 in PBS	3% BSA in PBS	10µl/ml	mouse monoclonal, prepared by T.Lutz (Universität Würzburg) according to standard protocols	recognizes aa 410-419 of human c-myc (EQKLISEEDL)

Antibody	Dilution for WB	Blocking	Conc. for IP	Type, origin	Epitope, recognition
first antibody					
p-P38 Promega (V1211)	1:2000 in TBS, 0,1% BSA	1% BSA in TBS	-	polyclonal antiserum, rabbit, affinity purified	raised against the phosphorylated form of human p38 (pTGpY), the catalytic core T180 and Y182.
pSmad1/5 from P. ten Dijke, Amsterdam (Persson et al., 1998)	1:1000 in $TBST_{0,5\%}$	5% milk in $TBST_{0,5\%}$	-	polyclonal antiserum, rabbit	recognizes the C-terminally phosphorylated form of Smad1 (KKKNPISSVS) (Ser463,465) and crossreacts with pSmad3
Smad1/5 (QWL) from P. ten Dijke, Amsterdam (Persson et al., 1998)	1:1000 in $TBST_{0,5\%}$	5% milk in $TBST_{0,5\%}$	-	polyclonal antiserum, rabbit	recognizes a region in the MH2 domain of Smad1 (QWLDKLTQMGSPHNPISSVS)
Smad1/5 (upstate)	1:200 in $TBST_{0,1\%}$, 3% milk	5% milk in$TBST_{0,1\%}$	-	rabbit polyclonal IgG	recognizes aa147-258 of Smad1, cross reacts with Smad5
Smad1 (sc-7965)	1:200 in $TBST_{0,1\%}$, 3% milk	3% milk in$TBST_{0,1\%}$	-	mouse, monoclonal IgG_1	raised against amino acids 1-465 representing full length Smad1 of human origin, recognizes human, murine, rat and mink protein
pTyr, PY99, (sc-7020)	1:200 $PBST_{0,1\%,}$ 1%BSA	$PBST_{0,5\%}$	-	mouse monoclonal IgG_{2b}, affinity purified	raised against phosphotyrosine conjugated to keyhole limpet hemocyanin

Antibody	Dilution for WB	Blocking	Conc. for IP	Type, origin	Epitope, recognition
secondary antibody					
goat anti rabbit Dianova	1:20000 in $TBST_{0,1\%}$ or $TBST_{0,5\%}$	-	-		goat anti rabbit, recognizes the Fc fragment of IgG, HRP coupled
goat anti mouse Dianova	1:20000 in $TBST_{0,1\%}$ or $TBST_{0,5\%}$	-	-		goat anti mouse, recognizes the heavy and light chain of IgG, HRP coupled
donkey anti rabbit Amersham	1:20000 in $TBST_{0,1\%}$	-	-		recognizing whole IgGs, HRP conjugated
sheep anti mouse Amersham	1:20000 in $TBST_{0,1\%}$	-	-		recognizing whole IgGs, HRP conjugated
donkey anti goat sc-2020	1:2000 in $TBST_{0,1\%}$, 5%milk	-	-	donkey anti-goat IgG	recognizes the F_c parts of goat IgG, conjugated to HRP

3. Methods

3.1 Microbiological methods

All the microbiological methods were performed according to standard protocols.

3.2 Molecular biological methods

3.2.1 Cloning of BRII variants and truncation mutants

To increase the efficiency of expression of the BMP receptor constructs, they were subcloned in pcDNA3. For generation of stable cell lines using retroviral transduction the BRII truncation variants, BRII, BRIa and BRIb were cloned into pczCFG5 IEGN and/or pczCFG5 IEYN. The proceeding is depicted in the appendix (A.4.2-A.4.6).

3.2.2 Generation of GST-fusion proteins

Three different GST fusion proteins of the BRII were created. The whole cytoplasmic part of BRII-LF was subcloned in pGEX-4T-1, introducing an HIS_6-tag at the C-terminus, and 5` a site for EcoRI and 3´ one for XhoI. For the shorter constructs BRII was subcloned in pGEX-KG by introducing restriction sites for BamHI (5`) and EcoRI (3´) as well as a N-terminal FLAG epitope using PCR. The SF contains the whole kinase domain plus 30 amino acids (amino acids L175-R530), the tail construct includes the amino acids M501 to L1038. For the vector maps and the sequence of the primers used see appendix (A.2 and A.4.1).

3.2.3 Primer design for the introduction of point mutations

Primer should be between 25 and 45 bases in length with a melting temperature T_m of ≥ 75°C (optimal is 78°C). Longer primers work more efficient. The mutation should be located in the middle of the primer with flanking sequences of 10-15 bases on each side. The minimum GC content is 40%. Besides that, the primer should terminate in one or more G or C bases at the 3`end.

T_m= 81,5 + 0,41x (%GC)-(675/N)-%mismatch
N = primer length in bases
%GC and %mismatch are whole numbers

3.2.4 Introduction of mutations in c-kit and BRIa

Mutations were introduced using the QuikChange™ site-directed mutagenesis system (Stratagene Cloning Systems) according to the manufacturer´s instructions. In brief, one complementary oligonucleotide containing the mutation of interest was synthesized and used in the mutagenesis PCR reaction with 100ng wild type c-kit or BRIa cDNA as a template, respectively. The amplification reaction mixture was then incubated with DpnI (37°C, 1h), which specificially cleaves methylated DNA, to remove template-DNA. The non-digested DNA was transformed in competent *E.coli* from the appropriate strain (MC1061/P3 for pcDNA1, XL1-Gold ultracompetent cells for pcDNA3) from which the mutated DNA could be recovered. The oligonucleotides used are described in the appendix (A.2).
It is important to perform the control reaction in parallel to test the efficiency of the mutagenesis reaction. Here, a LacZ control plasmid, containing three Stop codons is mutated to a wildtype one. Restoration of active β-galactosidase requires three reversion events occuring on the same molecule. After transformation, the colonies can be screened for the β-galactosidase phenotype, where a blue colony indicates the production of a triple mutated plasmid.

Conditions for the elongation reaction:

95°C	1min	
95°C	1min	⎫
55°C	1min	⎬ 30 cycles
65°C	2min/kb plasmid length	⎭
65°C	8min	
4°C	cooling	

3.2.5 Introduction of mutations in BRII

To mutate amino acids in BRII a home made QuikChange™ site-directed mutagenesis system was used. Hereby, two complementary oligonucleotides containing the mutation of interest were synthesized and used in the mutagenesis PCR reaction with 50ng wild type BRII cDNA as a template. It is important to use only 125ng of each oligonucleotide. The PCR- reaction mixture was then incubated with DpnI, which specifically cleaves methylated DNA, to remove BRII-DNA. The non-digested DNA was transformed in competent *E.coli* from the MC1061/P3 strain, from which the mutated DNA could be recovered. The used oligonucleotides are described in the appendix (A.2).

Conditions for the PCR reaction:

95°C	30sec	
95°C	30sec	12-16 cycles
55°C	1min	
68°C	2min/kb plasmid length	
4°C	cooling	

12 cycles	point mutations
16 cycles	single amino acid changes
18 cycles	multiple amino acid deletions or insertions

3.2.6 RNA extraction and Real time PCR

3.2.6.1 RNA extraction

Eukaryotic cells were seeded on a 6-well dish ($1x10^5$/dish) and treated with different ligands. The supernatant was removed, the cells were washed with PBS and subsequently the cells were lysed in 1ml TriFast (1ml for less than $10cm^2$ surface, if too little, then contamination of the RNA with DNA might occur). The lysed cells can be frozen at –70°C for several month. To extract RNA directly 200µl chloroform were added, vortexed for 15sec (important) and incubated 3-10min at RT. After a centrifugation (5min, 14000rpm, RT) the upper, aqueous phase, containing DNA and RNA, was transferred to a new tube and mixed with 500µl isopropanol. After mixing and incubation for 5-15min at RT the samples were centrifuged (10min, 12000rpm, 4°C). The pellet was washed 2 times with 75% ethanol (4°C) and air dried. Finally it was dissolved in 30µl RNAse free dH_2O and the OD_{260} was determined.

3.2.6.2 Reverse transcription

2µg RNA were subjected to cDNA synthesis using 0,5µg Oligo dT primers and RNAse free water. This mixture was denatured for 5min at 70°C and transferred on ice. Here, 10mM dNTPs, 5x MMLV Buffer, MMLV polymerase and 1µl RNase Inhibitor were added. The mixture was heated for 15min at 40C and subsequently at 70°C for 15min. Afterwards the sample can be stored at –20°C.

3.2.6.3 Real time PCR

The resulting cDNA was diluted 1:4 with dH_2O and used as a template for the Real time PCR. The PCR was performed with Taq-Polymerase, Taq-buffer, 25mM $MgCl_2$, 10mM of each oligonucleotide and 0,25x SybrGreen as well as FITC (Fluorescin, Biorad) 1:200000. SybrGreen and FITC are diluted 1:2000 (*i.e.* 1:10 in DMSO and 1:200 in dH_2O).

The program used is:

95°C	3min	
95°C	30sec	⎫ 40 cycles
60°C	30sec	⎭
4°C	cooling	

During this time the increase in fluorescent product was measured by monitoring the amount of fluorescent product every cycle.

3.3 Protein Chemical Methods

3.3.1 Protein purification

The bacteria used for GST-preparations should not be older than one week, so DNA had to be freshly transformed in BL21(DE3) cells. 6ml overnight culture of one clone were transferred to 300ml LB medium containing 100µg/ml ampicillin. Bacteria were grown to a optical density of OD_{600} = 0,4-0,5. Subsequently protein expression was induced by adding IPTG to a final concentration of 0,1mM. To obtain soluble protein the cultures were transferred to 30°C (BRII-SF, BRII-LF Annegret = U2L) or 20°C (BRII-tail), respectively. After 1-2 hours cells were transferred to ice for 10min and harvested by centrifugation (6000rpm, 4°C, 15min). The pellet was resuspended in 10ml STE and sonicated with a tip-sonicator (4°C, 40%, 3x30 sec with spacing) followed by centrifugation (15000rpm, 4°C, 25min). 660µl of swollen Glutathione-Sepharose-Slurry was added and the mixture was incubated for 3h on an end-over-end at 4°C. After incubation the sepharose was washed 3x with ice-cold STE and 2x with 20mM ice-cold Tris pH 7,4. 1/10 of the pellet was subjected to SDS-PAGE to check the amount of purified protein. For a large scale GST-pulldown (2D gel) ¼ of the pellet was used, for small scale experiments (mini- or midi-gel) 1/6-1/8 was used. The dry aliquots were stored at –80°C and are stable for up to 4 weeks.

STE: 10mM Tris pH 8,0
150mM NaCl
1mM EDTA pH 8,0
10µg/ml aprotinin
1mM PMSF
1mM DTT

3.3.2 GST-pulldown with ^{35}S labelled proteins

Labelling was performed over night, without previous starvation, in methionine/cysteine free medium with 20µCi/ml ^{35}S-labelling mix. The proteins were extracted with lysis buffer containing 1% TritonX-100, 20mM Tris, pH 7,5, 150mM NaCl, 10µg/ml aprotinin and 1mM

PMSF. Approximately equal amounts of GST-fusion-proteins were added to the cell lysate (lysate from $6x10^6$ cells per pull down) and incubated 6-8h at 4°C. After 3 washes with ice-cold lysis buffer the samples were washed once with 20mM Tris 7,4 and resuspended in 2D sample buffer.

2D Sample buffer:	8M urea
	4% CHAPS
	0,5% DTT
	IPG buffer pH 3-10

3.3.3 2D gels of GST-pulldowns

Samples were subjected to isoelectric focusing (IEF) using IPGDry strips with immobilized pH gradient, pH range 3-10, 18cm, linear. Samples were loaded by the in-gel rehydration technique, with active loading during the last 3h. IEF was performed in an IPGphor on a gold plate following the next protocol: rehydration 10h; 50V, 3h; 1000V, 1h; 8000V, 10h or to 50000Vh. After IEF, strips were equilibrated in equilibration buffer with 1% DTT (Roche) for 10min, and then for 10min in the same buffer without DTT but with 4% iodacetamide (Sigma). Equilibrated strips were placed on top of 10% or 12% polyacrylamide gels, and fixed with 0.5% agarose in a concentrating buffer. The SDS-PAGE was performed in the Ettan Dalt-Six gel chamber, following the manufacturer's recommendations (constant power 50 W, run for 6h to 8h; Amersham Biosciences).

Equilibration buffer:	50mM Tris, pH8,8
	6M urea
	2% SDS
	30% glycerol
	1% DTT (Roche, 709000) or
	4% iodactetamide (Sigma, 16125)
Concentrating buffer:	62,5mM Tris, pH6,8
	0,1% SDS

3.3.4 Gel analysis

Images of silver stained gels and images of ^{35}S-labeled proteins in the same gels were compared. As ^{35}S-labeled proteins originate from C2C12 cells, this comparison allows distinguishing on silver stained gels between co-precipitated C2C12 proteins and non-labelled proteins which originate from bacteria, including GST fragments. C2C12 cellular

proteins which specifically co-precipitated with BRII constructs and not with GST alone were selected for identification.

3.3.5 Silverstaining of 2D gels

After running the gel was transferred to destain solution and incubated over night while shaking. The following day 2 hours of incubation in destain followed (changing once). Subsequently the gel was incubated in high quality water (ddH_2O) for 1h. After sensitisation for 30min 4 washes (20-30min each) in high quality water (ddH_2O) were performed. The gel was incubated in silvernitrate for 30-45min. Rinsing with water removed traces of silvernitrate as well as short rinsing with developer. Subsequently the gel was stained during incubation in developer. The reaction was stopped by changing to destain and the gel could be stored in destain. For drying, the gel was incubated in drying solution for 30min before transferring to the dryer.

Destain	7,5% acetic acid (Riedel-de Haen, Order number 33209) 25% methanol in dH_2O
Sensitizer	25% ethanol (Solveco, UN1170) 0,2% sodium thiosulphate (Sigma, S1648) 3,4% sodium acetate (Merck, 1.06268.1000) in dH_2O
silver nitrate	0,25% silver nitrate dissolved in dH_2O
developer	2,5% sodium carbonate (Merck, 1.06398.1000) 0,015% formaldehyde (Merck, 1.04003.2500) in dH_2O
drying solution	25% methanol 4% Glycerol in dH_2O

3.3.6 Destaining of silver for MALDI analysis

according to F. Gharahdaghi et al, Rockefeller Protein/DNA center (Gharahdaghi et al., 1999)

Solution A and B were mixed 1:1 and the dried and cut gel piece was covered with 100µl of the mixture. Incubation was performed while shaking. When the gel was destained the destain was removed with four washes with water. During that step the cellophane covering

the gel was removed as well. Thus, the gel piece was covered with 100µl ambic and incubated for 20min at room temperature. The ambic was replaced with neat acetonitrile during two washes with acetonitrile, then the sample was dried under nitrogen to evaporate the acetonitrile. Subsequently the digestion was started by adding trypsin in ambic and the sample was incubated over night at 30°C with slow shaking.

solution A	30mM potassium ferricyanide dissolve in dH_2O
solution B	100mM sodium thiosulphate (Sigma, S1648) dissolve in dH_2O
ambic	0,2M ammonium bicarbonate (BDH, 10302) dissolve in dH_2O make fresh before use
trypsin	20µg porcine trypsin, sequencing grade (Promega) dissolved in 0,2M ammonium bicarbonate store in small aliquots at –20°C

3.3.7 Desalting and concentration of the sample

(according to N. Kalkinnen, Helsinki and R. Annan, King of Prussia) (Erdjument-Bromage et al., 1998)

To desalt and concentrate the samples containing submicrogram protein quantities a small column (nano-column) is needed. This was constructed by crimping the tip of a gel-loader pipette from eppendorf with narrow tweezers (air stream should be able to pass). Methanol was placed into the tip and about 1µl of column material was added to the methanol. The slurry was packed into the tip by passing methanol through. After a wash with 30µl of 0,1% TFA the sample was loaded, washed with 0,1% TFA and eluted with TFA/acetonitrile/matrix directly by making very small drops on the metal MALDI-target. Peptides were analyzed by matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry on a Bruker Autoflex MALDI TOF MS. Peptide spectra were internally calibrated using known autolytic peptides from trypsin.

Column material	30% slurry of POROS R2 20 (15-40µm reversed phase material) in methanol
TFA/acetonitrile	0,1% TFA (Perkin Elmer, 001279) 60% acetonitrile
matrix	α-cyano-4-hydroxycinnamic acid

3.3.8 MALDI-TOF

To identify proteins, we performed searches in the NCBInr sequence database using Mascot (www.matrixscience.com) and ProFound (http://65.219.84.5/service/prowl/ProFound.html) searching engines. One miscut and partial oxidation of methionine were allowed. Probability of identification was evaluated according to the "P" value (Probability Based Mowse Score; for searches via Mascot), and according to probability value, "Z" value, and sequence coverage (for searches using ProFound). Correspondence of experimental values of pI and molecular mass of proteins to the theoretical values was also considered for identification.

For ProFound: the list with the identified masses is copied and pasted in the sheet. The settings are: complete iodacteamide, partial methylation, sometimes partial phosphorylation, 0 to 1 missed cuts, monoisotopic masses, error 0,0 to 0,2 (normal 0,1).

3.4 Cell Biological Methods

3.4.1 Collagen coating

For better adhesion of 293T cells during transfection and in vivo labelling the cells were seeded on collagen-coated dishes. Vitrogen100 (Cohesion, FXP-019) was used in a concentration of 25µg/ml in PBS. For a 10cm dish 4ml collagen were added and the dish was incubated for one hour up to over night in the incubator. Prior to use, the dish was washed once with dH_2O and once with PBS and the cells were seeded in growth medium.

3.4.2 Transfection with Lipofectamine ™

Lipofectamine™ transfection was performed as to manufacturer's instructions (Life Technologies). Briefly, cells were seeded on 6-well plates at a density of $1x10^5$ cells per well, so that on the day of transfection, cell confluence reached ~60%. A total of 3µg DNA was mixed with medium containing 0,2% serum to achieve a volume of 100µl. In parallel, 10µl of Lipofectamine™ were added to 100µl of medium without supplements in a separate tube. The two solutions were combined, gently mixed and incubated for 30min at RT. Meanwhile, the cells were washed once with plain medium. 800µl of plain medium were added to the transfection mixture before it was distributed on the cells. Following incubation for 5h in the incubator, the transfection solution was replaced by 2-3ml of normal growth medium and 48h post transfection, cells could be used for further experiments.

3.4.3 Transfection using Calcium-phosphate coprecipitation

During this study, the calcium-phosphate coprecipitation method was used to transfect 293T cells. The day before transfection, $2x10^6$ cells were seeded on a 6cm plate. Prior to transfection, the medium was replaced by 4ml of fresh, preequilibrated growth medium and

the cells were returned to the incubator. 15µg of DNA were mixed with dH_2O to reach a final volume of 438µl and 62µl of 2M $CaCl_2$ were added. After vortexing, 500µl of 2x HBS were added by bubbling and the solution was immediately distributed on the cells drop by drop. Following incubation for 7-10h at 37°C, the transfection medium was replaced by fresh, preequilibrated growth medium and cells were returned to the incubator. 48h post transfection, cells could be used for further experiments.

2x HBS	50mM HEPES
	10mM KCl
	12mM α -D-Glucose
	280mM NaCl
	1,5mM Na_2HPO_4
	dissolve in dH_2O; adjust pH 7,0, 7,05 and 7,10; autoclave and store in aliquots at –20°C
	test the aliquots for transfection efficiency and use the best
2M $CaCl_2$	dissolve $CaCl_2$ in dH_2O; autoclave and store in aliquots at –20°C

3.4.4 Transfection using DEAE-dextran

The DEAE-dextran method is the method of choice to efficiently transfect COS cells.
Transfection was performed in 10cm dishes and the confluence of the cells should be in the range of 50%-70%. Thus, $1x10^6$ cells were seeded the day before transfection. 10-20µg of DNA were mixed with transfection buffer to reach a final volume of 1140µl and 60µl of DEAE-dextran (10mg/ml) were added. After washing three times with prewarmed transfection buffer, the cells were incubated with the DNA/DEAE-dextran mixture for 30min at 37°C while shaking in 5-10 min interval. Subsequently, 14ml Chloroquine-NS medium were added to the cells without aspirating the transfection mixture and the cells were returned to the incubator for 2,5-3h. The DMSO-shock is performed afterwards to facilitate the DNA uptake.
Therefore, the transfection medium was aspirated and cells were treated for exactly 2,5min with 3ml of growth medium containing 10% DMSO. The cells were washed once and then cultivated in normal growth medium until they could be used for further experiments (approx. 48h post transfection).

Transfection buffer	250mM NaCl
	2,3mM NaH_2PO4
	7,7mM Na_2HPO4
	dissolve in dH_2O; adjust pH 7,5; autoclave

DEAE-dextran	10mg/ml in dH_2O; sterilize by filtration
Chloroquine (40mM)	20mg/ml in dH_2O; sterilize by filtration
Chloroquine-NS medium:	10% NU-Serum 80µM Chloroquine in DMEM medium without supplements

3.4.5 Transfection with Electroporation

Another relatively harsh method to transfect cells is electroporation. A high number of cells is mixed with DNA and treated with an electric pulse at a certain voltage and a certain resistance.

MC3T3 or C2C12 cells, respectively were trypsinised, washed two times with growth medium and 3 times with plain medium and finally brought to a concentration of $6x10^6$ cells/ml. 350µl of the cell suspension were mixed with DNA in 50µl dH_2O (32µg DNA for reportergene assays and 12µg DNA for western blotting of the lysates) and quickly the pulse was performed. 5ml of growth medium were added and the cells were seeded on 2 wells of a 6-well dish.

MC3T3:	1800µF, 210V
C2C12:	1800µF, 240V

3.4.6 Transfection using PEI

Lipofectamine™ transfection was described above. Polyethylenimmine (Sigma) is a component of this transfection reagent and is appropriate for transfection of eucaryotic cells. C2C12, 293T or COS7 cells were seeded so that on the day of transfection, cell confluence reached ~60%. The DNA was mixed with medium containing 0,2% serum. In parallel, PEI was added to medium without supplements, in a separate tube. The two solutions were combined, gently mixed and incubated for 30min at RT. Meanwhile, the cells were washed once with plain medium. Plain medium was added to the transfection mixture before it was distributed on the cells. Following incubation for 5h in the incubator, the transfection solution was replaced by normal growth medium and 24 to 48h post transfection, cells could be used for further experiments.

PEI Stock solution	0,2µg/µl-2µg/µl in dH_2O, stored at 4°C in the dark, should be stable for up to one year

size of the dish	cells/dish	Amount of DNA	Amount of PEI	premix	plain medium added
COS7 cells					
10cm dish	$1x10^6$	10µg	10µg	500µl each solution	4ml
293T cells					
10cm dish	$2x10^6$	10µg	10µg	500µl each solution	1ml
6cm dish	$1x10^6$	5µg	5µg	500µl each solution	-
C2C12 cells					
10cm dish	$6x10^5$	10µg	5µg	500µl each solution	4ml
6 well plate	$1x10^5$	2,3µg	2µg	100µl each solution	0,8ml

3.4.7 Viral infection

To produce high titer replication deficient pseudotyped virus particles, the packaging cell line 293T was used to produce and to assemble virus particles. The calcium-phosphate coprecipitation method was used to transfect 293T cells with 5µg gag-pol (pHIT 60) DNA, 5µg envelope (eco, (pHIT123)) DNA and 5µg viral DNA (pczCFG5 IEGN). One day post transfection, the supernatant of the 293T cells was replaced by fresh media containing 10mM sodium-butyrate for 7-10h to increase the infection. Besides that, $4x10^4$ C2C12 cells per well are plated on a 6 well plate. The next day the medium was replaced by fresh media. Virus containing supernatant (3ml) was filtered through a 0,45µm filter and mixed with 24µg polybrene. The medium of the target cells was aspirated and the viral supernatant was added to the cells. Following incubation for 4-6h at 37°C, the viral supernatant was replaced by normal growth medium. To obtain a higher infection efficiency, the infection procedure can be repeated the next day. The infection rate should be monitored via GFP. The expression rate of the protein can be tested by western blotting.

Sodium-butyrate — 500mM n-Butyric acid sodium salt (= 50x stock solution)

Polybrene — 800µg/ml Hexadimethrine bromide (= 100x stock solution) dissolve in PBS; sterilize by filtration

3.4.8 Selection with G418

Cells, which have integrated the viral DNA into their genome are hereby resistant to neomycin. They can be selected by treatment with G418, a sodium salt which can be dissolved in PBS or in DMEM without any other supplements. The working concentration is

between 0,1mg/ml and 2mg/ml. The optimal concentration was determined performing a kill curve. The cells should be maintained in selection medium for at least two weeks or constantly.

G418	10mg/ml stock solution in DMEM adjust pH with NaOH sterile filter and aliquot store at 4°C
MC3T3	0,5mg/ml
C2C12	0,6mg/ml

3.4.9 β -Galactosidase assay

The β -galactosidase assay provides a tool to verify transfection efficiency. One dish of cells was transfected with a plasmid carrying the β-galactosidase construct. 48h post transfection, cells were fixed by addition of 200µl glutaraldehyde (25%) in 5,5ml PBS for 15min at 37°C. Subsequently, the fixation solution was removed and a mixture of 300µl 2% X-Gal and 2,7ml X-Gal incubation buffer was added to the cells. β-galactosidase is an enzyme that catalyzes hydrolysis of lactose. X-Gal, a colourless lactose-analogue, can be hydrolyzed by β-galactosidase as well, resulting in a blue staining of the β-galactosidase expressing cells. Transfection efficiency can thus be determined by estimating or counting the amount of blue stained cells.

Fixation solution:	0,9% Glutaraldehyde in PBS
2% X-gal:	5-bromo-4-chloro-3-indolyl-ß-D-galactosid dissolved in dimethylformamid store in aliquots at –20°C
X-gal staining buffer:	150mM NaCl 10mM NaH_2PO_4 pH 7,5 1mM $MgCl_2$ 3,3mM $K_3Fe(CN)_6$ 3,3mM $K_4Fe(CN)_6$ store at 4°C

3.4.10 Cell lysis

Depending on the following assay, different lysis buffers can be used for cell lysis. The use of the different buffers is mentioned in the description of the respective methods.
To produce lysates from mammalian cells, cells were washed twice with cold PBS. An adequate amount of lysis buffer containing protease inhibitors (Complete™, Roche), PMSF and phosphatase inhibitors, if necessary, was added to the cells which were subsequently scraped off using a cell scraper and transferred to an Eppendorf tube. Lysis was performed by incubation on ice for 10min. Cell debris was sedimented by centrifugation for 10min at 14000rpm and 4°C. The supernatant was transferred to a fresh tube and used for further analysis.

0,5% Tx-Buffer used for immunoprecipitation and binding and cross-linking assays

0,5% Triton lysis buffer	0,5% Triton
	1mM EDTA
	dissolved in PBS

TNE-buffer used for Smad Western-Blot

TNE lysis buffer	20mM Tris (pH7,4)
	150mM NaCl
	1% TritonX-100
	1mM EDTA

1% Tx-Buffer used for immunoprecipitation assays

1% Triton lysis buffer	20mM Tris, pH7,4
	150mM NaCl
	1% TritonX-100

RIPA lysisbuffer used for in vivo phosphorylation assays

RIPA-lysisbuffer	50mM Tris, pH 7,4
	150mM $NaCl_2$
	0,5% Sodium-desoxycholate
	0,1% SDS
	2mM EDTA
	1mM EGTA
	1% NP-40
	1mM PMSF

Protease inhibitors:

Protease Inhibitor cocktail (Roche)	dissolve 1 tablet in 2ml dH_2O (25x)
	store at –20°C

Trazylol (Bayer)	aprotinin (10mg/ml) (= 100x stock solution) store at 4°C
PMSF	100mM Phenylmethylsulfonyl fluoride dissolved in isopropanol (= 100x stock solution), store at –20°C

Phosphatase inhibitors:

NaF, Stock 1M in H_2O	working concentration 50mM
Na_3VO_4, Stock 100mM	working concentration 1mM
$Na_2P_2O_7$, Stock 0,2M in H_2O	working concentration 10mM

Preparation of Na_3VO_4, stock 100mM

184mg sodium orthovanadate were lysed in 9ml dH_2O in a 15ml Falcon tube. After addition of 25µl 5M HCl the colourless solution turned yellow. During boiling at 90°C the yellow colour disappeared and the solution was cooled on ice water. The addition of 25µl 5M HCl and the boiling/cooling cycle were repeated until the yellow colour was stable after 5min of boiling (approx. 200-300µl of 5M HCl are required). The volume was adjusted to 10ml and the solution can be stored in aliquots at –20°C and is stable for a few months.

3.4.11 Protein quantification using the BCA assay (Redinbaugh-method)

(Redinbaugh and Turley, 1986)

Cellular protein concentration was determined by the bicinchonic acid (BCA) method, calibrated against BSA, since this assay is compatible with detergent and linear for protein concentrations between 20µg/ml and 2000µg/ml. The determination was performed in doublets. The cell lysates of interest were diluted in dH_2O (1:10- 1:25; 50µl total volume) and 20µl of the dilution were added per well of a 96 well plate. BSA standards were used to create a standard curve: by pipetting 20µl of each standard per well. (Standards: 25µg/ml, 50µg/ml, 75µg/ml, 100mg/ml, 150mg/ml, 200µg/ml, 250µg/ml). Solution A and B were mixed prior to use and 200µl/well were added. After an incubation step (30-45 min at 60°C, cover the plate with a lid to avoid evaporation), the assay can be measured with a reference filter at 630nm and the test filter at 550nm. The protein concentrations are calculated referring to the standards.

Solution A:	1,35%$NaHCO_3$
	0,58% NaOH
	1% Bichinonic acid (Pierce)
	0,57% K-Na tartrate
	store in 50ml aliquots at –20°C

Solution B: 2,3% $CuSO_4*5H_20$

store at RT

3.4.12 Immunoprecipitation and westernblotting

To concentrate specific proteins from total cell lysates, immunoprecipitations were performed. Here, the specific proteins were isolated by incubating the lysate with antibodies specific for the respective protein and Protein A Sepharose (50µl/IP) to bind and to precipitate the antibodies. The Protein A specifically recognizes and binds to F_c parts of the antibodies. The immunoprecipitation was carried out at 4°C under rotation for at least 3h or over night. Sedimentation of immune-complexes was performed by a short centrifugation step. The sepharose beads were washed three times with ice-cold lysis buffer. Then, SDS sample buffer was added to remove the proteins form the sepharose beads. The sample can be stored at –20°C or directly boiled for 3min and subsequently the supernatant (with or without the beads) can be loaded on a SDS PAGE.

To detect single proteins out of a total cell lysate or an immunoprecipitate, proteins were separated by SDS-PAGE and subsequently transferred to a nitrocellulose membrane where they can be detected by using specific antibodies and visualized by enhanced chemoluminescence (ECL). To remove residual antibodies from the membrane for subsequent redetection with other primary antibodies, the membrane was treated with stripping buffer and incubated for 20-30 min at 56°C. The buffer was removed by extensive washes with PBS before new blocking and antibody incubation could follow.

Stripping buffer1: 5mM NaH_2PO_4
1% SDS
in dH_2O
add freshly before use 0,000135% β-MeOH

low stringency

Phosphate buffer: 0,2M NaH_2PO_4

Stripping buffer2: 16mM Tris, pH8,5
2% SDS
0,007% β-MeOH
in dH_2O

high stringency

3.4.13 Proliferation assay

Using the CellTiter 96® AQueous one solution (Promega), the amount of living cells is measured by adding a tetrazolium compound (MTS) and an electron coupling reagent PES. Proliferating cells reduce the MTS compound to a coloured formazan product whose absorbance can be easily recorded.

$1x10^3$ C2C12 or MC3T3 cells were plated on a 96-well dish and grown over night. 24h later cells were starved in medium containing 0,5% FCS for 5h and stimulated with different concentrations of BMP2 or 0,1µg/ml SCF or both. As a control stimulation with 4nM TGFβ2 was performed. 24h after stimulation 20µl of CellTiter 96® AQueous one solution (Promega) were added to the living cells and the plate was incubated for 1-4h at 37°C. The absorbance was recorded at 490nm with a 96 well plate reader.

3.4.14 Alkaline Phosphatase measurement

For quantitative analysis of alkaline phosphatase activity, cells were washed and extracted with a lysis buffer as described (Asahina et al., 1993), (Nishitoh et al., 1996). Alkaline phosphatase activity was determined using p-nitrophenyl phosphate (Sigma) as a substrate.

$1,5x10^4$ C2C12 or MC3T3 cells were plated on a 96-well dish and grown over night. 24h later cells were starved in medium containing 0,5% FCS for 5h and stimulated with 50nM BMP2 or 0,1µg/ml SCF or both. 72h after stimulation cells were rinsed with PBS, lysed with 100µl ALP1 (1h rocking plate, RT) and the enzymatic reaction was started by adding 100µl of ALP2. Enzymatic activity was measured in an ELISA reader with test filter 405nm and reference filter at 550nm.

ALP1:	0,1M Glycine pH 9,6
	1%NP40
	1mM $MgCl_2$
	1mM $ZnCl_2$
ALP2:	0,1M Glycine pH 9,6
	1mM $MgCl_2$
	1mM $ZnCl_2$
	20mg/ml p-nitrophenyl phosphate (pNPP)
pNPP:	1 tablet (20mg), dissolved in
	0,1M Glycine pH 10,4
	1mM $MgCl_2$
	1mM $ZnCl_2$

3.4.15 Alkaline Phosphatase staining

$4{,}5x10^5$ C2C12 or MC3T3 cells were plated on a 6-well dish. The next day, cells were starved in medium containing 0,5% FCS for 5 hours and stimulated with 50nM BMP2 or 0,1µg/ml SCF or both, respectively. 72h after stimulation histochemical analysis of alkaline phosphatase activity was performed as described (Katagiri et al., 1994). Briefly, cells were fixed for 10min with 3,7% formaldehyde at room temperature. After washing with PBS, cells were incubated for 20-30min with staining solution at room temperature and stopped with addition of 87% glycerol, followed by histochemical analysis using phase-contrast microscopy. The percentage of blue cells per dish can be quantified by using the Metamorph offline software from Visitron

3,7% formaldehyde:	3,7% formaldehyde in PBS
Staining solution:	0,1mg/ml Naphtol AS-MX phosphate 0,5% N,N-dimethylformamid 2mM $MgCl_2$ 0,6mg/ml fast blue BB salt in 0,1M Tris-HCl, pH 8,5 mix directly before use
Naphtol AS-MX phosphate	50mg/ml in EtOH, store at –20°C
fast blue BB salt	10mg/ml in dH_2O, store at –20°C

3.4.16 Reportergene Assay

Reporter gene assays are a rapid and convenient method to study transcriptional activity of specific promoter elements, induced by external stimuli. The responsive promoter element of choice is cloned in front of a luciferase gene (here: firefly luciferase, from *Photinus pyralis*) and transfected into responsive cells. The "dual luciferase assay system" (Promega) additionally includes an internal control which is represented by a constitutively active luciferase gene (here: renilla luciferase, from *Renilla reniformis*, sea pansy) which is cotransfected. So, the renilla luciferase serves as a control for transfection efficiency and can be used to normalize the firefly signal, which is based on external signals (i.e. ligand).
In this study, different reporter gene constructs were used (see Appendix A3). The pRLTK construct from Promega encodes the *renilla* luciferase gene under the control of a thymidine kinase promotor. The pSBE reporter consists of four Smad binding elements that are cloned in front of an adenoviral minimal promoter and the luciferase gene (Jonk et al., 1998). In $p(GCCG)_{12}$-luc, three repeats of the GCCGCCG element are cloned in front of the luciferase reporter (Ishida et al., 2000). Whereas pSBE-luc serves as a readout for TGF-β as well as for

BMP signalling, the p$(GCCG)_{12}$-luc and pBRE-luc constructs respond specifically to BMP-mediated signals.

Cells were transfected with Lipofectamine™ or electroporated with the reporter construct, the renilla pRLTK and with one or two DNAs for the genes of interest. The total amount of DNA was kept constant by addition of empty vector (pcDNA3). For Lipofectamine™ transfection 1µg of reporter DNA, 0,3µg renilla DNA and 2µg additional DNA were used. For electroporation 9µg of reporter, 2µg of renilla DNA and 5µg of DNA of interest were taken.

The next day, cells were starved in medium containing 0,5% serum for 5h, followed by stimulation with either 20nM BMP2 or 0,1µg/ml SCF for additional 24h. The "Dual Luciferase Assay System" from Promega was used for the assay and the measurements which were performed using the FB12 Luminometer (Berthold). Luciferase activity was recorded in relative light units (RLU).

3.4.17 Copatching

At day one cells were plated on chamber slides $2x10^4$ cells per chamber. After attaching over night, they were transfected with Lipofectamine (400µl total volume, 4µl Lipo in 40µl DMEM plus 1µg DNA in 40µl DMEM with 0,2% FCS). The next day, cells were washed 2x with ice-cold HANKS with 20mM Hepes and 1% BSA and incubated in that solution at 4°C for 2h. After that the first antibody, in this case anti-HA (Roche), 1:100 in HANKS/Hepes/BSA (200µl/Chamber), was incubated for 1h at 4°C and, after three washes with HANKS/Hepes/BSA the secondary Cy3-mouse 1:200 in with HANKS/Hepes/BSA, 1h at 4°C as well. Subsequently the cells were washed three times with HANKS/Hepes/BSA and fixed for 5min at –20°C with 200µl ice-cold methanol and for 2min at -20°C with ice-cold acetone.

As control, the nucleoli were counterstained. For this, Hoechst was diluted 1:1000 (1µg/ml) in PBS and incubated for 2min at RT. After washing with PBS the immunofluorescence was covered with Kaiser`s glycerin gelatine.

3.4.18 In vitro kinase assay

After immunoprecipitation the sepharose beads were washed 1x with kinase buffer and incubated with 10µCi (γ-^{32}P) ATP for 30min at RT. Residual radioactivity was removed by washing the beads 4x with kinase wash buffer, the bound proteins were eluted form the beads with 2x sample buffer and subjected to SDS-PAGE. The dried gel was exposed to a phospho imager screen.

Kinase buffer	20mM Hepes, pH 7,4
	10mM $MgCl_2$
	2mM $MnCl_2$
	1mM Na_3VO_4
	2mM NaF

kinase wash buffer
20mM Tris, pH 7,4
150mM NaCl
20mM EDTA
0,5% TritonX-100
1mM Na_3VO_4

3.4.19 ^{35}S-methionine/cysteine incorporation for detection of expressed proteins

48h post transfection COS7 cells were washed 3x with DMEM without methionine and cysteine and starved for 90min. Subsequently, the cells were labelled with 0,7mCi Labelling-Mix with 0,2mM oxidised glutathion for 2h (with or without ligand). After lysis of the cells using 0,5% Triton-lysisbuffer preclear followed by immunoprecipitation was performed. The samples were subjected to SDS-Page and the dried gel was exposed on a phospho-imager screen.

0,2mM oxidised glutathion
0,2mM oxidised glutathion in dH_2O
make fresh before use

3.4.20 In vivo Phosphorylation

For in vivo phosphorylation 24h post transfection one 10cm dish with cells (transfected COS7 or transfected 293T or non-transfected C2C12) was washed 4 times with phosphate-free medium without FCS and incubated for 2h. Labelling took place with 1mCi/ml orthophosphate in 4ml phosphate-free medium for 4h. After 3h of incubation ligand was added. After aspiration of the medium the cells were lysed while shaking for 10min with 1ml RIPA-lysisbuffer (COS7, C2C12) or Triton-lysisbuffer for 293T cells, respectively. Preclear with sepharose for 30min at RT is performed and respective receptors are immunoprecipitated with specific antibodies as described above (2.12).

RIPA-lysisbuffer
50mM Tris, pH 7,4
150mM $NaCl_2$
0,5% Sodium-desoxycholate
0,1% SDS
2mM EDTA
1mM EGTA
1% NP-40
1mM PMSF
1:100 Trazylol (= aprotinin) (Bayer AG)
50mM NaF
1mM Na_3VO_4

Triton-lysisbuffer	20mM Tris, pH 7,4
	150mM $NaCl_2$
	1% TritonX-100
	1:100 Trazylol (= aprotinin) (Bayer AG)
	1mM PMSF
	1mM Na_3VO_4

3.4.21 Phosphopeptide maps

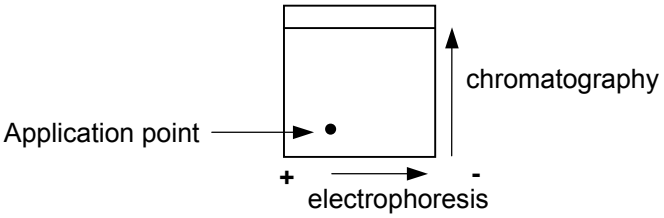
SDS-PAGE was performed using a big gel (approx. 40cm x 25cm) (ON, 15mA) and the proteins were subsequently transferred to a Hybond C-extra nitrocellulose membrane (Amersham) using a big westernblot chamber (Biorad) and performing the transfer in prechilled buffer while stirring in the cold room (400mA, 4h). The filter was cut on the edges and put on top of a wet whatman paper in a sealed plastic pouch. After exposure on the phospho imager the protein band of interest was excised (do not let the membrane dry) and soaked in 200µl PVP for 30 min at 37°C. After 3 washes with 500µl water 200µl ambic with 1µg modified trypsin was added to the filter piece. After vortexing the filter was incubated over night at 37°C.

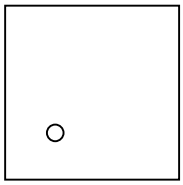
The supernatant and one subsequent wash with 200µl ambic were transferred to a new tube. After freezing at –80°C the sample was dried in the speedvac connected to an oil pump. The unvisible pellet was dissolved in 50µl performic acid and oxidised for less than 1h on ice. The sample was diluted with 500µl water, frozen (!) and lyophilised over night. The pellet should not be visible. Brown pellets indicate too much oxidation. 50µl ambic was added to re-dissolve the peptides and 1µg trypsin was added followed by a digest over night at 37°C. 140µl of pH1,9 buffer was added, the sample was vortexed and spun down. The supernatant was transferred carefully to a new tube avoiding all insoluble material. After freezing, the sample was dried in the speed-vac. If there was no white pellet visible the sample was dissolved in 10µl pH1,9 buffer and applied in very, very small portions to a cellulose thinlayer chromatography plate (use a fan without heating); the application point for BRII-LF is 6,5cm from the left and 3cm from the bottom. In case of a white pellet it was re-dissolved in 100-200µl pH1,9 buffer, frozen and dried again. This step has to be repeated until there are no white ambic traces visible.

If the thinlayer plate was dried, high voltage electrophoresis was performed (27min, 2000V) using pH1,9 buffer to soak the electrodes, to wetten the plate and to concentrate the sample with a specially prepared Whatman paper (see picture).

After electrophoresis, the very well dried plates were marked with a sharp knife 3cm from the top and ascending chromatography over night (for exactly 12h!) was performed in isobutyric acid buffer.

The maps were dried extensively in the fume hood, labelled with radioactive ink and exposed on the phospho-imager screen stored in a led box to minimise background radiation (exposition 3-7d).





Whatman paper with a hole to wetten the plate

PVP	0,5% Polyvinylpyrrolidon K30 (Aldrich, 85656-8) Dissolved in 0,6% acetic acid Store at RT
Ambic	50mM ammoniumbicarbonate (BDH, 10302) dissolved in dH_2O make fresh before use
Trypsine	20µg is dissolved in 200µl 1mM HCl store in aliquots at –20°C
Performic acid	90% formic acid (1.00264.2500) 10% 30% H_2O_2 incubate 1h at room temperature before use
pH1,9 buffer	50ml formic acid 156ml acetic acid 1794ml dH_2O make fresh before use
Isobutyric acid buffer	1250ml isobutyric acid 38ml n-butanol 96ml pyridine (Merck, 1.09728.0500) 58 ml acetic acid 558ml dH_2O
thin layer chromatography plate	Merck (1.05716)

(Boyle et al., 1991)

3.4.22 Analysis of the 2D phosphopeptide spots

3.4.22.1 Extraction of the 2D phosphopeptide spots

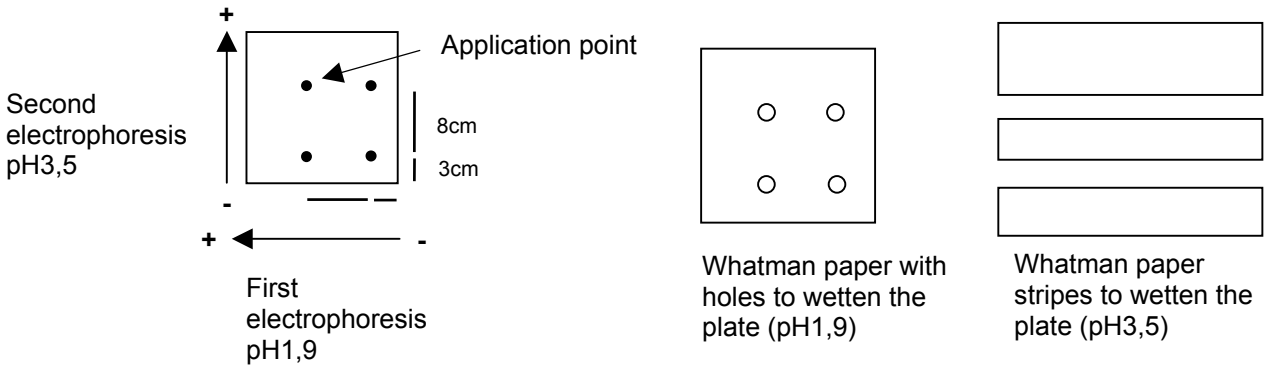
Spots of interest were scraped off the plate and the peptides were extracted by adding 2x vol of the powder of pH1,9 buffer, followed by vortexing and shaking for 30min. After spinning down (10min, 14000 rpm, RT), the supernatant was transferred to a new tube, carefully avoiding any powder. After a second extraction the liquid was dried in the speed-vac.

3.4.22.2 Edman degradation

To determine the position of the radioactive amino acids, the peptide was subjected to Edman degradation (Christer Wernstedt, LICR, Uppsala) and the single fractions, containing one amino acid were collected and dried (20 per peptide). The samples were dissolved in 10µl 100% TFA (Perkin Elmer) and spotted on a cellulose thin layer chromatography plate (not on glas, but on plastic background). Afterwards the plate was exposed on a phospho-imager screen (>5 days).

3.4.22.3 Phosphoamino acid analysis

200µl 7,5N HCl were added to the dried phosphopeptide and incubated in a metal block for 1h at 100°C. After centrifugation for 2 min at 140000 rpm the supernatant was transferred to a new tube and lyophilised. With 5µl of a standard of phosphorylated amino acids the pellet was resuspended and after centrifugation (5min, 14000 rpm, RT) the samples could be spotted in very tiny portions on a cellulose thin layer chromatography plate, using a fan to enhance evaporation.



The electrophoresis was performed after wettening the plate with pH1,9 buffer (2000V, 27min). The plate had to be dried very well before it was wettened again with pH3,5 buffer. The plate was rotated 90° counter clock wise and electrophoresis was performed at 1800V for 20min with pH3,5 buffer. The plate was dried at 65°C in the oven and sprayed with 0,25% ninhydrin in acetone (w/v) (BDH) to make the standards visible through incubation at 65°

(pattern like a cat foot). The plate was marked with radioactive ink and exposed to a phospho-imager screen (>5 days). After scanning the plate the amino acids could be identified via overlay with the standards.

7,5N HCl	2 parts 37% HCl 1 part dH_2O
Phosphoamino acid standard	1mg/ml phospho– serine (Sigma) 1mg/ml phospho- threonine (Sigma) 1mg/ml phospho- tyrosine (Sigma) dissolved in dH_2O
pH3,5 buffer	100ml acetic acid 10ml pyridine (Merck) 1890ml dH_2O

Some of the methods described here are nicely explained in Bart´s cookbook http://pingu.salk.edu/~sefton/Hyper_protocols/Tryptic.html and in Chapter 11 of Methods in Enzymology, v. 201, 1991, Academic Press.

4. Results

4.1 BRII - receptor oligomerization studies (Nohe et al., 2002), (Hassel et al., 2003)

4.1.1 BMP receptor oligomerization

Receptor mediated signal transduction is initiated by ligand-induced homo- or hetero oligomerization of the receptors (Schlessinger, 2000), (Vivien et al., 1995). Many tyrosine kinase receptors are constitutively active kinases. Ligand addition is inducing dimerization of the receptors and the constitutively active kinases can transphosphorylate and activate each other (Schlessinger, 2000). The members of the TGF-β superfamily transmit their signal via receptors which have intracellular serine/threonine kinase activity. Whereas TGF-β itself binds with high affinity to the TGF-β type II receptor (TβRII), and the low affinity receptor, type I (TβRI) is recruited, the binding of BMP2 to its receptors occurs in reverse order (Knaus and Sebald, 2001), (Kirsch et al., 2000b), (Vivien et al., 1995).

Several investigations suggest both heteromeric and homomeric complex formation among the TGF-β receptors (Wrana et al., 1992), (Moustakas et al., 1993), (Chen and Derynck, 1994), (Wells et al., 1999). In the absence of ligand, TβRI and TβRII were shown to form homodimers (Gilboa et al., 1998). A low degree of heterooligomers were detected which was enhanced upon ligand addition (Wells et al., 1999). It has been shown that a dimer of TβRI as well as a dimer of TβRII is required for efficient signal transduction (Luo and Lodish, 1997), (Vivien and Wrana, 1995), (Anders and Leof, 1996), (Okadome et al., 1994).

Our lab has shown that the BMP receptor oligomerization pattern shows a higher degree of flexibility, compared to the TGF-β system (Gilboa et al., 2000). Preformed homo- and heteromeric complexes of the receptors (BRII, BRIa, BRIb) exist at the cell surface. Ligand addition increases the percentage of BRI homooligomers, as well as heterooligomers of BRII and the BRI. Whereas the latter could be observed for the TGF-β receptors as well, but in a lower percentage, the first observation is specific for the BMP receptors. In the absence of ligand BRI and BRII monomers as well as homomers and heterodimers can be observed. When ligand is added, some monomers are recruited in the homodimers or in the heteromeric signalling complex (Fig 4.1).

However, it is still unclear how many BMP receptor molecules are forming the heterooligomeric complex.

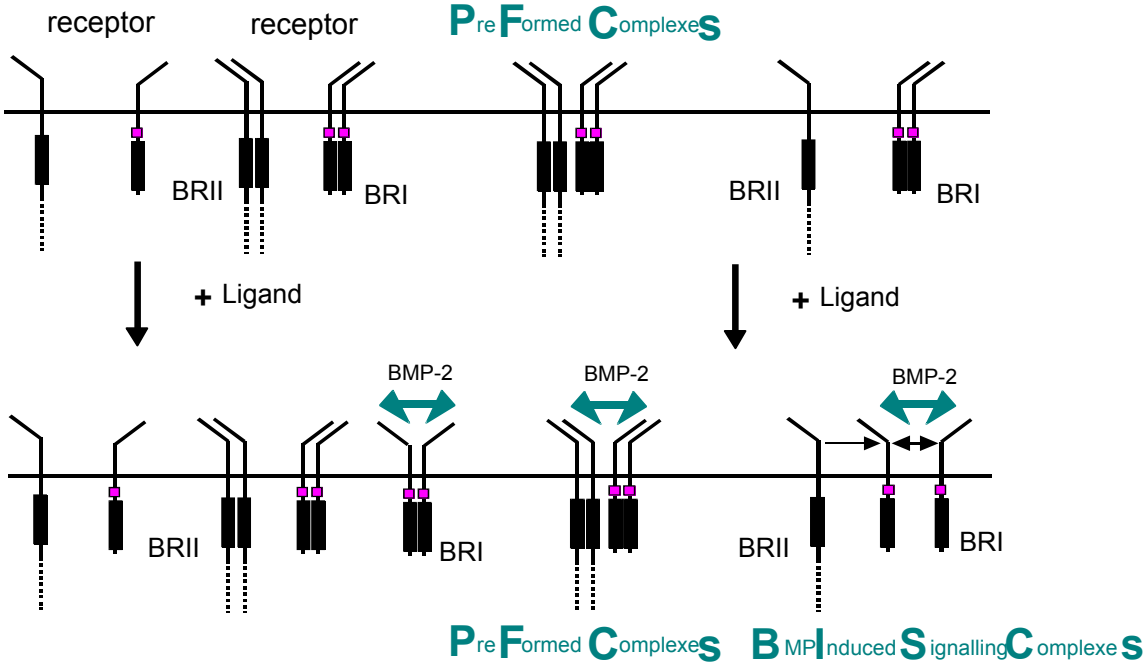


Fig. 4.1 Scheme of the flexibility of the BMP receptors
In the absence of ligands, the BMP receptors can be found on the cell surface as receptor monomers, homo-oligomers and preformed complexes as depicted above. Addition of ligand increases the amount of BRI homo-oligomers, but still monomers can be found. Besides, ligand binds to the preformed complexes or to the type I receptor and recruits the type II, resulting in signalling complexes (PFCs + ligand and BISCs).

Based on these findings of receptor oligomerization, it is of interest which domain of the BMP receptors is important and necessary for the formation of the ligand-independent heterooligomeric complex.

Besides that, the signal transduction of the ligand-bound preformed receptor complex (PFC) and the BMP-induced signalling complex (BISC) and their differences should be investigated.

To address these questions, truncation mutants of the BRII were created, named TC1 to TC8 according to their size (Fig. 4.2).

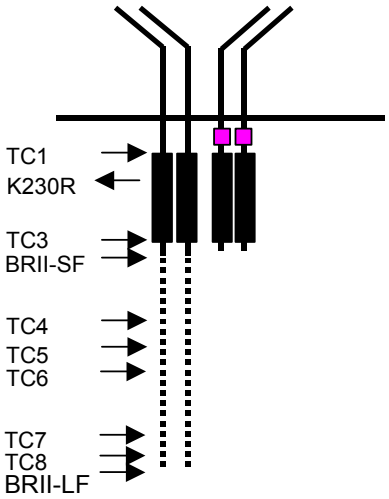
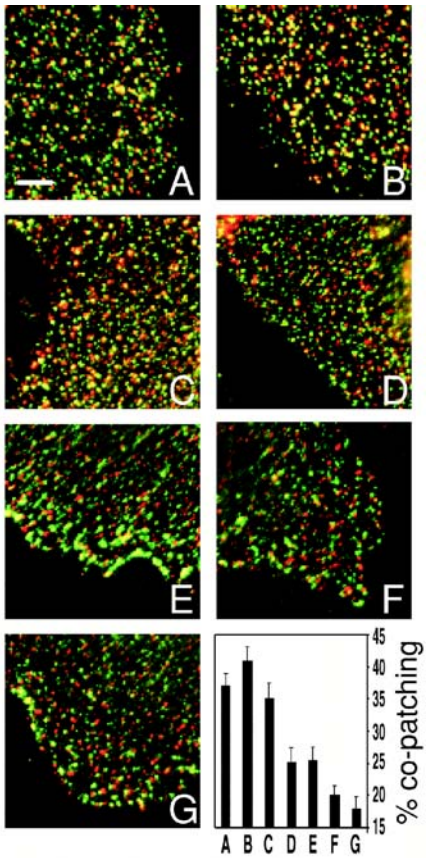


Fig. 4.2 Truncation mutants of the BRII
To dissect BMP receptor oligomerization and BMP receptor mediated signal transduction, BRII truncation mutants were generated. TC1 is the shortest one, TC8 the longest. BRII-SF and BRII-LF are the naturally occurring splice variants.

All these truncations were cloned in pcDNA1 (Anja Nohe) and tested for equal expression. To check which one can interact with BRIa or BRIb in a ligand-independent manner, immunofluorescence co-patching of tagged receptor constructs was performed on live cells (Fig. 4.3) (Yoav Henis). Another approach was to metabolically label receptor transfected cells with ^{35}S-methionine/cysteine and to perform immunoprecipitations of one interaction partner to visualize the other (Fig. 4.4) (Anja Nohe, Sylke Hassel).



	Truncation Mutant	Ability to form PFCs
A	BRII-HA + BRIa-myc	14%
B	BRII-myc + BRIb-HA	23%
C	TC3-HA + BRIa-myc	14%
D	TC3-myc + BRIb-HA	6%
E	TC1-HA + BRIa-myc	6%
F	TC1-myc + BRIb-HA	1%
G	BRIa-HA+Tfr	1%

Fig. 4.3 Immunofluorescence copatching of the BRII receptor variants with BRIa and BRIb
COS7 cells were transiently transfected with the different tagged BMP receptors. When performing immunofluorescence on live cells HA-tagged receptors were labelled with Cy3 (red) and myc-tagged receptors or the transferrin receptor (control) with FITC (green). Patches containing both receptors in close proximity appear yellow when the two fluorescent images (red and green) are overlapped. The panel H shows the percentage of merged receptors, G is the control (non specific interaction, technical errors). The table on the right side shows the percentage of preformed complexes in the absence of ligand, i.e. the percentage of copatching over background level.

With the method used above, receptor oligomerization can be directly measured on the surface of living cells. If differently tagged receptors appear in close proximity, then the staining can overlap and result in a different colour. HA-tagged receptors are labelled with a red secondary antibody, whereas myc-tagged receptors are labelled with a green secondary antibody. If both receptors are close near by, mutual patches appear yellow. Not to measure non specific interaction, the background signal (proximity of BRIa to a non-related human transferrin receptor, panel G) is subtracted. As depicted above, a subpopulation of the BRII was interacting with the BRIa (14%), and even stronger with the BRIb (23%) (Fig. 4.3, A and B). The shorter BRII-variant, TC3 which stops directly after the kinase domain, could still interact

with BRIa (14%), pointing out, that the oligomerization domain is in the kinase (Fig. 4.3.C). The interaction of TC3 with BRIb was with 6% slightly over background level and thus nearly lost. TC1 showed marginal interaction with BRIa, but none with BRIb (Fig. 4.3.E and F).
Concluding, a subpopulation of BRII was forming preformed complexes with both BRIa and BRIb. TC3 could form complexes only with BRIa and TC1 was not recruited in preformed complexes at all.
To confirm these results, in vivo labelling with ^{35}S-methionine/cysteine was performed in COS7 cells, transiently transfected with the HA- and myc-tagged BMP receptors (Anja Nohe, Sylke Hassel).

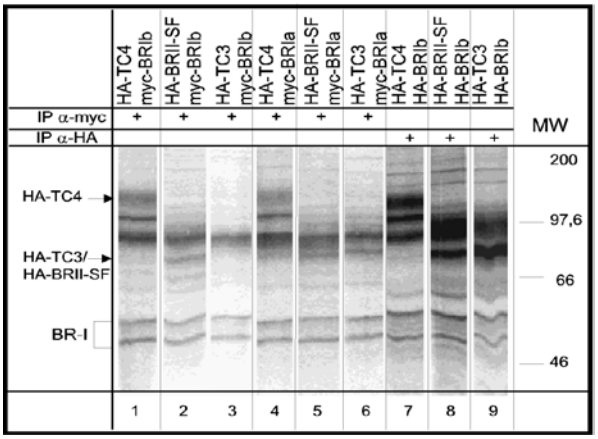


Fig. 4.4 Co-immunoprecipitation of 35S labelled BMP receptors
COS7 cells were transiently transfected with HA-tagged BRII receptor variants, TC3, BRII-SF and TC4 together with BRIa or BRIb, tagged with HA or myc. An immunoprecipitation anti myc was performed to detect preformed complexes. The immunoprecipitation anti HA shows BRIb and the BRII-TCs as control.

In the in vivo labelling experiment all cellular proteins with a certain turnover are radioactively labelled, just as the transfected receptors. With an immunoprecipitation anti-myc the BRIb-myc was precipitated together with it the TC4-HA or BRII-SF (Fig. 4.4, lanes 1-2). TC3-HA was not detectable, due to the marginal amount of interacting receptors (Fig. 4.4, lane 3, compare Fig. 4.3,D). With BRIa-myc all three BRII receptor variants could be precipitated, showing preformed complexes of BRIa with TC3, BRII-SF and TC4 (Fig. 4.4, lanes 4-6). In lanes 7-9 BRIb-HA and the TC3-HA, BRII-SF-HA and TC4-HA were precipitated with anti-HA, indicating the migration positions of the receptors on the gel.

As TC1 could not be detected in preformed complexes the question was whether it can be recruited in BMP induced signalling complexes, the BISCs. A very sensitive method is the binding of ^{125}I-BMP2 and subsequent crosslinking with an appropriate crosslinker, here DSS (Tanja Geissendörfer). COS7 cells were transfected with the TCs together with BRI. After addition and binding of iodinated BMP2, the ligand is chemically crosslinked to the receptors and the receptors are immunoprecipitated. On a gel the ligand-bound receptors can be visualized. So cell surface BMP receptor hetero complex formation can be detected by ligand-binding and crosslinking .

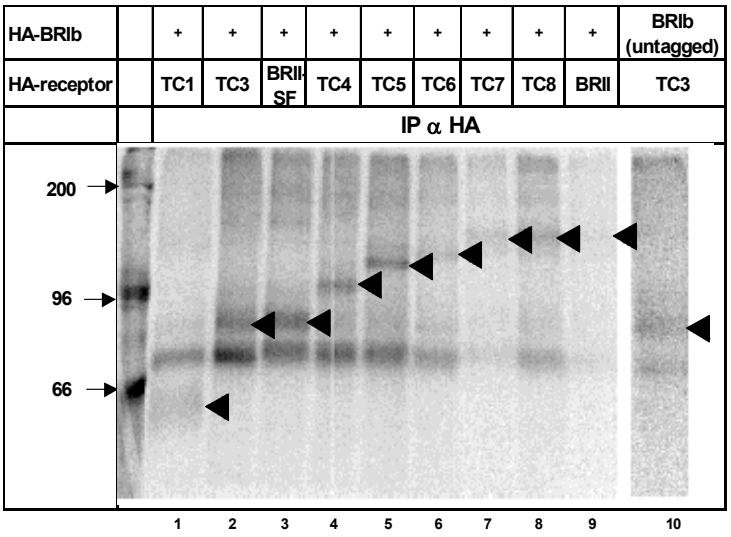


Fig. 4.5 Binding and crosslinking of 125I-BMP2 to the BMP receptors
COS7 cells were transiently transfected with the HA-TCs together with BRIb-HA. 5nM ^{125}I-BMP2 was added and after binding of the ligand and crosslinking to the receptors the receptor was immunoprecipitated with anti HA. Ligand-bound receptor is visualised by autoradiography.

As depicted above, all the TCs are expressed on the cell surface and thus can be detected by crosslinking. All BRII variants can be recruited in the ligand-induced signalling complex, BISC, composed of BRI and BRII. Concerning ligand-dependent interaction, there is no difference between BRIa and BRIb (crosslinking for BRIa not shown).

4.1.2 Influences of the different BMP receptor complexes on signal transduction

From the data obtained, it can be concluded that there are two different scenarios of ligand binding on the cell surface. BMP2 can either bind to free or homodimerized BRI and the low affinity receptor BRII is subsequently recruited. Or BMP2 can bind to preformed complexes of BRI and BRII (see Fig. 4.6).

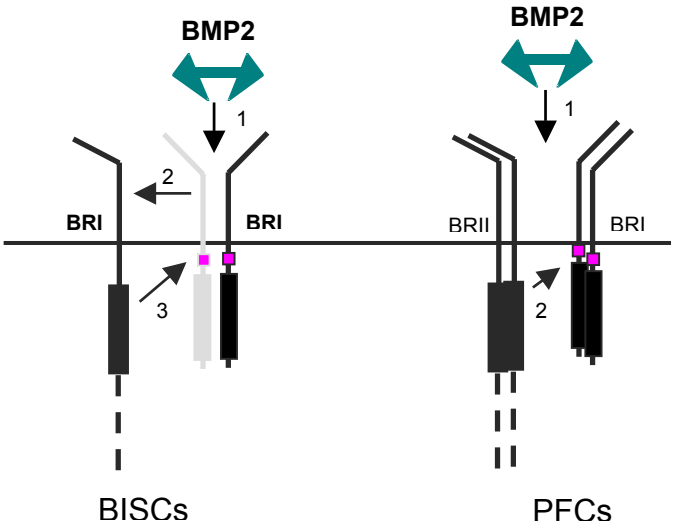


Fig. 4.6 Binding mode of BMP2 to PFCs or BISCs

Due to the different binding mode the events might happen in different cellular compartments and therefore induce different signalling events. The next goal was to identify differences in signal transduction referring to the two complexes.

To address this question we were focusing on two mutants, which were deficient in the BMP2 mediated signal transduction. BRII-SF-KR bears a mutation in the kinase domain and is therefore unable to bind ATP and to transphosphorylate BRI. The other mutant examined, TC1, has no kinase domain and cannot phosphorylate the BRI as well.

Both mutants behave differently during complex formation. Whereas BRII-SF-KR can form preformed complexes as well as ligand induced complexes, TC1 is lacking the kinase, which is important for complex formation (see Fig. 4.4 and 4.5). Despite the short intracellular domain TC1 is expressed on the cell surface (Fig. 4.5) and can be recruited in the BMP-induced signalling complex.

So, BRII-SF-KR can block signal transduction from PFCs and BISCs whereas TC1 can only block BISC mediated signal transduction.

The best characterized pathway is the Smad-pathway. After activation of the BRI via trans-phosphorylation mediated by the BRII, the BRI phosphorylates intracellular mediators of signal transduction, the Smad-proteins. For BMPs we find Smad1/5/8, which are phosphorylated at a SSXS motif at their C-terminus. After a conformational change they oligomerize with the common mediator, Smad4, and translocate to the nucleus where they bind to specific DNA motifs, alone or in combination with other transcription factors (Zhang et al., 1997), (Shi et al., 1999), (Kusanagi et al., 2000).

To examine the activation of Smad1/5/8 a westernblot with antibodies specifically recognizing the activating, C-terminal phosphorylation of Smad1/5/8 can be performed.

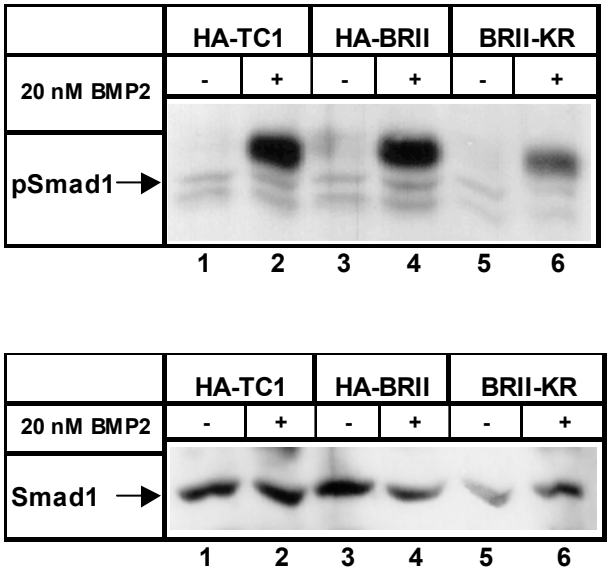
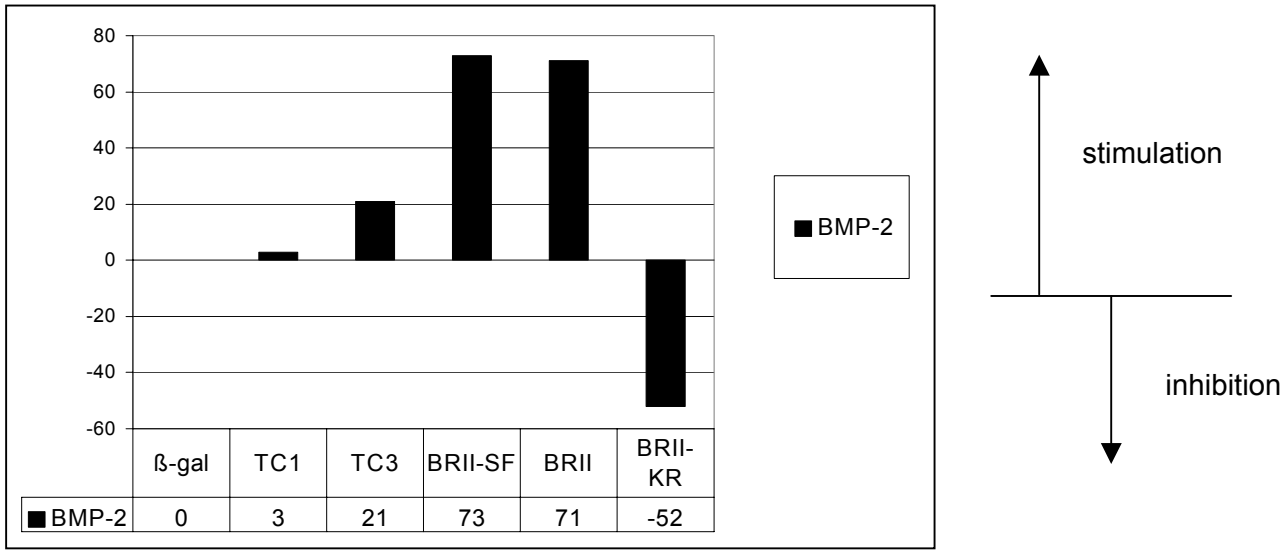


Fig 4.7 Smad-phosphorylation in BMP2 and SCF stimulated C2C12 cells
MC3T3 cells were transfected with the indicated receptor constructs by electroporation. The following day, cells were starved for 24h and subsequently stimulated with 20nM BMP2 for 30 min. Equal amounts of protein were subjected to gel electrophoresis and western blotting.

MC3T3 as well as C2C12 cells are mesenchymal precursor cells with an intact BMP2 mediated signal transduction pathway. Therefore it is easier to look for inhibition of signals via dominant negative receptor constructs than for activation. As depicted above, activation of Smad1/5/8 was observed in cells transfected with all receptor constructs after stimulation with BMP2. Whereas the kinase deficient receptor TC1 and the wildtype BRII-LF are not inhibiting the ligand-induced Smad-phosphorylation, BRII-SF-KR is strongly reducing Smad1/5 activation by BMP2. (Fig 4.7). Blotting of equal amounts of cell lysates with antibodies to endogenous Smad1/5 (gift from Peter ten Dijke, described in (Korchynskyi et al., 1999), (Persson et al., 1998)) showed that the cells expressed equal amounts of Smad1/5 (Fig. 4.7).

Smads, as signal transducers for BMP go to the nucleus and start transcription by binding to specific elements on the DNA, alone or together with other transcription factors. For TGF-β as well as for BMP specific responsive elements on the DNA are characterized. A common DNA element is the Smad binding element (SBE), which is composed of four Smad binding elements (Jonk et al., 1998). The reporter gene construct used is depicted in the appendix (A3).



	ß-gal	TC1	TC3	BRII-SF	BRII	BRII-KR
BMP-2	0	3	21	73	71	-52

Fig 4.8 Smad-reportergene assay in transfected MC3T3 cells
MC3T3 cells were transfected with pSBE-luc and as reference pRLTK as well as with empty vector (ß-gal), different BMP receptor constructs. Cells were stimulated with 10nM BMP2 for 24h. Luciferase activity was measured using SBE as a reporter and RLTK to normalise the signals. Similar results were obtained within four independent experiments.

In the experiment depicted above all lanes show stimulated cells, transfected with different BMP receptor constructs. The signalling level of mock transfected cells was set to baseline and the data obtained from other transfections represent differences to the baseline. TC1 was slightly enhancing the signal over mock level, whereas TC3, BRII-SF and BRII-LF

strongly enhanced the signal over mock. BRII-SF-KR is reducing the signal more than 50 percent.
BRII-SF-KR was inhibiting Smad-mediated signal transduction as expected, in contrast to TC1 which did not reduce the Smad pathway. These data reflect the results obtained in the phospho-Smad1/5/8 westernblot.

To further examine the signal transduction mediated via PFCs and BISCs and inhibited by the kinase negative receptors, alternative pathways were investigated. As described previously, BMP2 is inducing other pathways, distinct from the Smad-pathway, resulting in activation and phosphorylation of p38 MAPK and JNK (Kimura et al., 2000), (Iwasaki et al., 1999).

The next goal was to examine the influence of BRII-SF-KR and TC1 on the p38 pathway.

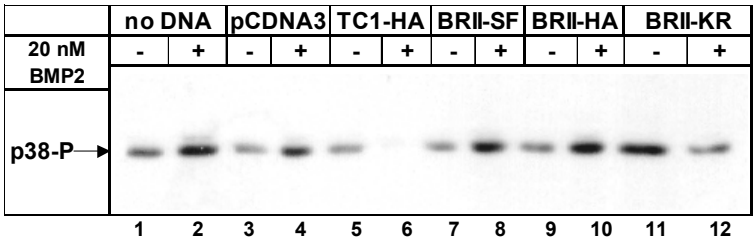


Fig 4.9 BMP2 dependent p38 phosphorylation
$1x10^6$ MC3T3 cells/well were transfected by electroporation and after attaching over night, starved for 5h with DMEM supplemented with 0,5% FCS. Subsequently they were stimulated with 20nM BMP2 for 90min. Equal protein amounts were subjected to westernblotting with anti phospho-p38 antibodies.

BMP2 is enhancing p38 phosphorylation in C2C12 as well as MC3T3 cells, but, in contrast to pSmad1/5/8 some phospho-p38 can be observed, even in the absence of ligand (Fig. 4.9, lanes 1, 3, 8). Cells transfected with mock, BRII-SF or BRII-LF showed BMP2 dependent increase in p38 phosphorylation. Transfection with BRII-SF-KR resulted in a significant decrease in p38 phosphorylation in response to BMP2. TC1 transfection induced nearly complete inhibition of the p38 phosphorylation in response to BMP2, indicating a dominant negative effect of TC1 on the p38 pathway.

An early marker protein which is induced during osteoblastic differentiation is alkaline phosphatase (ALP). The pathway leading to ALP expression is still under investigation, but it is presumably Smad-independent (Guicheux et al., 2003) , (Suzuki et al., 1999). To check the influence of TC1 and BRII-SF-KR on the ALP induction and to get a closer insight into the ALP pathway, C2C12 cells were transiently transfected with the different BMP receptor variants and ALP activity was measured 3 days after BMP2 stimulation.

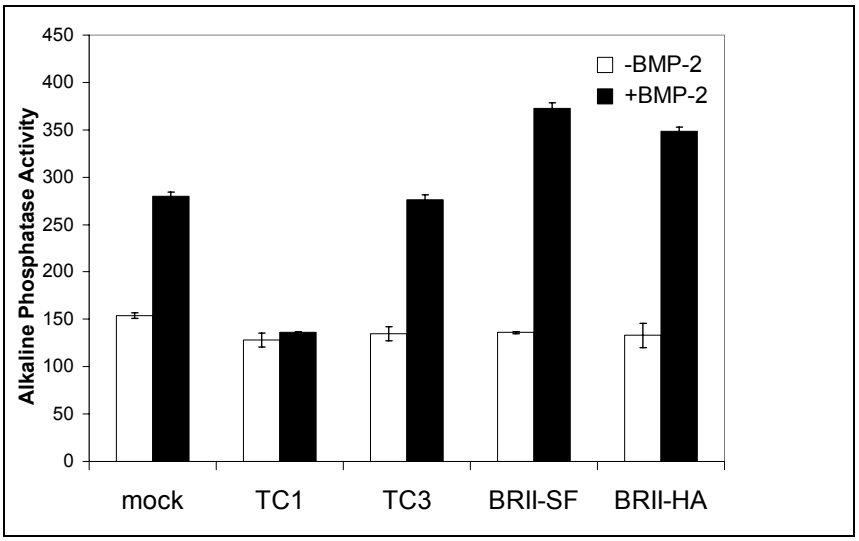


Fig 4.10 Enzymatic measurement of Alkaline phosphatase in C2C12 cells
1x10^6 C2C12 cells/ well were transfected and after attaching over night, starved for 5h in DMEM with 0,5% FCS and subsequently stimulated with 10nM BMP2 for 72h. After washing with PBS, the cells were lysed and the activity of Alkaline Phosphatase was measured, normalized on equal protein amounts.

In the ALP assay, BMP2 stimulation led to an increase of ALP production in mock transfected cells . Whereas TC3 did not show any significant effect, BRII-SF and BRII-LF enhanced the BMP2 dependent stimulation. Cells transfected with TC1 did not show any BMP2 effect, indicating the dominant negative effect of TC1 on the ALP pathway.

4.1.3 Cells stably expressing BRII truncation variants

Due to the low transfection rate of MC3T3 and C2C12 cells with about 30% maximum of transfected cells, cells stably expressing these BRII truncations are an important tool to further examine BMP mediated signal transduction.

To generate stable cell lines, retroviral transduction was performed.

Therefore, DNA-fragments encoding TC1 to TC8, the BRII-SF, BRII-SF-KR, the BRII-LF and the BRII-LF-KR were cloned into a retroviral vector. BRII-SF-KR could be directly cloned into pczCFG5 IEGN (Knodel et al., 1999), but for all the other constructs shuttle constructs were created. The 5´ poly-T-stretch at the BRII and TC-constructs was removed via PCR (using the primer BRII-34) and the resulting receptor DNAs were subcloned in pcDNA3 for expression studies and further in pcDNA3 with a modified linker (TC-Linker) for cloning into the viral vector. The BRII variants carrying an HA-tag were subcloned in pcDNA3 and pczCFG5 IEGN, the myc-tagged variants only in pcDNA3, but they easily could be subcloned in pczCFG5 IEGN as well (see Appendix A4.3).

BRIa and BRIb were cloned into a modified pcDNA3 (BRI-Linker) at first, before they were ligated into the viral vector. For further details see appendix. Only BRIb-HA was subcloned in the retroviral construct, but both HA-and myc-tagged BRIa are in pczCFG5 IEGN. Both

shuttle vectors, TC-Linker and BRI-Linker are described in more detail in the appendix (A4.3.1 and A4.5.1).

Vector details:

The pczCFG5 vectors are retroviral vectors, who are based on the murine leukaemia virus (MuLV). The vector backbone is pcDNA3.1+zeo (pcz). The gene expression in the host cell is driven by the MuLV promoter. The construct contains an internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) which permits both genes, the BRII and EGFP-Neomycin, to be translated from a single bicistronic mRNA. See references: pczCFG5 IEYZ (Kuss et al., 1999) and pczCFG5 IEGN (Knodel et al., 1999).

For infection of C2C12 cells, VSV-G or ecotrophic pseudotyped virus particles were used. The infection was carried out once or two times.

The characteristic of pseudotyped viruses is that the envelope protein (env, *e.g.* VSV-G) originates from another virus strain than the retroviral vector that is used (*e.g.* MuLV-derived retroviral vector) (Witte and Baltimore, 1977), (Emi et al., 1991). Advantages of these pseudotyped viruses are that they produce very high virus titers and allow multiple integration events in the genome of the target cell (Yee et al., 1994a), (Yee et al., 1994b). After cloning of the retroviral constructs, the packaging cell line, 293T, was transiently transfected to produce infectious virus particles. The cells were cotransfected with the retroviral DNA as well as with separate expression vectors for gag-pol (pHIT 60) and env (VSV-G (pczVSV-G wt) or eco (pHIT123)). Because the coding sequences for the viral structure proteins gag-pol and env are separated from the retroviral DNA, they are exclusively used for production of infectious virus particles by the packaging cell line but the resulting virus particles themselves are replication-deficient due to the lack of the genetic information for the gag-pol and env proteins. Harvest of the virus containing supernatant and infection of the target cells were carried out as described in Pear et al. and Knaus et al. (Pear et al., 1993), (Knaus et al., 1996).

Since the use of different envelope proteins resulted in a different success regarding infection rate, all the constructs were used for infection with the VSV-G and the ecotropic envelope protein. Moreover, single as well as multiple infections were carried out.

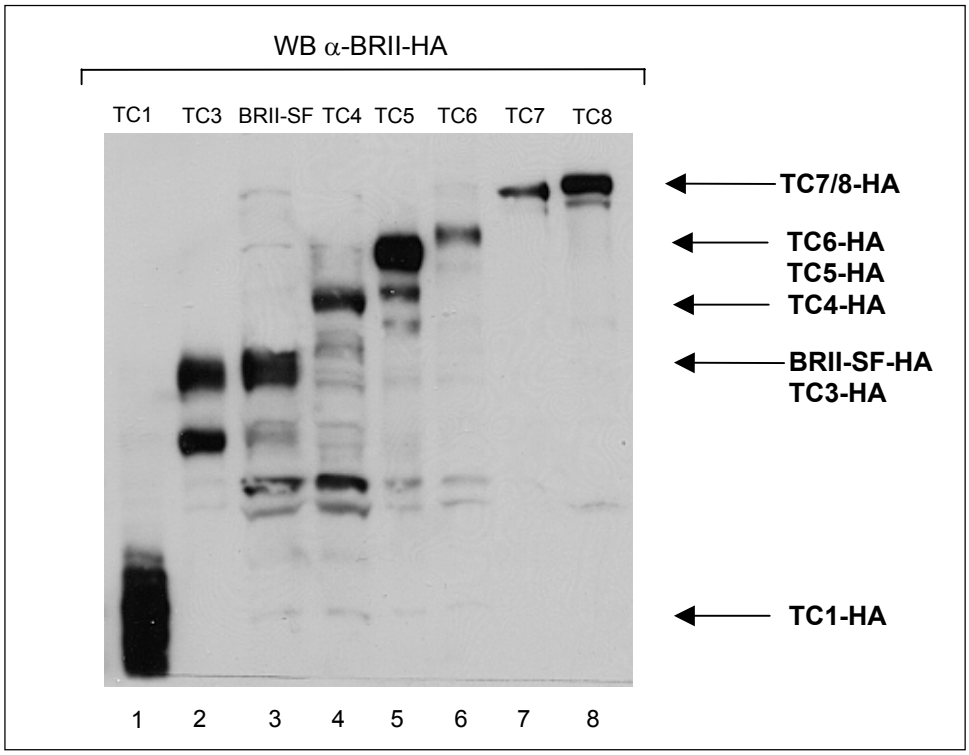


Fig 4.11 Western blot of the stable BRII-TC cell lines
Cells stably expressing the BRII receptor variants were seeded, harvested and subsequently lysed. Equal amounts of protein were subjected to SDS-PAGE and transfer on a nitrocellulose membrane. The overexpressed receptors were detected with anti-HA.

The luciferase assays depicted above as well as the phospho-Smad and phospho-p38 blots were repeated using the stable cell lines, confirming the data obtained so far. The stable cell line expressing TC3 (C2C12/TC3) showed the same induction of p38 phosphorylation as the wildtype C2C12 cells (Fig. 4.9 and 4.12).

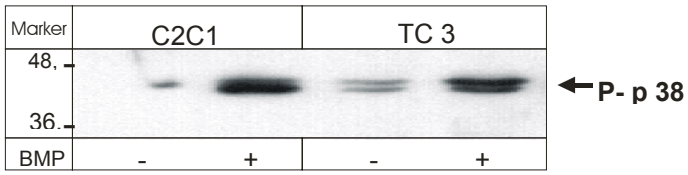


Fig 4.12 BMP2 dependent p38 phosphorylation in the TC3 cell line
$1x10^5$ C2C12 cells were plated on a 6-well plate and after attaching over night starved for 3h with DMEM supplemented with 0,5% FCS. Subsequently they were stimulated with 20nM BMP2 for 60min. Equal protein amounts were subjected to westernblotting with anti P-p38 antibodies.

For generating the cell line stably expressing TC1-HA two infections were performed to get a higher infection rate, resulting in a higher expression rate. The line C2C12/TC1 which was obtained after treatment with pseudotyped viruses encapsulated with the VSV-G envelope twice, resulted in C2C12 cells expressing high amounts of TC1-HA protein and behaving like transiently transfected cells, C2C12/TC1 (2xVSV-G). In the phospho-p38 western blot, the line C2C12/TC1 (2xVSV-G) abolished p38 phosphorylation whereas the line C2C12/TC1 (2xeco) generated with 2x ecotrophic infection was behaving like the wildtype cell line (Fig.

4.13). This indicated that the infection rates using the different envelopes differ, resulting in higher or lower protein expression.

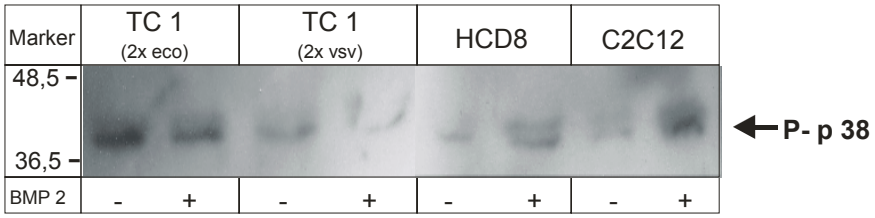


Fig 4.13 BMP2 dependent p38 phosphorylation in different C2C12/TC1 cell lines
1x10^5 C2C12 cells were plated on a 6-well plate and after attaching over night, starved for 3h with DMEM supplemented with 0,5% FCS. Subsequently they were stimulated with 20nM BMP2 for 60min. Equal amounts of protein were subjected to westernblotting with anti P-p38 antibodies.

To examine the ALP pathway, the cells can either be lysed followed by measuring the enzymatic activity of ALP with pNPP as a substrate. Another approach a histochemical analysis of ALP activity with Naphtol AS-MX phosphate and fast blue BB salt. Here all ALP expressing cells appear blue.

To address the question whether C2C12/TC1 (2xVSV-G) cells would suppress ALP production compared to wildtype C2C12 cells, cells were stained for ALP and the percentage of blue cells was measured (Metamorph offline, Visitron).

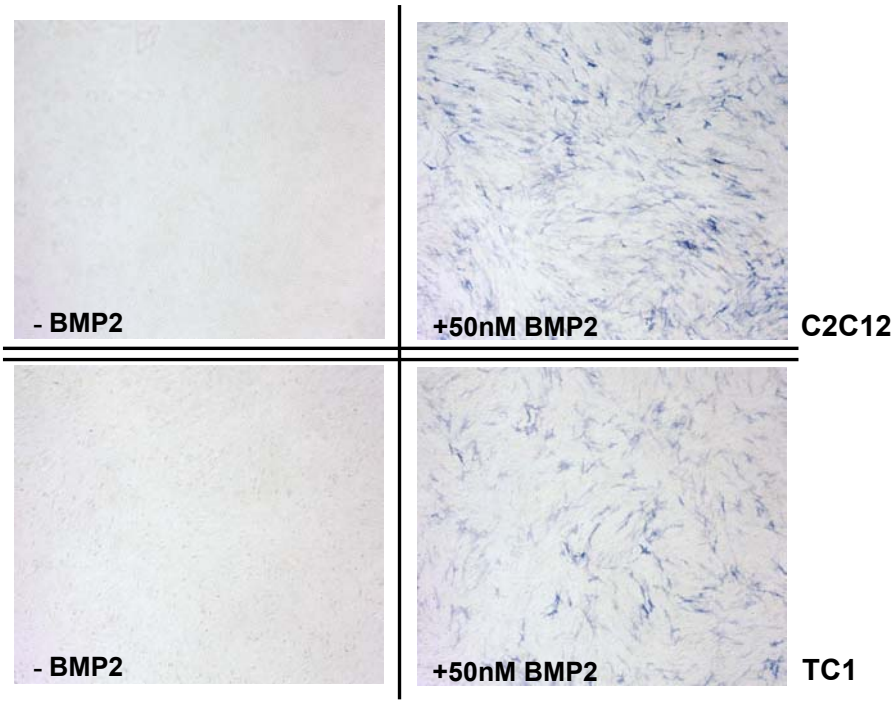
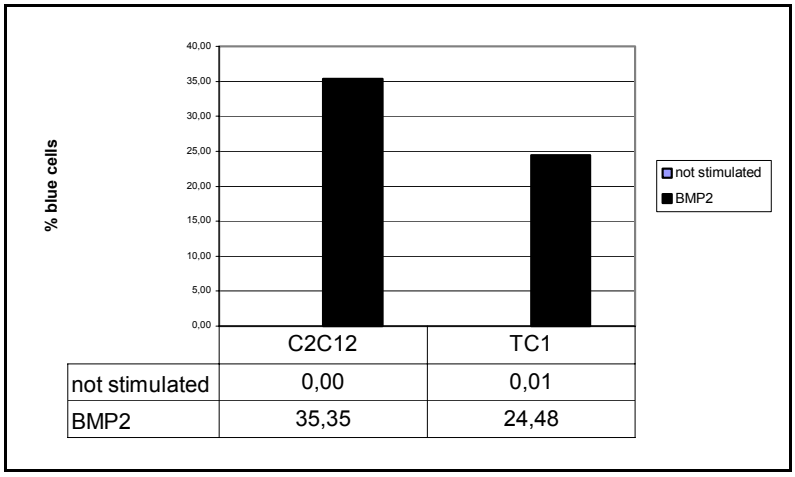


Fig 4.14a Alkaline phosphatase stain in C2C12 and C2C12/TC1 cells
5x10^5 C2C12 cells were seeded on a 6-well plate, after attaching over night, starved for 5h in DMEM with 0,5% FCS and subsequently stimulated with 50nM BMP2 for 72h. After washing with PBS, the cells were fixed and stained with Naphtol AS-MX phosphate and fast blue BB salt.

As depicted above, the ALP stain in the C2C12/TC1 cell line was reduced compared to wildtype C2C12. The residual ALP activity in the C2C12/TC1 cell line results from non transduced cells because the cell line was not sorted.



	C2C12	TC1
not stimulated	0,00	0,01
BMP2	35,35	24,48

Fig 4.14b Quantification of the Alkaline phosphatase stain in C2C12 and TC1 cells
The percentage of blue stained cells per dish was evaluated using Metamorph offline from Visitron.

Fig. 4.14b shows a quantification of the percentage of blue cells. Whereas in the wildtype cells 35% of all the cells were blue, in the C2C12/TC1 line only 24% produced ALP. Therefore the data obtained with the transiently transfected cells could be confirmed working with the stable lines.

4.1.4 Location of the BRII and its influence on signal transduction

The question arises why PFCs and BISC do induce different signal transduction pathways. One possible explanation for that is the presence of different receptor associated proteins. This question will be addressed in chapter 4.2. Another explanation is the different location of BISCs and PFCs, or of BRIa and BRII, respectively. Data from ICS (image correlation spectroscopy) hint to a redistribution of BRIa in the presence of BRII (Nohe et al., 2003). To prove this observation, MC3T3 cells were transfected with BRIa-Q233D or BRIb-Q203D, constitutively active BRI mutants together with Smad5, to enhance the signal. As the BRI-QD is constitutively active, the cells were not stimulated. To test for need of the type II receptor, BRII was cotransfected (Fig. 4.15).

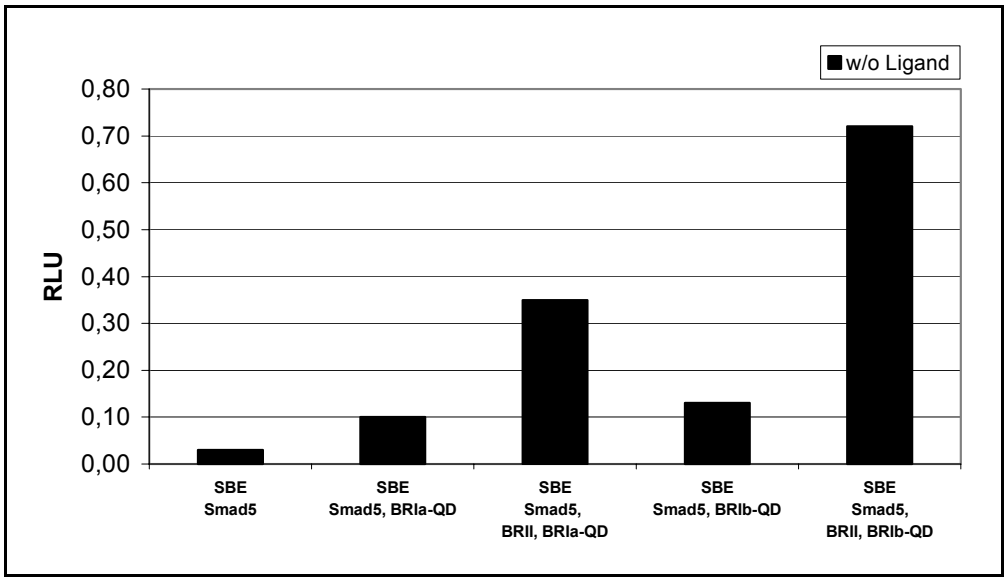


Fig 4.15 Smad-reportergene assay in transfected MC3T3 cells
MC3T3 cells were transfected with pSBE-luc and pRLTK and different constructs. Cells were stimulated with 10nM BMP2 for 24h. Luciferase activity was measured using SBE as a reporter and RLTK to normalise the signals. Similar results were obtained within two independent experiments.

It could be observed that the presence of BRII strongly enhanced the ligand-independent signal mediated by BRI. For BRIa the observed signal was lower, but the factor of induction mediated by BRII was in both cases about five fold. This might be due to the formation of heterooliogomeric complexes. BRII is recruiting BRI to specific domains where more efficient Smad-mediated signal transduction can occur.

4.2 BRII associated proteins (Hassel et al., 2004)

4.2.1 GST - protein purification

For the GST pulldown we used GST-fusion proteins of the BRII-LF. Three different GST fusion proteins of the BRII were created. The whole cytoplasmic part of BRII-LF was subcloned in pGEX-4T-1 and C-terminally fused with a His-tag. For the shorter constructs BRII was subcloned in pGEX-KG by introducing restriction sites for BamHI (5`) and EcoRI (3´) as well as a N-terminal FLAG epitope using PCR. The SF contains the whole kinase domain plus 30 amino acids (amino acids L175-R530), the tail construct includes the amino acids M501 to L1038 and the long form comprises the amino acids 173 to 1038 (for the clone cards see appendix A4.1). The proteins were expressed in BL21(DE3) cells and were purified as described in methods (chapter 3.3.1) using Glutathione-Sepharose (Amersham).

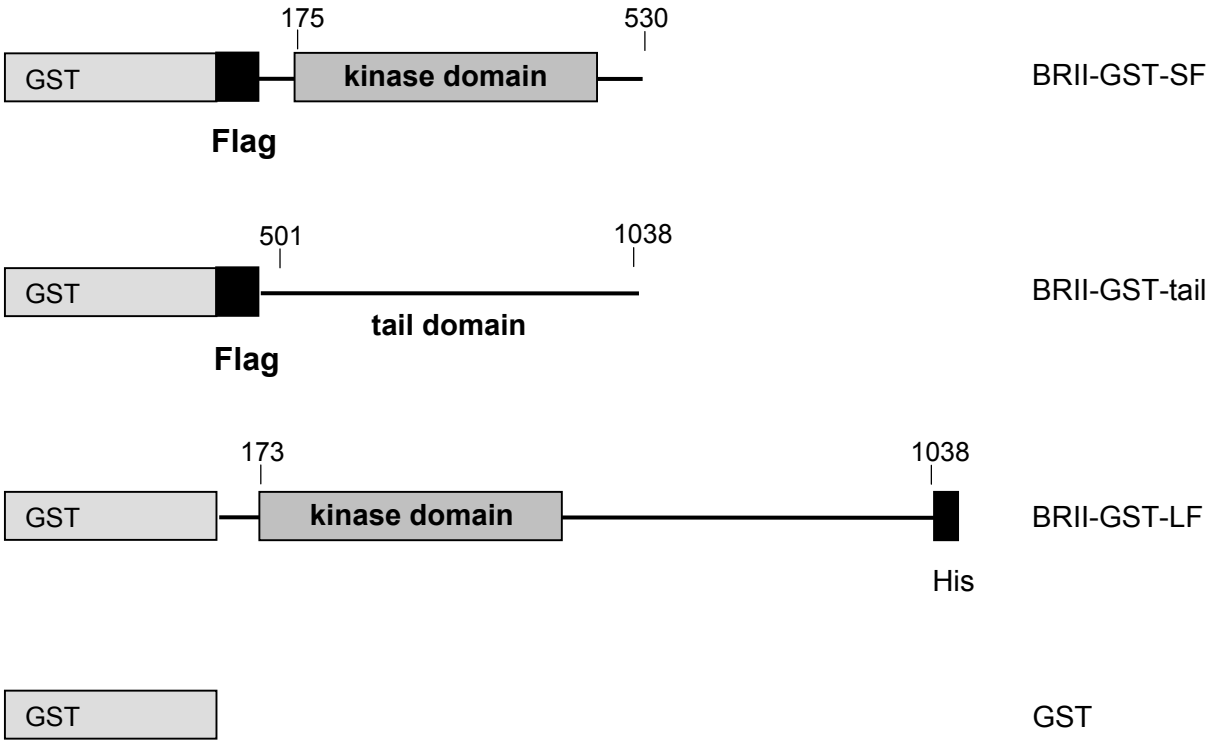


Fig. 4.16 GST-BRII fusion proteins
Schematic representation of full-length and truncated BRII-GST constructs. GST (light grey), the epitopes (black), the kinase domain (grey) and the tail (black line) are presented. The numbers of the amino acid residues are indicated above each structure.

Briefly, BL21(DE3) were grown over night and the next day a large scale culture was inoculated 1:50 and incubated at 37°C. At OD_{600} between 0,4 and 0,5 the fusion proteins were induced and the culture was transferred to 20°C (GST-BRII-tail) or 30°C. The cells were spun down, sonified and the supernatant was incubated with Glutathione Sepharose. After subsequent washes the peptide-bound beads were stored at –80°C.

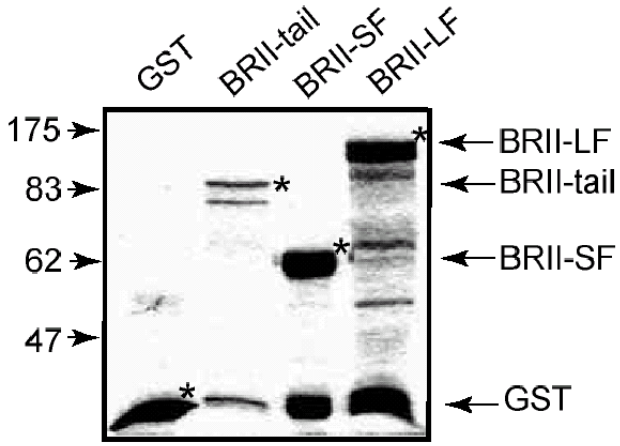


Fig. 4.17 Purification of the BRII-GST fusion proteins
Coomassie gel of the purified GST-BRII fusion proteins. GST-BRII-LF was purified by Annegret Eichner.

Whereas GST-BRII-SF shows a clear band, the GST-BRII-tail protein has some degradation products (lane 3). GST-BRII-LF was purified by Annegret Eichner according to the same protocol, resulting in many degradation products as depicted above.

4.2.2 In vitro kinase assay of GST proteins

To test the correct folding of the purified proteins, we performed an in vitro kinase assay (IVKA), to check the kinase activity and, besides that the correct folding of the protein, of GST-BRII-SF and GST-BRII-LF. The purified proteins, coupled to Glutathione -Sepharose, were subjected to an in vitro kinase assay. 10µl of the Sepharose were used. GST-BRII-SF as well as GST-BRI-LF show auto-phosphorylation, demonstrating an intact kinase and a conserved structure of the protein during the purification process. As a control neither GST alone nor GST-BRII-tail showed a phosphorylation signal.

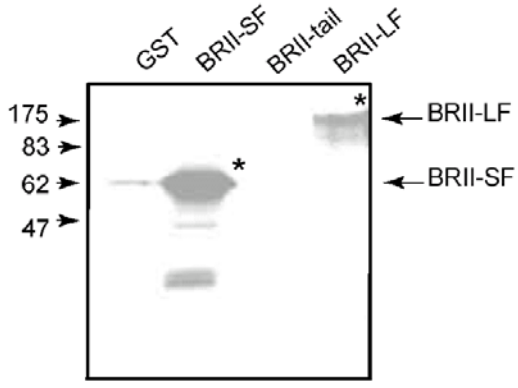
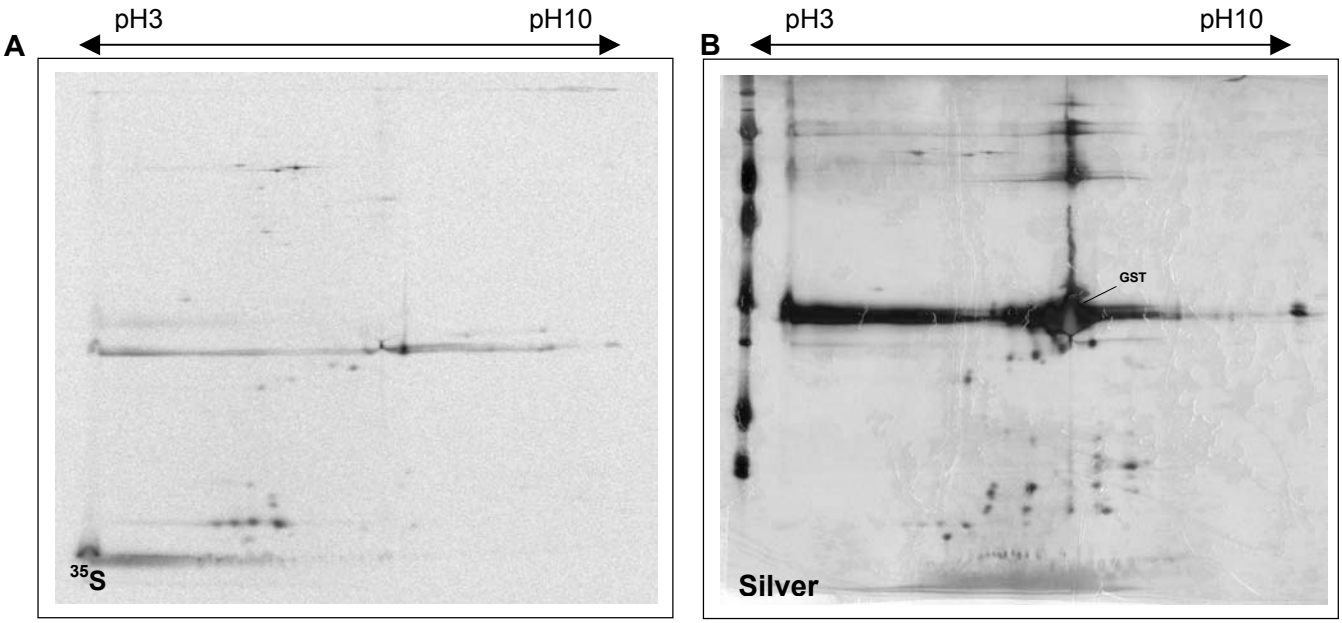


Fig 4.18 In vitro kinase assay of the BRII-GST fusion proteins
The proteins from Fig. 4.17 were subjected to an in vitro kinase assay with 10µCi (γ-^{32}P)ATP per sample. As depicted, GST-BRII-SF as well as GST-BRII-LF are active, auto phosphorylating kinases.

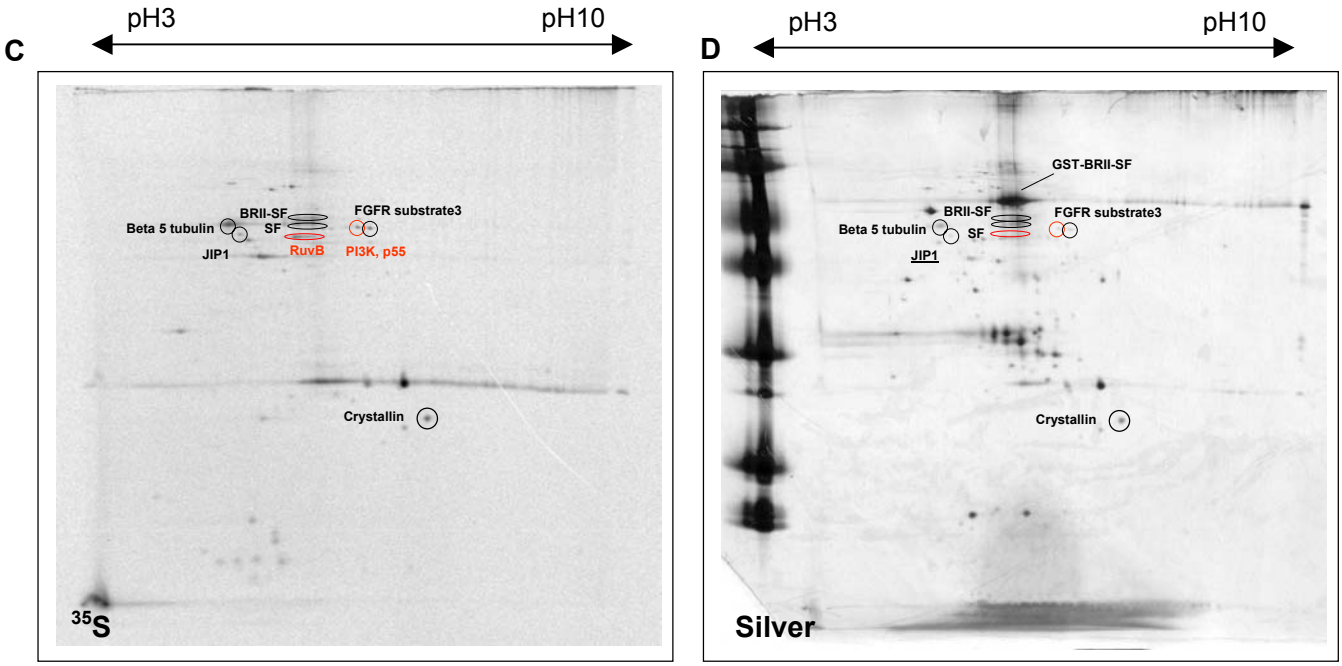
4.2.3 2D-gels

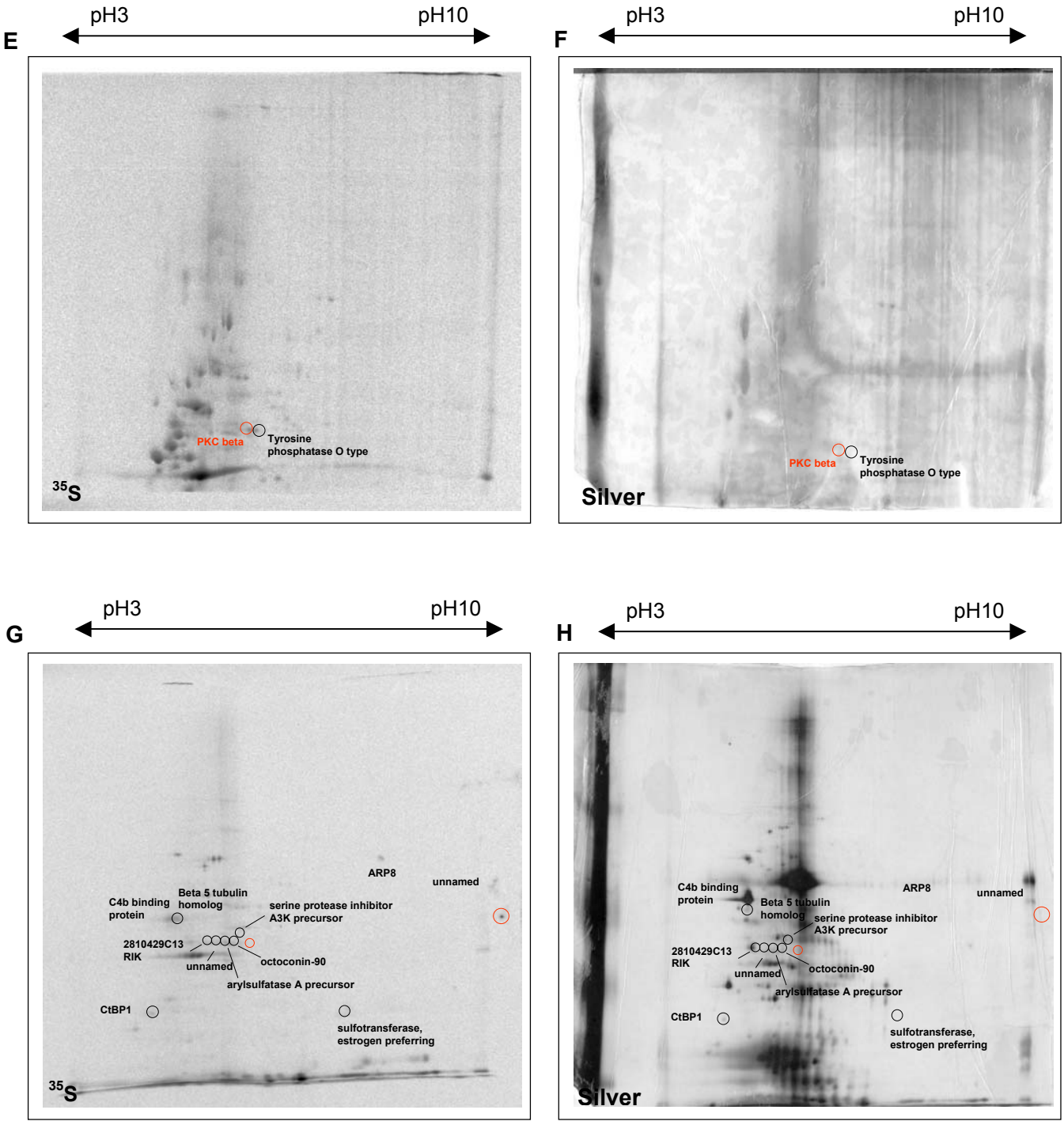
BRII associated proteins should be isolated from the murine myoblast cell line C2C12, which is endogenously expressing BRII-LF as well as BRII-SF (data obtained from Anja Nohe).
The GST-beads were incubated with cell lysate of ^{35}S-methionine/cysteine labelled cells over night at 4°C (lysate from 6x10^6 cells per pull down). After 3 washes with ice-cold lysis buffer the samples were washed once with 20mM Tris pH 7.4 and resuspended in sample buffer (8M urea, 4% CHAPS, 0.5% dithiothreitol (DTT), IPG buffer pH 3-10). After two dimensional gel electrophoresis (Isoelectric focusing and SDS-PAGE) the gel was stained with silver and exposed to a phosphoimager screen. As ^{35}S-labelled proteins originate from C2C12 cells, this comparison allows distinguishing on silver stained gels between co-precipitated C2C12 proteins and non-labelled proteins which originate from bacteria, including GST fragments. C2C12 cellular proteins, which specifically co-precipitated with BRII constructs and not with GST alone, were selected for identification.

Interacting with GST:



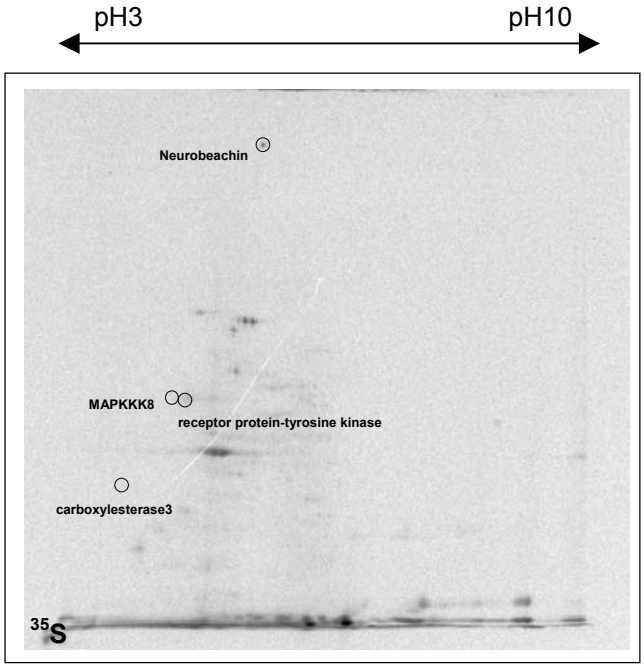
Interacting with BRII-SF:



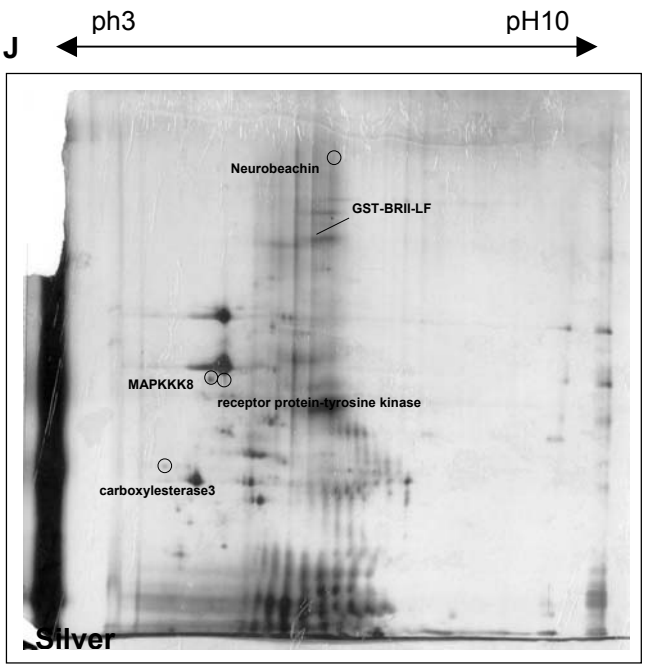

E
pH3
pH10
PKC beta
Tyrosine phosphatase O type
35S
F
pH3
pH10
PKC beta
Tyrosine phosphatase O type
Silver
G
pH3
pH10
ARP8
unnamed
C4b binding protein
Beta 5 tubulin homolog
serine protease inhibitor A3K precursor
2810429C13 RIK
unnamed
octoconin-90
arylsulfatase A precursor
CtBP1
sulfotransferase, estrogen preferring
35S
H
pH3
pH10
C4b binding protein
ARP8
unnamed
Beta 5 tubulin homolog
serine protease inhibitor A3K precursor
2810429C13 RIK
unnamed
octoconin-90
arylsulfatase A precursor
CtBP1
sulfotransferase, estrogen preferring
Silver

Interacting with BRII-LF:

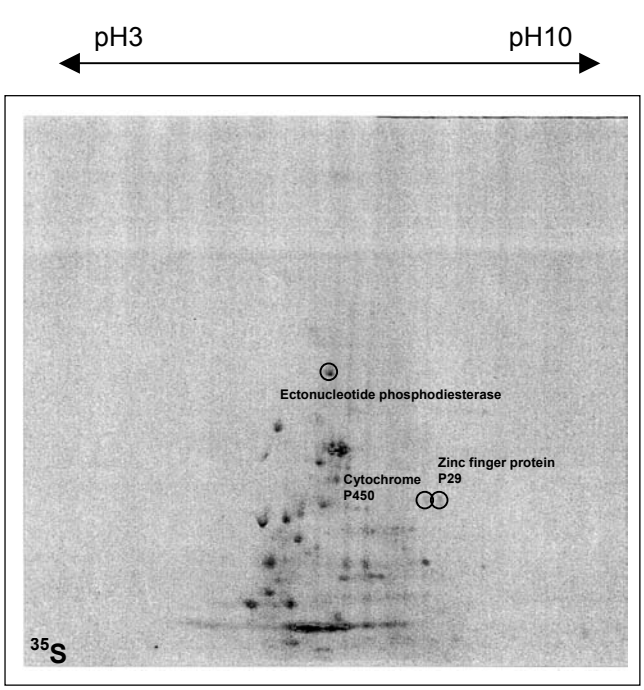
I



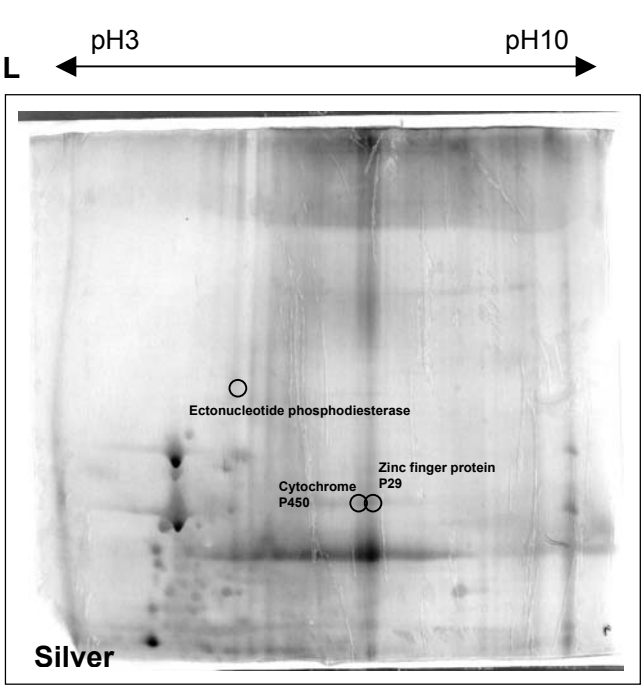
J



K



L



Interacting with BRII-tail:

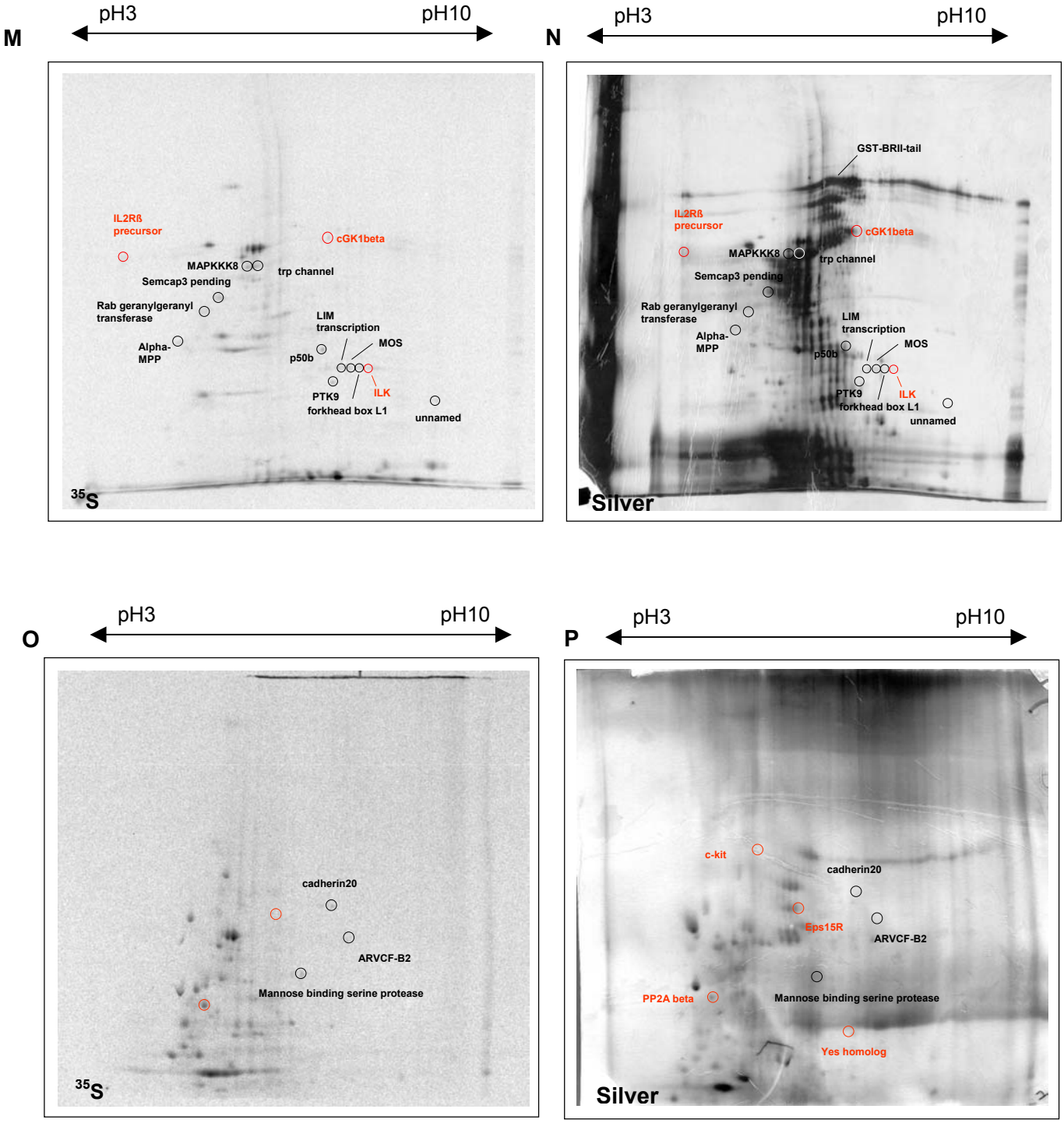


Fig 4.19 Two dimensional gels of the GST-pulldowns
Here the two-dimensional gels of the identified associated proteins are shown. Three sets of BRII-GST-SF gels, and two sets of GST-BRII-LF and GST-BRII-tail sets are depicted here. Each set is composed of a silver stained and a ^{35}S-labelled gel. On the silver gel all proteins are visualised (B, D, F, H, J, L, N, P), on the ^{35}S-gel only the cellular proteins pulled down with the GST-protein are shown (A, C, E, G, I, K, M, O).
As a control pulldowns with GST alone were performed. Therefore, GST interacting proteins could be identified and neglected for identification.

Representative gels of the BRII or interacting GST proteins are shown here. On the right hand the silver stained gels are depicted, visualizing BRII interactors and bacterial proteins (B, D, F, H, J, L, N, P). The corresponding gels, exposed on a phosphoimager and showing the cellular BRII interactors, labelled with ^{35}S-methionine and cysteine, are on the left hand (A, C, E, G, I, K, M, O). On one silver stained gel each the GST fusion protein is marked. The GST and the GST-BRII-SF are migrating as compact spots, the GST-BRII-LF and the GST-BRII-tail show degradation, which is visualised on the 2D gel as a smear.

The MALDI method to identify proteins is very sensitive, even proteins, which are weak spots on the ^{35}S gel and are not visible on the silver stain could be identified. All protein spots which were cut, were visible on the radioactive gel. Processing with the computer sometimes led to decrease in the intensities.

Further, it can be observed, that many proteins interact with the kinase domain of the BRII. For the BRII-tail approximately the same number of associated proteins could be isolated. Compared to this, only very few proteins interact with the BRII-LF. This might be due to the lower amount of protein or a different conformation of the BRII-LF compared to the kinase or the tail domain.

4.2.4 Identification of the associated proteins with MALDI TOF MS

The protein spots from the gel were cut, destained, digested with trypsin and subsequently the peptides were eluted from the gel. After concentration and desalting the peptides were eluted with matrix (α-cyano-4-hydroxycinnamic acid) directly on the MALDI TOF MS target (see chapter 3.3.6-8). The masses of the peptides were determined by their time of flight in an electric field after ionisation with a laser-beam. The resulting masses were calibrated using autolytic peptides from trypsin (842,51; 870,54; 1045,56; 1940,93; 2211,10). Before and after subtraction of known masses from human keratin peptides (1179,59; 1234,679; 1475,76; 1707,698; 1993,969), the masslists were pasted into the Mascot and ProFound search engines to identify proteins in the NCBI sequence database. When checking the mass lists for autolytic trypsin or digested keratin peptides, it has to be remembered that the given masses and the masses plus one additional C-atom (+14Da) might occur.

The proteins with the highest probability and highest Z-Value were chosen for further validation. The technique where proteins are identified according to their combination of flying peptides is called peptide mass fingerprinting (Shevchenko et al., 1997), (Henzel et al., 2003).

Identified proteins interacting with BRII-SF (table 4.1), BRII-LF (table 4.2) and BRII-tail (table 4.3), respectively are listed below. In total, 115 proteins were excised from the gel, purified and spectra were measured. 52 BRII interacting proteins could be identified, 24 were interacting with the BRII-SF, 7 with BRII-LF and 21 with the BRII-tail (6 were identified to

interact with BRIb, data not shown). The masslists from the peptides SH01 to SH20 were subjected to searches in masscot and ProFound. As the results were comparable we chose only ProFound due to better handling for further experiments. In case of more protein the peptides can be sequenced, using Chemically Assisted Fragmentation-MALDI chemistry (CAF chemistry) (Amersham, Biosciences).

Interacting with BRII-SF:

Identified with BRII-	ID	Protein	accession No	Probability	Z-Value	Sequ. Coverage	Experimental value		Theoretical value	
							pI	MW (kDa)	pI	MW (kDa)
SF	SH04	tubulin, beta 5	NP_035785	1.0e+000	2,29	19%	5,0	66	4,8	50,11
SF	SH49	arylsulfatase A precursor (ASA) (Cerebroside-sulfatase)	P50428	1.0e+000	2,25	14%	5,7	55	5,5	54,56
SF	SH44A	sulfotransferase, estrogen-preferring	P49891	1.0e+000	2,2	24%	7,5	40	6,6	35,86
SF	SH50	otoconin-90	AAC99455	1.0e+000	2,2	12%	5,9	55	4,7	51
SF	SH56	similar to heterogeneous nuclear ribonucleoprotein R (Rub B-like), Hnrpr protein	AAH04679	1.0e+000	2,09	6%	10,0	66	9,1	67,51
SF	SH40	protein tyrosine phosphatase, receptor type O	NP_035346	1,00E+00	1,92	11%	6,0	60	6	47,39
SF	SH43	C-terminal binding protein 1, Ctbp1	AAH13702	1.0e+000	1,85	23%	5,5	40	6,2	47,05
SF	SH01	Crystallin alpha-B	NP_034094	1.0e+000	1,71	30%	7,0	25	6,8	20,05
SF	SH57	unnamed protein product	BAB28688	9,80E-01	1,42	19%	10,0	75	10,2	77,86
SF	SH58	ARP8, actin-related protein 8 homolog	NP_081769	7,70E-01	1,38	14%	8,0	80	7,9	71,34
SF	SH11	BRII-SF	NP_031587	1.0e+000	1,36	10%	6,5	66	5,8	116,31
SF	SH10	BRII-SF	NP_031587	1.0e+000	1,2	9%	6,5	66	5,8	116,31
SF	SH55	unnamed protein product, tubulin 5 beta homolog	BAB27292	9,90E-01	0,98	20%	5,5	63	4,8	50,08
SF	SH09	Rub B-like protein 2 (RuvB)	NP_035434	9,10E-01	0,76	14%	6,5	60	5,5	51,27
SF	SH53	Serine proteinase inhibitor A3K precursor (Contrapsin)	P07759	9,70E-01	0,72	27%	6,0	56	5	47,03
SF	SH47	2810429C13Rik protein	AAH06707	9,80E-01	0,71	12%	5,5	55	6,7	69,48
SF	SH39	PKC beta	NP_032881	9,60E-01	0,61	10%	6,0	60	6,6	78,07
SF	SH52	IL2Rß	NP_032394	9,00E-01	0,48	8%	6,1	55	5,4	61,49
SF	SH54	C4b binding protein	NBMSC4	7,10E-01	0,42	12%	5,5	63	6,9	52,99

Identified with BRII-	ID	Protein	accession No	Probability	Z-Value	Sequ. Coverage	Experimental value		Theoretical value	
							pI	MW (kDa)	pI	MW (kDa)
SF	SH07	Fibroblast growth factor receptor substrate 3	AAH14819	7,10E-01	0,33	10%	6,5	66	6,3	52,38
SF	SH48	unnamed protein product	BAB26511	7,10E-01	0,3	10%	5,6	55	9,4	64,89
SF	SH06	PI3K, P85gamma/p55	Q64143	5,80E-01	0,26	11%	6,5	66	5,7	54,74
SF	SH41	cdc37	NP_058022	4,60E-01	0,24	13%	5,5	30	5,2	45,09
SF	SH08	JNK interacting protein, JIP1	Q9WVI9	5,00E-02	-	6%	5,0	60	4,8	78,12

Tab 4.1 GST-BRII-SF interacting proteins
The GST-BRII-SF interacting proteins are listed according to their probabilities. Probability, Z-value, coverage and theoretical pI and MW were obtained from the ProFound search. The calculation of the experimental pI and MW was based on the migration of proteins on a 2D-gel.

Interacting with BRII-LF:

Identified with BRII-	ID	Protein	accession No	Probability	Z-Value	Sequ. Coverage	Experimental value		Theoretical value	
							pI	MW (kDa)	pI	MW (kDa)
LF	SH32	ectonucleotide pyrophosphatase/ phosphodiesterase 1 allotype b	AAK84174	1.0e+000	1,38	6%	5,3	95	6,1	105,16
LF	SH80	Neurobeachin	CAC18812	9,90E-01	0,7	4%	6,0	200	5,8	325,64
LF	SH76	carboxylesterase3 (triacylglycerol hydrolase)	NP_444430	9,80E-01	0,67	21%	4,3	40	6,3	62,15
LF	SH35a	zinc finger protein 29	NP_033579	8,60E-01	0,42	13%	5,8	60	6,9	70,41
LF	SH79	receptor protein-tyrosine kinase	AAA85355	7,90E-01	0,36	22%	5,0	66	5,1	58,08
LF	SH35	cytochrome P450 CYP2J9	AAK59868	3,30E-01	0,16	12%	5,8	60	7,9	58,37
LF	SH78	MAPKKK8	NP_031772	2,10E-01	0,08	16%	4,8	66	5,8	52,92

Tab 4.2 GST-BRII-LF interacting proteins
The GST-BRII-LF interacting proteins are listed according to their probabilities. Probability, Z-value, coverage and theoretical pI and MW were obtained from the ProFound search. The calculation of the experimental pI and MW was based on the migration of proteins on a 2D-gel.

Interacting with BRII-tail:

Identified with BRII-	ID	Protein	accession No	Probability	Z-Value	Sequ. Coverage	Experimental value		Theoretical value	
							pI	MW (kDa)	pI	MW (kDa)
tail	SH61	integrin linked kinase	AAH03737	1.0e+000	2,34	10%	7,6	42	8,7	51,87
tail	SH74	IL2Rß precursor	NP_032394	1.0e+000	2,24	24%	4,0	80	5,4	61,49
tail	SH26	cadherin20, cadherin 7	NP_035930	1.0e+000	1,45	9%	7,0	90	4,5	89.32
tail	SH64	LIM homeobox transcription factor 1 alpha	NP_387501	1.0e+000	1,37	29%	7,0	42	7,9	42,84
tail	SH29	mannose-binding lectin associated serine protease-2	BAA34674	1.0e+000	0,89	11%	6,5	75	5,8	76,96
tail	SH67	Mitochondrial processing peptidase alpha subunit, mitochondrial precursor (Alpha-MPP) (P-55)	Q9DC61	9,90E-01	0,73	13%	5,2	60	6,4	58,77
tail	SH63	proto-oncogene serine/threonine Protein kinase MOS	P00536	8,20E-01	0,73	17%	7,2	42	9,9	43,56
tail	SH65	p50b	BAA08541	9,70E-01	0,69	16%	6,8	45	4,8	36,82
tail	SH73	CGMP dependent protein kinase1, beta isozyme (CGK1 beta)	NP_035290	9,80E-01	0,66	20%	6,9	82	5,3	78,34
tail	SH28	ARVCF isoform B2	AAK64217	9,70E-01	0,64	4%	7,5	85	6,3	97,62
tail	SH72	transient receptor potential cation channel, subfamily C, member 1; transient receptor protein 1	NP_035773	9,80E-01	0,64	10%	6,3	75	8,8	93,79
tail	SH27	EGFR substrate 15 related (EPS15R)	NP_031970	9,10E-01	0,58	6%	6,3	90	4,9	99,47
tail	SH59	unnamed protein product	BAB24410	9,70E-01	0,57	31%	8,5	35	6,1	29,74
tail	SH70	Semcap3 pending protein (= Similar to semaF cytoplasmic domain associated protein 3)	AAH10329	8,90E-01	0,54	13%	5,8	66	5,1	75,21
tail	SH71	MAPKKK8	NP_031772	9,20E-01	0,54	9%	6,1	75	5,8	53,38
tail	SH62	forkhead box L1, forkhead homolog 6	NP_032050	9,20E-01	0,47	18%	7,4	42	9,9	36.52
tail	SH31	viral oncogene yes homolog	NP_033561	8,70E-01	0,46	8%	7,0	55	6,2	61,07

Identified with BRII-	ID	Protein	accession No	Probability	Z-Value	Sequ. Coverage	Experimental value		Theoretical value	
							pI	MW (kDa)	pI	MW (kDa)
tail	SH30	unnamed protein product; homolog to serine/threonine Protein phosphatase 2A, 55 KDA regulatory subunitB beta isoform (PP2A,beta)	BAB31079	8,30E-01	0,4	9%	4,5	66	5,9	52.,32
tail	SH60	PTK9, protein tyrosine kinase 9	AAH15081	7,80E-01	0,35	22%	7,0	35	6,2	40,3
tail	SH69	Rab geranylgeranyl transferase, a subunit; gunmetal	NP_062392	7,00E-01	0,34	11%	5,5	64	5,5	66,23
tail	SH25	c-kit, steel factor	NP_066922	4,30E-01	0,16	7%	5,0	100	6,6	110,24

Tab 4.3 GST-BRII-tail interacting proteins
The GST-BRII-tail interacting proteins are listed according to their probabilities. Probability, Z-value, coverage and theoretical pI and MW were obtained from the ProFound search. The calculation of the experimental pI and MW was based on the migration of proteins on a 2D-gel.

The green highlight shows a very high identification probability. The proteins marked in red were chosen for further validation.

4.2.5 Grouping of BRII associated proteins

The BMP receptor interacting proteins can be grouped according to their functionality. The scheme below visualizes which proteins interact with which part of the BRII. The proteins interacting with the BRII-SF are indicated above the dashed line, on the left hand of the receptor signal transducing molecules are listed, on the right hand structural, transcriptional and metabolic proteins are shown. Below the receptor BMP non-related proteins are depicted.

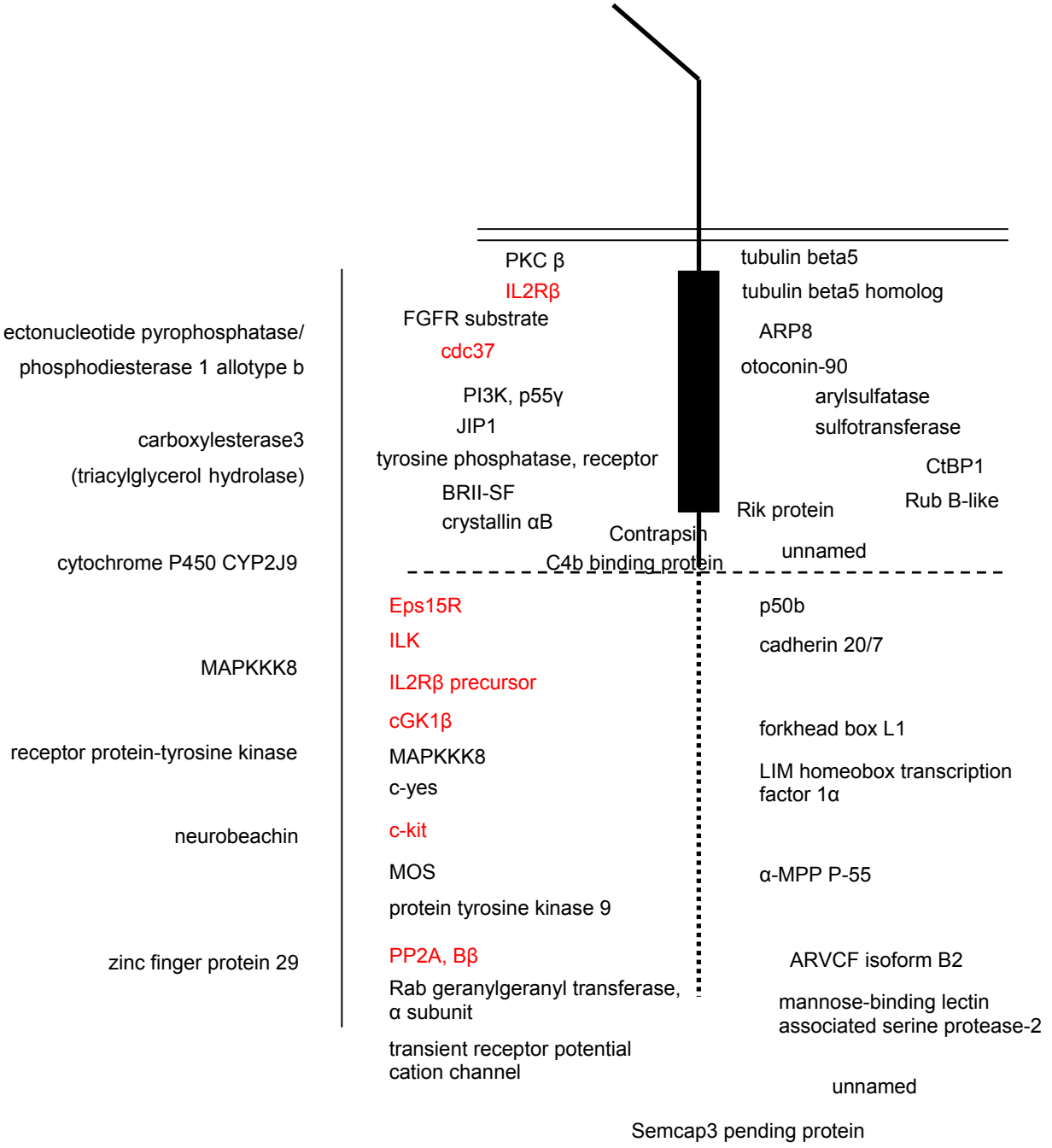


Fig 4.20 Schematic representation of BRII-interacting proteins
Summary of all BRII interacting proteins. The proteins depicted on the left side of the lane are BRII-LF interactors, whereas the proteins associated with BRII-SF are above the dashed line and the proteins associated with BRII-tail below, respectively.

Below the dashed line BRII-tail interacting proteins are listed, on the left hand signal transducing molecules, on the right hand proteins involved in metabolic processes and transcription and more on the bottom non-related proteins can be found. On the very left BRII-LF interactors are depicted,

All the proteins identified can be grouped according to their functionality. The majority of the interactors are proteins involved in signal transduction processes. Most of them have been found with the BRII-SF, containing the kinase domain of BRII.

signalling group

BRII-SF: PKCβ, IL2Rβ, FGFR substrate, cdc37, PI3K p55γ, tyrosine phosphatase receptor type O, BRII-SF, JIP1, Crystallin αB

BRII-LF: Neurobeachin, MAPKKK8, receptor protein-tyrosine kinase

BRII-tail: Eps15R, ILK, IL2Rβ precursor, cGK1β, MAPKKK8, c-yes, c-kit, MOS, protein tyrosine kinase 9, PP2A Bβ, Semcap 3 pending protein, transient receptor potential cation channel, Rab geranylgeranyl transferase, α subunit

PKC (α/β) and phospholipase D have been shown to participate in growth factor-induced signalling in C3H10T1/2 fibroblasts (Thorsen et al., 2003). Besides that PKCβ has a role in regulation of osteoclast formation and function, potentially by participating in the ERK signalling pathway (Lee et al., 2003b). PKC-dependent signalling is required for BMP-dependent induction of apoptosis (Hay et al., 2001) as well as for αI-collagen expression (Palcy and Goltzman, 1999). IL2Rβ is an essential signalling subunit that is shared exclusively by IL-2 and IL-15 (Petitto et al., 2002). Corrigall and colleagues have shown that IL2Rβ is not only expressed on immune cells but on other cells as fibroblast-like synoviocytes (Corrigall et al., 2001). The polypeptides of the IL-2 receptor do not themselves have intrinsic catalytic activity, but interact with cytoplasmic signalling proteins to transduce signals. Different regions of the cytoplasmic domain of the IL-2 receptor β chain interact and couple with distinct signalling pathways and cellular responses, as JAK1, the adaptor Shc (which leads to Ras activation via Grb2 and Sos-1), PI3K and many other phosphorylated tyrosine residues can be recognized through src homology 2 (SH2) domains (Friedmann et al., 1996), (Gaffen et al., 1996), (Gaffen, 2001). Besides that, the IL2R is efficiently internalized through a clathrin-independent pathway (Lamaze et al., 2001). Another identified protein, FRS3, is an adapter protein that is activated by fibroblast growth factor and NeurotrophinTyrosinReceptor Kinase1 (Ranzi et al., 2003), also named suc-1 associated neurotrophic factor target 2 (SNT2) (Xu et al., 1998). This protein has been shown to be involved in FGF signalling (Goldfarb, 2001), (Marie et al., 2002). The mammalian Cdc37 is a protein kinase-targeting

subunit of the molecular chaperone Hsp90 (Stepanova et al., 1996). Cdc37 plays a general role in signalling pathways dependent on intrinsically unstable protein kinases by being a co-chaperone that binds to numerous kinases and promotes their interaction with the Hsp90 complex. This results in stabilization and promotion of their activity. p55PI3K γ, is a new regulatory subunit which might be important for receptor signalling (Pons et al., 1995). It binds to activated (phosphorylated) protein tyrosine kinases through its SH2 domain and regulates their kinase activity. The active molecule is a heterodimer of a p110 (catalytic) and a p55 γ (regulatory) subunit. Two major signals emerge from PI3Kγ: phosphoinositides that target PKB and protein phosphorylation that activates MAPK (Bondeva et al., 1998). Tomemori and colleagues cloned the murine protein tyrosine phosphatase receptor type O (PTPRO) (Tomemori et al., 2000), a type III receptor protein tyrosine phosphatase with an extracellular region containing eight fibronectin type III domains. Proteins of that subgroup regulate proliferation, differentiation and viability of epithelial cells. mPTPRO is involved in the differentiation and axonogenesis of central and peripheral nervous system neurons, where it is in a position to modulate intracellular responses to neurotrophin-3 and/or nerve growth factor (Beltran et al., 2003). BMP2 signals via two types of receptors, type I and type II and is important for the regulation of cell growth, differentiation, and apoptosis. The BRII is the low affinity receptor for BMP2. Alternative splicing of the BRII gene can yield a longer, BRII-LF, and a shorter form of the BRII, BRII-SF (Liu et al., 1995), (Rosenzweig et al., 1995). BMP2 transmits signals via the Smad-, p38- and ERK-pathway. So far no difference between BRII-SF and –LF in the onset of these pathways has been described yet [Nohe, 2002 #57). The identification of BRII-SF as interactor of BRII-SF is confirming the data obtained by Gilboa et al., demonstrating the formation of ligand-independent homo-oligomers of BRII (Gilboa et al., 1998). JIP1 is a MAPKKK8 interacting protein, which is shown to be JNK interacting and a potential inhibitor of JNK pathway (Mooser et al., 1999). Yasuda et al. [Yasuda, 1999 #61] concluded that the JIP proteins function by aggregating components of a MAP kinase module, including MLK, MKK7, and JNK, and facilitate signal transmission by the protein kinase cascade. JIP1 caused cytoplasmic retention of JNK and inhibition of JNK-regulated gene expression. In addition, JIP1 suppressed the effects of the JNK signalling pathway on cellular proliferation (Dickens et al., 1997). Crystallin αB is a chaperone belonging to the family of small heat shock proteins (Bullard et al., 2004), (Jordan and Wilson, 1998). These proteins form oligomers that bind to partially unfolded substrates and prevent denaturation.

Neurobeachin is a PKA anchoring protein, responsible for membrane trafficking (Wang et al., 2000). It targets PKA to certain cellular compartments (Moss and Gerton, 2001). The MAPKKK8, also named COT proto-oncogene serine/threonine-protein kinase is a not very well characterized protein, but it has been shown by Gandara et al. that it is involved in

cellular activation and proliferation (Gandara et al., 2003), (Chen et al., 2001). The tyrosine kinase, that we found is similar to c-mer, but hardly known (Graham et al., 1995).

Eps15 and its related partner Eps15R play a key role in clathrin-mediated endocytosis of transmembrane receptors (Klapisz et al., 2002), as they are constitutive components of clathrin-coated pits (Poupon et al., 2002). Eps15/Eps15R, epsins and Hrs may function as adaptors between ubiquitinated membrane cargo and either the clathrin coat or other endocytic scaffolds. In addition, through their own ubiquitination, they may further contribute to the amplification of this network in the endocytic pathway (Polo et al., 2002).

ILK is a serine/threonine kinase, interacting with the beta 1-integrin cytoplasmic domain (Hannigan et al., 1996) and thus regulating integrin-mediated signal transduction. Integrin-linked kinase (ILK) is an ankyrin repeat containing serine-threonine protein kinase whose activity is rapidly and transiently stimulated by cell-fibronectin interactions as well as by insulin stimulation. ILK activates AKT/protein kinase B and inhibits the glycogen synthase kinase 3 (GSK-3) activity in a PI3K-dependent manner (Troussard et al., 1999). It may act as a mediator of the inside-out integrin signalling. ILK interacts also with PINCH and PARVIN proteins as well as other intergrin subunits on focal adhesions, forming a large complex in the cytoplasm ((Tu et al., 1999) , (Yamaji et al., 2001); (Grashoff et al., 2003)). Cyclic GMP and cyclic GMP-dependent protein kinases (cGK) play important roles in physiologic processes such as relaxation of vascular smooth muscle and inhibition of platelet aggregation. Two main forms of cGK have been identified: a soluble form designated type I and an intrinsic membrane-bound form designated type II. Orstavik et al. (Orstavik et al., 1997) noted that type I cGK is a homodimer, with each monomer containing a regulatory cGMP-binding domain and a catalytic domain. The proto-oncogene c-yes, that was cloned by Klages (Klages et al., 1993), belongs to the tyrosine family of protein kinases and with 80% homology to src, to the SRC subfamily. c-Yes itself is poorly characterized. The transmembrane kinase c-kit (Yarden et al., 1987) is the receptor for stem cell factor, SCF (stem cell growth factor). It has a tyrosine-protein kinase activity, and binding of the ligand leads to dimerization and autophosphorylation of c-kit and its association with substrates such as PI3K, JAK2 and shc (Deberry et al., 1997), (Lennartsson et al., 1999). Mutations in c-kit are the cause of the white-spotting phenotype (W). White-spotting variants show severe effects in pigmentation, gametogenesis and haematopoiesis (Tan et al., 1990). MOS, is a proto-oncogene, encoded on chromosome 8 (Neel et al., 1982), (Chen et al., 2001). The functions of this serine/threonine kinase, also called oocyte-maturation factor are still not well explored. Another BRII-tail interacting protein is the tyrosine kinase 9, Twinfilin-1, an highly conserved actin monomer-binding protein that regulates cytoskeletal dynamics in organisms from yeast to mammals (Vartiainen et al., 2003) and colocalizes with the activated forms of small GTPases Rac1 and Cdc42 to membrane ruffles and to cell-cell contacts, respectively

(Vartiainen et al., 2000). The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm (McCright et al., 1996). It is one of the major serine/threonine phosphatases and together with PP1 responsible for >50% of all the de-phosphorylation events in the cell (reviewed in (Millward et al., 1999)), one important function is cell cycle control where it blocks G2-M transition. The transient receptor potential cation channel (trp), belongs to a novel mammalian gene family which is essential for agonist-activated capacitative Ca^{2+} entry (Zhu et al., 1996). Capacitative calcium entry (CCE) describes Ca^{2+} influx into cells that replenishes Ca^{2+} stores emptied through the action of IP3 and other agents. It is an essential component of cellular responses to many hormones and growth factors. The molecular basis of this form of Ca^{2+} entry is complex and may involve more than one type of channel. One of them is trp (Montell, 2001). Rab geranylgeranyl transferase, α subunit is described by Detter (Detter et al., 2000) as an enzyme that attaches geranylgeranyl groups to Rab proteins. Posttranslational modification with the geranylgeranyl moiety is essential for the ability of Rab GTPases to control processes of membrane docking and fusion (Rak et al., 2002), (Seabra et al., 2002). Semcap 3 pending protein or 1110020C07RIK contains one PDZ/DHR domain (Strausberg et al., 2000), according to Gene Ontology ™ it has zinc ion binding activity and electron transporter activity, which is important for cell communication. The related protein, SemaF, is a protein involved in axon guidance (Adams et al., 1996).

transcriptional related proteins:

BRII-SF: CtBP1, two RubB like proteins

BRII-LF: zinc finger protein 29

BRII-tail: forkhead L1 transcription factor, LIM homeobox transcription factor

The corepressor CtBP (C-terminal binding protein) is targeting diverse transcription regulators and it is expressed throughout the developmental stages and in a wide range of adult tissues (Postigo et al., 2003). CtBP1 is sumoylated; if the sumoyl group is attached, the originally cytoplasmic protein is found in the nucleus (Lin et al., 2003c). Smad6 represses bone morphogenetic protein-induced Id1 transcription through recruiting CtBP. A consensus CtBP-binding motif, is in the linker region of Smad6 (Lin et al., 2003b). With the BRII-tail two nuclear proteins were fished: the forkhead L1 transcription factor and the LIM homeobox transcription factor. Two identified proteins were RuvB-like, a DNA helicase like protein with DNA unwinding functions in the nucleus (Gohshi et al., 1999).

With the BRII-LF zinc finger protein 29 is associated, a nuclear protein that belongs to the krueppel family of C2H2-type zinc finger proteins (Denny and Ashworth, 1991).

Whereas the forkhead L1 transcription factor is poorly described as being a nuclear, winged helix transcriptional regulator (Kaestner et al., 1993), (Fukuda et al., 2003), the LIM homeobox transcription factor (Lmx1a) is much better explored. It is a transcriptional activator that contains one homeobox domain and two LIM zinc-binding domains. Lmx1a is required for development of the roof plate and, in turn, for specification of dorsal cell fates in the CNS and developing vertebrae, as described in the dreher mouse mutant (Millonig et al., 2000).

structural proteins:

BRII-SF: tubulin β5, tubulin β5 homolog, otoconin-90, ARP8
BRII-tail: cadherin20/7, p50b

Among the structural proteins connected to the BMP signal transduction pathway tubulin β5 is found. Microtubules of the eukaryotic cytoskeleton perform essential and diverse functions. The alpha and beta tubulins represent the major components of microtubules, while gamma tubulin plays a critical role in the nucleation of the microtubule assembly (Lewis et al., 1985), (Oakley, 1992). ARP8 (Actin-related protein 8), belongs to the large family of actin and actin-like proteins. Recently, the function of ARPs in the chromatin-remodeling process have been described (Shen et al., 2003). Otoconin-90 is an extracellular matrix component, the principal matrix protein of mammalian calcitic otoconia, which was found to be a structural homologue of the phospholytic enzyme phospholipase A2 (Thalmann et al., 2001).
Cadherins, among them Cadherin20, are calcium dependent cell adhesion proteins. They preferentially interact with themselves in a homophilic manner in connecting cells; cadherins may thus contribute to the sorting of heterogeneous cell types (Faulkner-Jones et al., 1999). A putative calcium-binding and actin-binding protein is p50b, that is identical with LSP1, with unknown function (Matsumoto et al., 1995).

metabolism :

BRII-SF: sulfotransferase, estrogen preferring, arylsulfotransferase
BRII-LF: carboxylesterase3 (triacylglycerol hydrolase), ectonucleotide pyrophosphatase/ phosphodiesterase 1 allotype b
BRII-tail: Mitochondrial processing peptidase alpha subunit, mitochondrial precursor (α-MPP, P-55)

The sulfotransferase, estrogen preferring is responsible for the sulfation of estradiol and estrone. This enzyme may control the level of the estrogen receptor by sulfurylating free estradiol (Kakuta et al., 1997). It has been found in testis and placenta, later on in other tissues as well (Song et al., 1995). Metachromatic leukodystrophy (MLD) is a lysosomal

storage disease caused by the deficiency of arylsulfatase A (Gieselmann et al., 1991), a lysosomal enzyme that is involved in the degradation of sulfated glycolipids. It exists both as a single chain of 58 kDa (component A) or as a chain of 50 kDa (component B) linked by disulfide bond(s) to a 7 kDa chain (component C).

The carboxylesterase3 (triacylglycerol hydrolase), is an enzyme that is involved in mobilization of triacylglycerol from storage pools in hepatocytes (Dolinsky et al., 2001). Ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) have been implicated in bone mineralization, signalling by insulin and by nucleotides (Bollen et al., 2000) as well as and tumour invasion (Banakh et al., 2002). The ectonucleotide pyrophosphatase/ phosphodiesterase 1 allotype b is a transmembrane protein, bearing the catalytic site extracellular with a structure similar to ALP.

Mitochondrial processing peptidase (MPP), where we pulled down the alpha subunit, cleaves the vast majority of mitochondrial proteins (Gakh et al., 2002).

non-related proteins:

BRII-SF: Rik-protein, Contrapsin, C4binding protein, two unnamed proteins

BRII-LF: cytochrome P450 CYP2J9

BRII-tail: ARVCF isoform B2, mannose-binding lectin associated serine protease-2, unnamed protein

Many of these proteins grouped here are not known or not identified yet. Among them here are three unnamed proteins and one Rik-protein, which was recently cloned (Strausberg et al., 2002). From the characterized proteins, some are involved in the defense against pathogens, like C4binding and protein mannose-binding lectin associated serine protease-2. The C4binding protein (C4bp) is a regulatory component of the serum complement system (Kristensen et al., 1987), the mannose-binding lectin associated serine protease-2, MASP2, is involved in host defense against pathogens through a novel system of complement activation, designated the lectin pathway (Endo et al., 2003).

Besides them we find associated with the BRII-SF Contrapsin, a protein that inhibits trypsin-like proteases, being a serine protease inhibitor, belonging to the serpin family (Ohkubo et al., 1991).

Cytochrome P450 CYP2J9 is a new mouse arachidonic acid omega-1 hydroxylase, distinct from other cytochrome monooxygenases. Arachidonic acid products play important functional roles in the brain (Qu et al., 2001).

ARVCF isoform B2 is a candidate gene for velo-cardio-facial syndrome (Saint-Jore, B., Puech, A., Merscher, S., Xu,H., Kucherlapati, R. and Skoultchi, A., unpublished)

Grouping of BRII associated proteins:

signal transduction			
Identified with BRII-	**Protein**	**accession No**	**function**
transmembrane proteins			
SF	BRII-SF	NP_031587	short splice variant of BRII, low affinity receptor for BMP2, signal transduction via phosphorylation of BRI, Smad- p38- and ERK-pathway, regulation of cell growth, differentiation, and apoptosis (Liu et al., 1995), (Nohe et al., 2002)
SF, tail	IL2Rß	NP_032394	shared by IL-2 and IL-15, no intrinsic catalytic activity, but interaction with cytoplasmic signalling proteins to transduce signals, as JAK1, the adapter Shc (which leads to Ras activation via Grb2 and Sos-1), PI3K and many other proteins via SH2 domains (Friedmann et al., 1996), (Gaffen, 2001) (Gaffen et al., 1996)
tail	c-kit, steel factor	NP_066922	receptor for SCF, tyrosine-protein kinase activity, binding of the ligand leads to dimerization and autophosphorylation, association with substrates as PI3K, JAK2 and shc. Mutations result in white-spotting phenotype (Lennartsson et al., 1999), (Deberry et al., 1997)
SF	protein tyrosine phosphatase, receptor type O	NP_035346	type III receptor protein tyrosine phosphatase, extracellular region containing eight fibronectin type III domains, regulates proliferation, differentiation and viability of epithelial cells (Tomemori et al., 2000), (Beltran et al., 2003)
proteins linked to MAPK pathways			
SF	JNK interacting protein, JIP1	Q9WVI9	MAPKKK8 interacting protein, JNK interacting, potential inhibitor of JNK pathway (Mooser et al., 1999)
LF, tail	MAPKKK8	NP_031772	cellular activation and proliferation (Gandara et al., 2003)
SF	PKC beta	NP_032881	PKC-beta has a role in regulation of osteoclast formation, potentially participating in the ERK pathway (Lee et al., 2003b)
tail	integrin linked kinase	AAH03737	ankyrin repeat containing serine-threonine kinase, interacting with the beta 1-integrin cytoplasmic domain , thus regulating integrin-mediated signal transduction, stimulated by cell-fibronectin interactions, activates AKT/protein kinase B and inhibits the glycogen synthase kinase 3 (GSK-3) activity in a PI3K-dependent manner (Hannigan et al., 1996), (Troussard et al., 1999)
SF	PI3K, P85gamma/p55	Q64143	catalytic subunit of PI3K, together with p110, the regulatory subunit, binding and regulating activated protein tyrosine kinases (Pons et al., 1995), PI3Kgamma regulates phosphoinositides that target PKB and protein phosphorylation that activates MAPK (Bondeva et al., 1998)
chaperones, membrane trafficking			
tail	PTK9, protein tyrosine kinase 9	AAH15081	Twinfilin-1, actin-monomer binding protein, colocalizes with activated forms of Rac1 and Cdc42 to membrane ruffles and to cell-cell contacts, regulates cytoskeletal dynamics (Vartiainen et al., 2000)
SF	cdc37	NP_058022	subunit of the molecular chaperone Hsp90, protein kinase targeting (Stepanova et al., 1996), involved in signal tranduction processes of unstable proteins
SF	Crystallin alpha-B	NP_034094	small heat shock proteins, forming oligomers binding to partially unfolded substrates to prevent denaturation (Bullard et al., 2004)
tail	Rab geranylgeranyl transferase, a subunit; gunmetal	NP_062392	attaches geranylgeranyl groups to Rab proteins, attaching is essential for ability of Rab GTPases to control processes of membrane docking and fusion (Detter et al., 2000)
tail	EGFR substrate 15 related (EPS15R)	NP_031970	clathrin-mediated endocytosis of transmembrane receptors (Klapisz et al., 2002)
LF	Neurobeachin	CAC18812	PKA anchoring, membrane trafficking (Wang et al., 2000)

other signal transducing molecules			
tail	CGMP dependent protein kinase1, beta isozyme (CGK1 beta)	NP_035290	soluble form, cyclic GMP-dependent protein kinase, important roles in physiologic processes as relaxation of vascular smooth muscle and inhibition of platelet aggregation (Orstavik et al., 1997)
tail	proto-oncogene serine/threonine Protein kinase MOS	P00536	proto-oncogene (Neel et al., 1982)
LF	receptor protein-tyrosine kinase	AAA85355	similar to c-mer (Graham et al., 1995)
tail	viral oncogene yes homolog	NP_033561	proto-oncogene (Klages et al., 1993), 80% homology to src
SF	Fibroblast growth factor receptor substrate 3	AAH14819	adapter protein activated by FGF and NTRK1 (Neurotrophin Tyrosine Receptor Kinase1) (Ranzi et al., 2003)
tail	unnamed protein product; homolog to serine/threonine Protein phosphatase 2A, 55 KDA regulatory subunitB beta isoform (PP2A,beta)	BAB31079	regulatory subunit encodes proteins that target PP2A to both nucleus and cytoplasm (McCright et al., 1996), PP2A is one of the major Ser/Thr phosphatases
tail	transient receptor potential cation channel, subfamily C, member 1; transient receptor protein 1	NP_035773	channel, agonist-activated capacitative Ca^{2+} entry (Zhu et al., 1996)
tail	Semcap3 pending protein (= Similar to semaF cytoplasmic domain associated protein 3)	AAH10329	contains one PDZ/DHR domain, may have zinc ion binding activity and electron transporter activity ((Strausberg et al., 2002), Gene Ontology ™), SemaF is a protein involved in axon guidance (Adams et al., 1996)
proteins with nuclear function			
Identified with BRII-	**Protein**	**accession No**	
SF	similar to heterogeneous nuclear ribonucleoprotein R (Rub B-like), Hnrpr protein	AAH04679	DNA helicase like protein (Gohshi et al., 1999)
SF	Rub B-like protein 2 (RuvB)	NP_035434	DNA helicase like protein (Gohshi et al., 1999)
tail	LIM homeobox transcription factor 1 alpha	NP_387501	transcriptional activator, development of the roof plate and specification of dorsal cell fates in the CNS (Millonig et al., 2000)
SF	C-terminal binding protein 1, CtBP1	AAH13702	transcription corepressor, Smad6 can recruit CtBP to repress Id1 transcription (Lin et al., 2003b)
tail	forkhead box L1, forkhead homolog 6	NP_032050	nuclear, winged helix transcriptional regulator (Fukuda et al., 2003)
LF	zinc finger protein 29	NP_033579	belongs to the krueppel family of C2C2-type zinc-finger proteins (Denny and Ashworth, 1991)

proteins with structural functions			
Identified with BRII-	**Protein**	**accession No**	**function**
SF	tubulin, beta 5	NP_035785	microtubules, eukaryotic cytoskeleton, major component (Lewis et al., 1985)
SF	unnamed protein product, tubulin 5 beta homolog	BAB27292	microtubules, eukaryotic cytoskeleton, major component (Lewis et al., 1985)
tail	cadherin20, cadherin 7	NP_035930	calcium dependent cell adhesion proteins, connecting cells, sorting of heterogeneous cell types (Faulkner-Jones et al., 1999)
SF	otoconin-90	AAC99455	extracellular matrix component, component of otoconia (Thalmann et al., 2001)
SF	ARP8, actin-related protein 8 homolog	NP_081769	involvement in the chromatin-remodelling process (Shen et al., 2003)
tail	p50b	BAA08541	identical with LSP1, a putative calcium-binding and actin-binding protein, (Matsumoto et al., 1995)

proteins with metabolic functions			
Identified with BRII-	**Protein**	**accession No**	
LF	ectonucleotide pyrophosphatase/ phosphodiesterase 1 allotype b	AAK84174	transmembrane protein, catalytic site extracellular, implicated in bone calcification, type II diabetes, control of purinergic signalling and tumour invasion (Banakh et al., 2002), structure similar to ALP
LF	carboxylesterase3 (triacylglycerol hydrolase)	NP_444430	involved in mobilization of triacylglycerol from storage pools (Dolinsky et al., 2001)
SF	arylsulfatase A precursor (ASA) (Cerebroside-sulfatase)	P50428	lysosomal enzyme that is involved in the degradation of sulfated glycolipids (Gieselmann et al., 1991)
SF	sulfotransferase, estrogen-preferring	P49891	Sulfation of estradiol and estrone, may control the level of the estrogen receptor by sulfurylating free estradiol (Kakuta et al., 1997)
tail	Mitochondrial processing peptidase alpha subunit, mitochondrial precursor (Alpha-MPP) (P-55)	Q9DC61	Mitochondrial processing peptidase (MPP) cleaves the vast majority of mitochondrial proteins (Gakh et al., 2002)

proteins with non-related functions			
with BRII-	**Protein**	**accession No**	**function**
SF	2810429C13Rik protein	AAH06707	
SF	unnamed protein product	BAB28688	
SF	unnamed protein product	BAB26511	
tail	unnamed protein product	BAB24410	
SF	C4b binding protein	NBMSC4	regulatory component of the serum complement system (Kristensen et al., 1987)

tail	mannose-binding lectin associated serine protease-2	BAA34674	involved in host defense against pathogens through a novel system of complement activation, designated the lectin pathway (Endo et al., 2003)
LF	cytochrome P450 CYP2J9	AAK59868	a new mouse arachidonic acid omega-1 hydroxylase, arachidonic acid products play important functional roles in the brain (Qu et al., 2001)
tail	ARVCF isoform B2	AAK64217	candidate gene for velo-cardio-facial syndrome (Saint-Jore,B., Puech,A., Merscher,S., Xu,H., Kucherlapati,R. and Skoultchi,A., unpublished)

SF	Serine proteinase inhibitor A3K precursor (Contrapsin)	P07759	inhibits trypsin-like proteases, extracellular, belongs to the serpin family (Ohkubo et al., 1991)

Tab 4.4 Functional groups of BRII interacting proteins
Above the BRII interactors are grouped according to their functions.

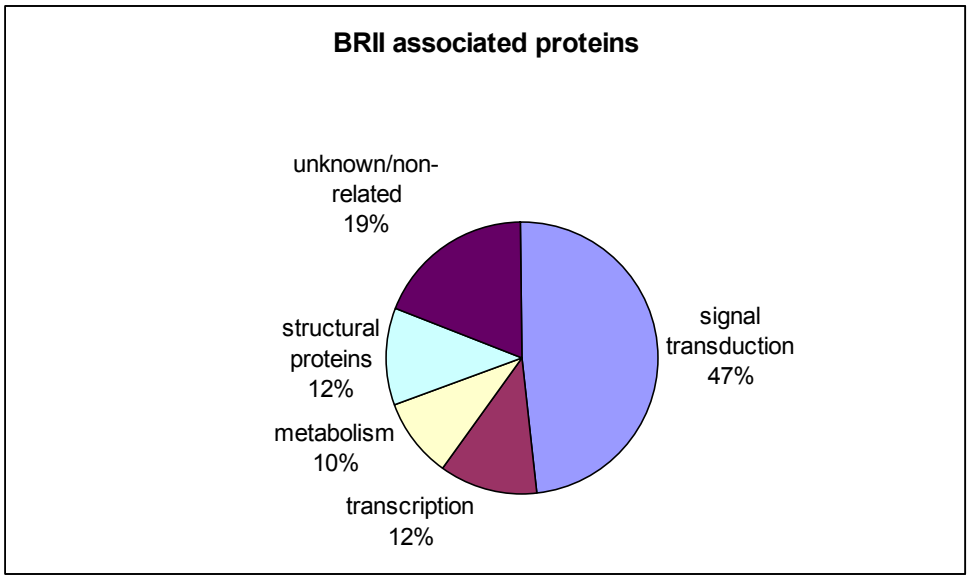


Fig 4.21 Functional groups of BRII-interacting proteins
Schematic representation of the grouping of BRII interactors. The majority of all interacting proteins is involved in signal transduction, some proteins are so far unknown or can not be related to the BMP pathway. A relatively small percentage are structural and metabolic proteins as well as proteins involved in transcriptional regulation.

The majority of the BRII interactors are molecules involved in signal transduction processes. Besides that we find some proteins involved in transcriptional processes, some structural proteins as well as some metabolic proteins. This leads to the conclusion that the BRII is in addition to signal transduction involved in other cellular procedures as regulation of cellular stability, membrane shape and metabolic processes.

Figure 4.22 is explaining how the BRII is embedded in the cellular network, regulating and influencing many important cellular processes.

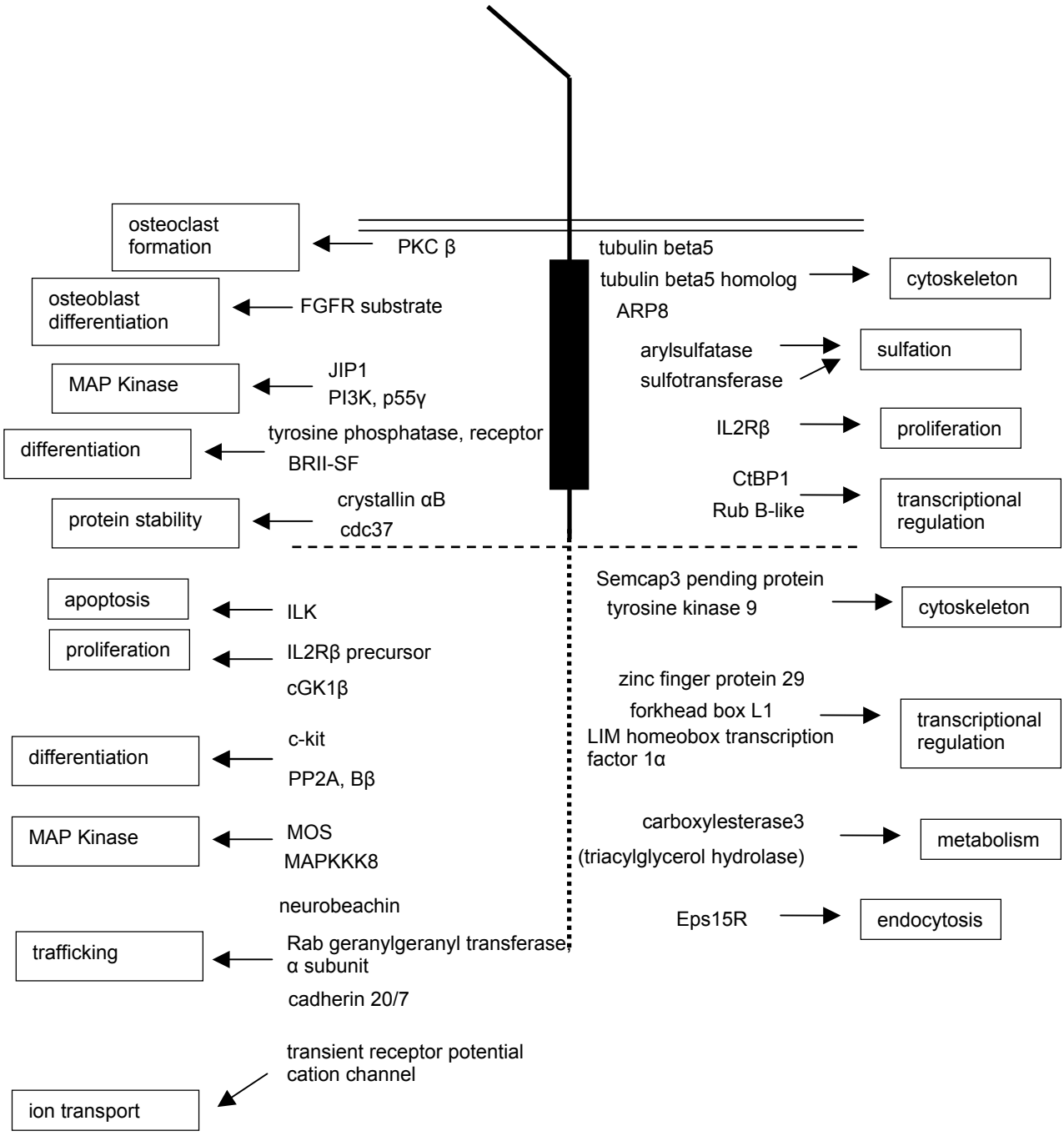


Fig 4.22 Functional groups of BRII-interacting proteins
Grouping of BRII interactors. Some of the BRII interacting proteins can be linked to regulation of bone maintenance and growth. Others regulate important cellular processes like apoptosis, endocytosis and differentiation, whereas some are involved in the maintenance of the cell.

To confirm some interactions uncovered we performed GST-pulldowns with C2C12 lysates followed by western blotting with specific antibodies. For PKCβ and for CtBP the interaction could be confirmed. PKCβ was identified as a BRII-SF interacting protein, but in the specific pulldown it precipitated with the BRII-SF as well as the BRII-LF. No interaction was proven with the BRII-tail, demonstrating that the interaction site is in the kinase domain.

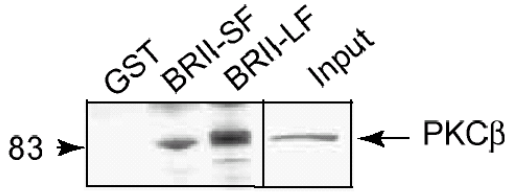


Fig 4.23a PKCβ interacted with BRII-GST-SF and -LF
$2x10^6$ C2C12 cells were lysed and mixed with GST-beads or GST-BMP receptor fusion proteins coupled on beads. The precipitate was washed and subjected to SDS-PAGE and western blotting. PKCβ was detected with antibodies specific for PKCβ. While GST alone (lane 1) did not interact, the GST-BRII-SF and GST-BRII-LF showed interaction with PKCβ (lanes 2-3). PKCβ could be detected in the lysate (lane 4).

CtBP could be precipitated with BRII-SF as well as BRII-LF and no interaction was observed with the tail. So the BRII can be directly linked to transcriptional regulation via its kinase domain interacting with CtBP.

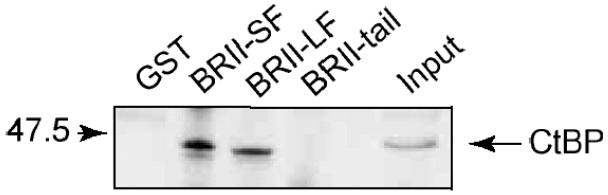


Fig 4.23b CtBP interacted with BRII-GST-SF and -LF
C2C12 cell lysate was mixed with GST-beads or GST-BMP receptor fusion proteins coupled on beads. CtBP could be detected using specific antibodies. GST alone (lane 1) did not co-precipitate, GST-BRII-SF and GST-BRII-LF showed interaction with CtBP (lanes 2-3), whereas the tail did not (lane 4). As control, CtBP was detected in the lysate (lane 5).

To further exclude non-specific interactions using bacterially expressed proteins, we chose to examine the BRII-PKCβ interaction with transiently overexpressed proteins. COS7 cells were transfected with the indicated receptor constructs and either left untreated or stimulated with 80μM BMP2 for 1h. BRII-His_6 was precipitated with Ni-NTA agarose and bound proteins were detected by western blotting.

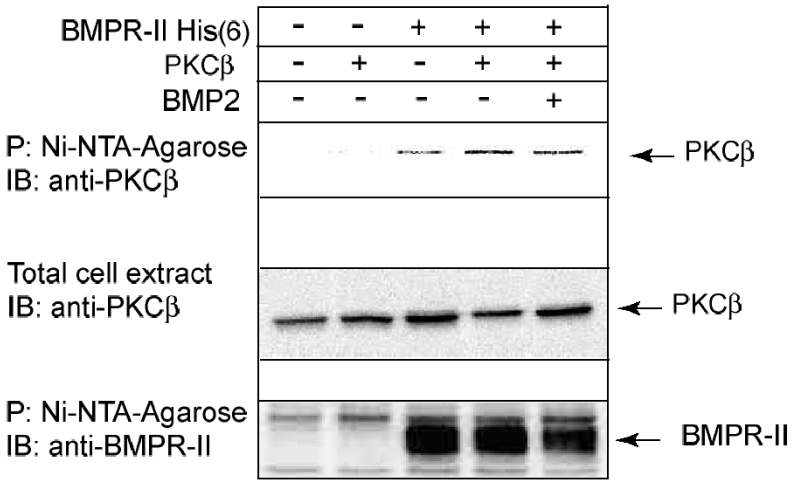


Fig 4.23c PKCβ interacted with overexpressed His-BRII-LF
COS7 cells were transfected with the indicated constructs and immunoprecipitation using Ni-NTA-Agarose was performed. Here, BRII-His_6 was precipitated. Associated PKCβ was detected with antibodies specifically recognizing PKCβ. Endogenous PKCβ did not bind the Ni-NTA-Agarose (lane 1), as well as overexpressed did not (lane 2). PKCβ was co-precipitated with BRII-LF, independent of overexpression of PKCβ (lanes 3-4) or stimulation with BMP2 (lane 5). Endogenous as well as overexpressed PKCβ could be detected in the lysate (blot2). Overexpressed BRII-LF was detected with specific antibodies (blot3).

As depicted above, cellular BRII still interacts with PKCβ, stimulation with BMP2 did not change the intensity of the proven interaction. Surprisingly the cellular level of PKCβ is that high that overexpression showed only marginal differences in the expression level. Endogenous as well as overexpressed PKCβ interacted with the BRII.

Besides, the interaction could be confirmed for ILK, IL2Rβ, cGK1β and cdc37 (Diploma Thesis Raphaela Schwappacher) and for Eps15R as well (Christina Sieber).

4.3 c-kit as a BRII-associated protein (Hassel et al., submitted)

One of the identified BRII-LF interacting proteins was c-kit, a receptor tyrosine kinase, belonging to the split-kinase domain subfamily, which is important for haematopoiesis, melanogenesis and germ cell development (Chian et al., 2001), (Linnekin, 1999).

4.3.1 MALDI of c-kit

For the GST pulldown we used GST-fusion proteins of the cytoplasmic part of BRII-LF. As described above, three different BMP receptor constructs were generated and used for the pulldown, the GST-BRII-SF, the GST-BRII-LF and the GST-BRII-tail, GST alone served as a control. BRII interacting proteins were isolated from the mesenchymal, myoblast precursor cell line C2C12, which is expressing BRII-LF as well as BRII-SF and BRIa (A. Nohe, personal communication). Proteins bound to GST fusion constructs were resolved by two-dimensional gel electrophoresis and identified by peptide mass fingerprinting using MALDI TOF MS. Using the search engine ProFound, c-kit was identified as a protein bound to BRII-GST-tail, the migration of the spot was at pH 5,0 and the size was about 100kDa.

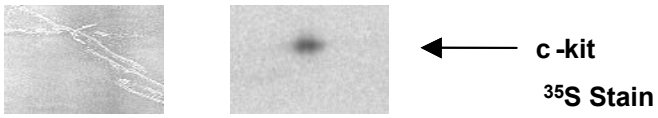


Fig. 4.24 c-kit on the 2D gels
Pulldown with GST-tail was performed from C2C12 cell lysate. The left picture shows part of the silver-stained 2D gel of the pulldown, the right picture an image of the radioactive gel where c-kit was detected as a ^{35}S labelled, cellular protein, precipitated with GST-tail.

As depicted above, c-kit was not visible on the silver-stained gel but could be detected as a ^{35}S-labelled protein. This shows the sensitivity of the method. Even very low amounts of protein could be visualized using the metabolic label. Moreover with the MALDI-TOF very tiny amounts of protein could be measured and identified.

ID	Protein	accession No	Probability	Z-Value	Sequ. Coverage	Experimental value		Theoretical value	
						pI	MW (kDa)	pI	MW (kDa)
SH25	c-kit, steel factor	NP_066922	4,30E-01	0,16	7%	5,0	100	6,6	110,24

Tab. 4.5 Identification of c-kit
Using MALDI TOF MS we identified c-kit being a BRII-tail interacting protein. The masslist was pasted into the search engine ProFound. c-Kit was identified with 43% probability and 7% coverage. There was only a slight difference between the experimental pI and molecular weight from the theoretical ones.

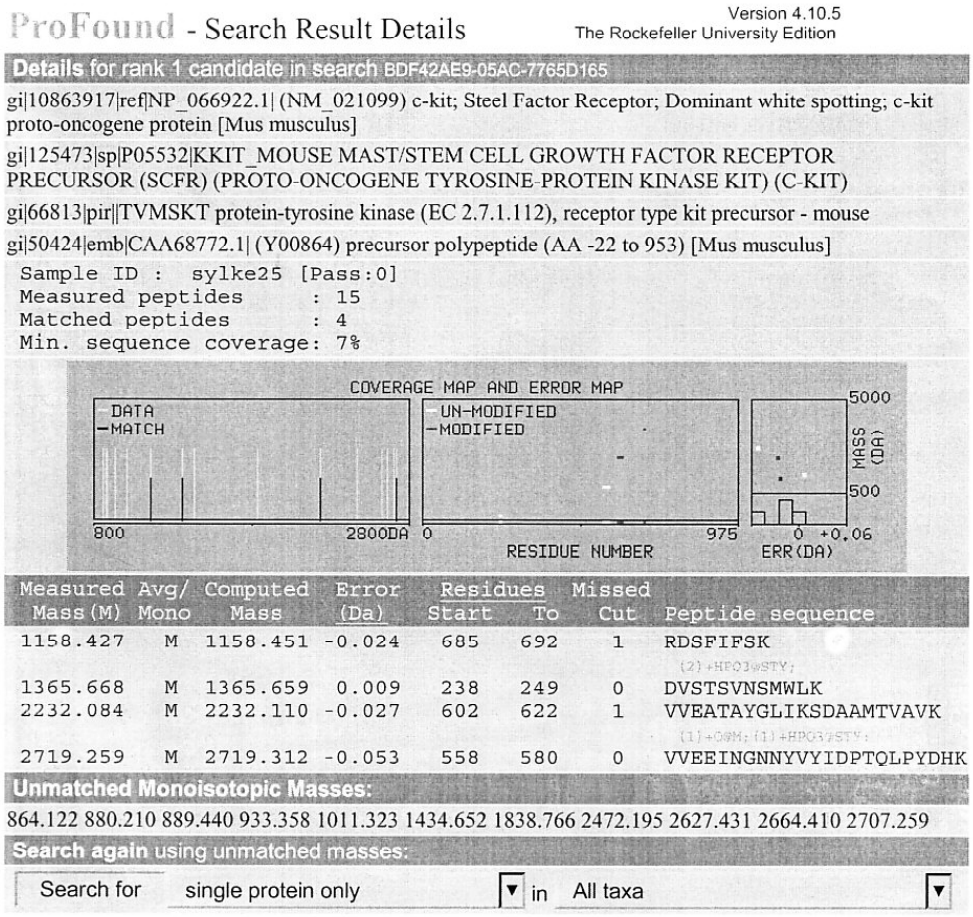


Fig. 4.26 Search results of the identification of c-kit
Identification of c-kit as BMP receptor tail interacting protein. The ProFound program identified c-kit according to the masslist of flying peptides.

4.3.2 Interaction of BRII and c-kit

4.3.2.1 Interaction of BRII and c-kit in a pulldown assay

The interaction of BRII and c-kit was confirmed by a pull-down assay with the BRII-GST constructs or GST alone, coupled to Glutathion-Sepharose beads, using C2C12 cell lysate, followed by one dimensional gel electrophoresis and western blotting with antibodies specific for endogenous c-kit.

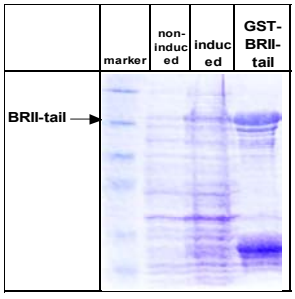


Fig. 4.27 Purification of GST-BRII-tail
Purification of GST-BRII-tail using Glutathion-Sepharose. Lane 3 shows the purified GST-BRII-tail and the additional tail breakdown products.

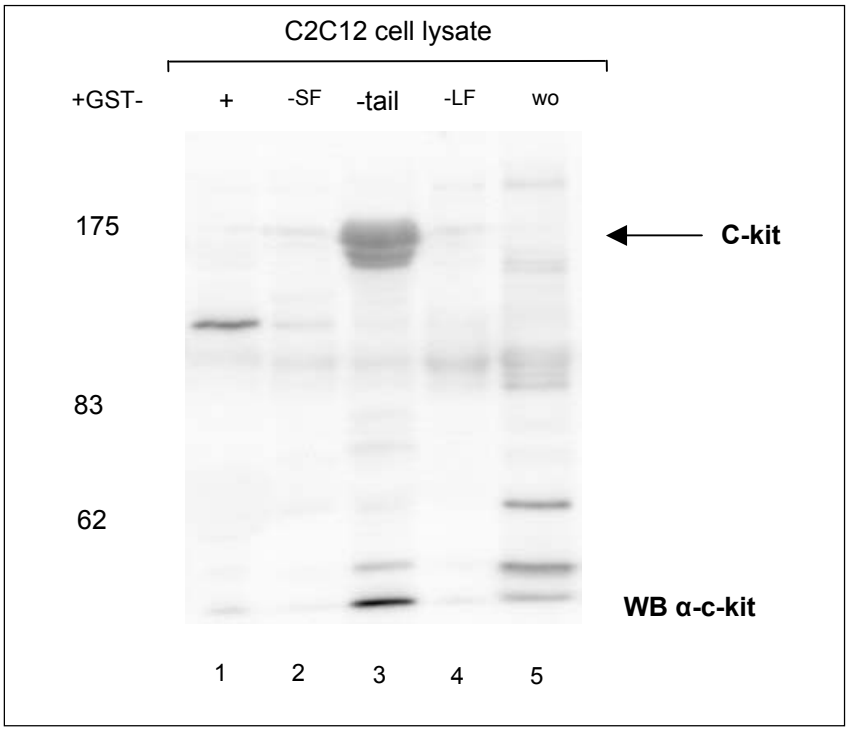


Fig 4.28 BRII-GST-tail interacts with c-kit
$2x10^6$ C2C12 cells were lysed and mixed with GST-beads or GST-BMP receptor fusion proteins coupled on beads. The precipitate was washed and subjected to SDS-PAGE and western blotting. c-Kit was detected with antibodies specific for c-kit. While GST alone (lane 1) and the GST-BRII-SF and GST-BRII-LF did not interact, only the tail showed interaction with c-kit (lane 3). In the lysate, c-kit could not be detected (lane 5).

BRII-GST-tail bound c-kit (lane 3), while GST alone (lane 1) or BRII-GST-SF or BRII-GST-LF (lanes 2, 4) did not. Due to its low endogenous expression level, c-kit could not be detected in the C2C12 cell lysate (lane 5).

4.3.2.2 Co-Immunoprecipitation of BRII-LF and c-kit in transfected 293T cells

The interaction between c-kit and BRII-tail was confirmed in transfected mammalian cells. Here, c-kit interacted with BRII-LF, in contrast to the GST-pulldown, assuming that the conformation of the BRII-GST-LF is different from the BRII-LF in vivo, exposing different epitopes.

c-Kit was coexpressed with HA-tagged BRII-LF in 293T cells. After 2 hours of starvation the cells were either left untreated or stimulated with 20nM BMP2, 0,1µg/ml SCF or both for 1 hour. c-Kit was immuno-purified with anti c-kit antibodies and Protein A Sepharose. Interacting proteins were detected with anti-HA antibody (Fig. 4.29). As a control, BRII-LF-HA was precipitated with anti-HA-antibody (lane 2). BRII-LF interacted with c-kit when coexpressed, in the absence as well as in the presence of different ligands as 20nM BMP2, 0,1µg/ml SCF or a combination of both (lanes 4-7). BMP2 enhanced the interaction (lanes 5, 7).

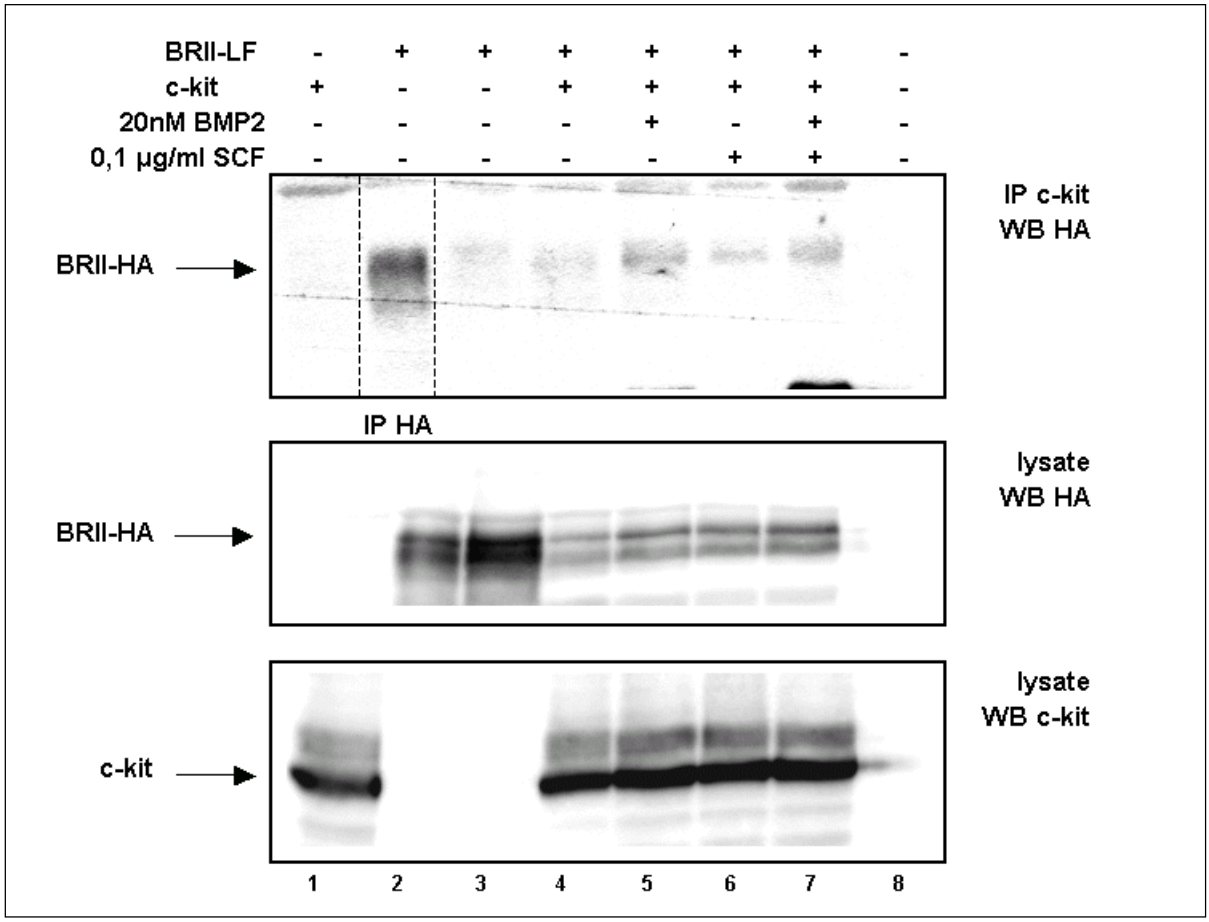


Fig 4.29 BRII-LF interacts with c-kit in an overexpression system
293T cells were transfected with the indicated receptor constructs. After stimulation for 1h or not and lysis of the cells, the lysate was precipitated with polyclonal rabbit antibodies directed against c-kit and protein A Sepharose. The washed beads were subjected to SDS-PAGE and western blotting. Blotting with anti-HA antibody detects c-kit bound BRII-LF. As a control the lysates were blotted against c-kit and BRII-LF-HA, respectively .

To investigate whether the interaction region specifically occurs in the BRII-tail region we performed co-immunoprecipitation experiments in overexpression systems (293T or COS7 cells) using BRII-SF, which does not posses the C-terminal tail. In contrast to BRII-LF, BRII-SF was unable to bind c-kit or c-kit mutants (data not shown). These results suggest that the interaction region is located in the tail region.

The demonstrated interaction of BRII-LF and c-kit was proven to be c-kit kinase independent. In an in vitro kinase assay with c-kit or c-kit KN, a kinase deficient mutant where K623 was exchanged to arginine resulting in a receptor unable to bind ATP, it was tested whether BRII-LF and c-kit K623N can interact. 293T cells were transiently transfected with c-kit, c-kit KN or c-kit KN together with BRII-LF and either left untreated or stimulated with the appropriate ligands. In Fig. 4.30, the lanes 1-4 show the kinase assay of c-kit, demonstrating that both ligands did not influence c-kit auto phosphorylation. In the lanes 5-8 it can be observed that c-kit KN was not able to phosphorylate itself, independent of any stimulation. When c-kit KN

was cotransfected with kinase active BRII-LF, the BRII-LF could be co-precipitated with c-kit antibodies, resulting in a phosphorylation signal on the autoradiogramm (lanes 9-12). The kinase deficient c-kit KN did not give any signal. Equal expression levels are demonstrated on the lysate westernblots. Due to the same apparent molecular weight on an SDS-PAGE, BRII-LF and c-kit interaction could not be detected using this method.

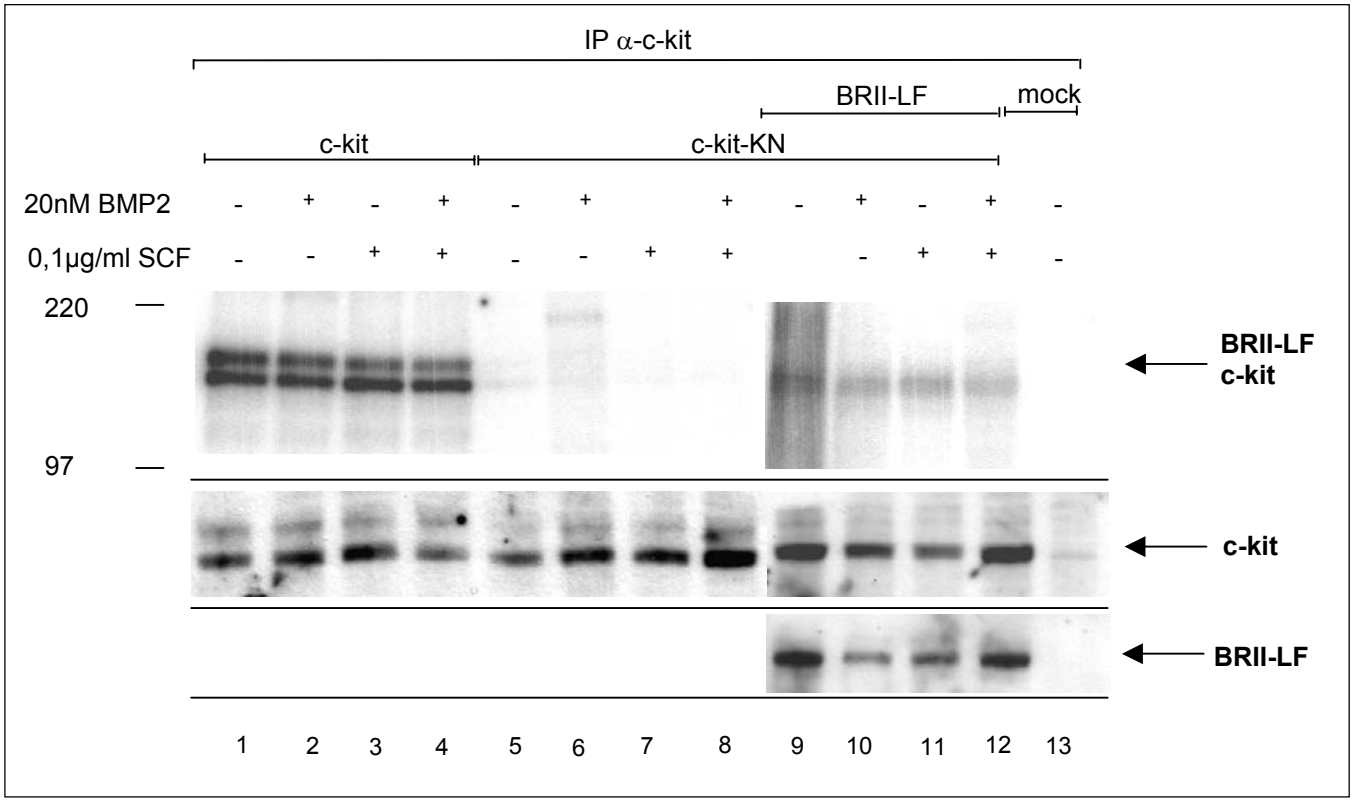


Fig 4.30 BRII-LF interacts with c-kit KN in an overexpression system
293T cells were transfected with the indicated receptor constructs. After starvation, stimulation and lysis of the cells, the lysate was precipitated with polyclonal rabbit antibodies directed against c-kit and Protein A Sepharose. The washed beads were subjected to in vitro kinase assay. Auto-phosphorylated proteins are visualised with a phosphoimager. Equal protein expression is shown by blotting the lysate with anti-c-kit antibody (lane 2) or anti HA-antibody (lane 3), respectively.

As demonstrated in Fig 4.29 and 4.30, BRII-LF interacts with c-kit as well as with c-kit KN. Because different techniques were used to prove this, the interaction should be compared. Therefore 293T cells were transfected with BRII-LF alone or in combination with c-kit or c-kit KN. After immuno-purification of c-kit or c-kit KN bound BRII-LF was detected with anti-HA antibody (Fig. 4.31). As a control BRII-LF was immuno-purified with anti-HA (lane 1). BRII-LF was co-precipitated with c-kit (lane 3).

Even if the expression of BRII-LF and c-kit KN together was weaker than the one of BRII-LF and c-kit, the interaction of BRII-LF with c-kit KN was still detectable. To demonstrate that c-kit KN is kinase-deficient and is not able to undergo tyrosine phosphorylation the immunoblot with c-kit was stripped and reprobed with PY99, an anti-phospho tyrosine antibody. As

expected (Fig. 4.31, panel D) overexpressed c-kit showed auto-phosphorylation whereas c-kit KN showed only a very weak band.

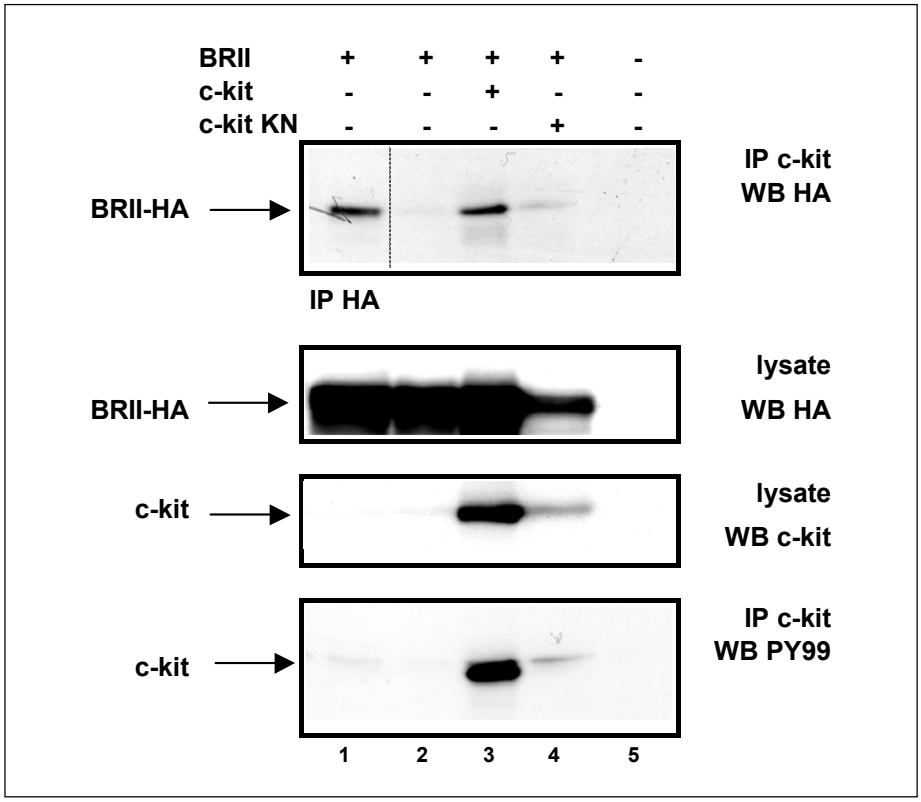
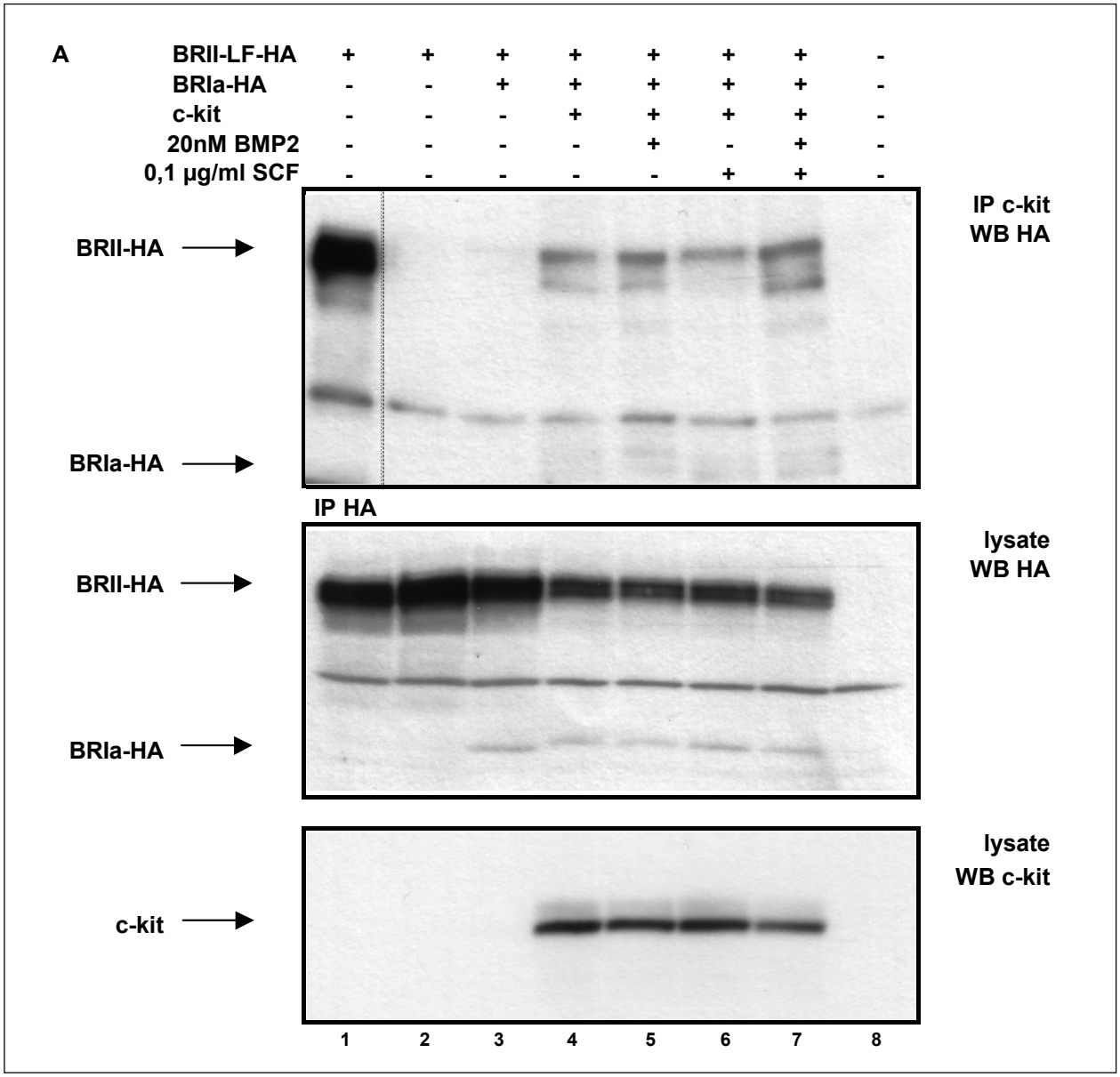


Fig 4.31 BRII-LF interacts with c-kit as well as with c-kit KN in an overexpression system
293T cells were transfected with the indicated receptor constructs. After lysis of the cells, the lysate was precipitated with polyclonal rabbit antibodies anti c-kit and protein A Sepharose. Probing the blot with anti-HA antibody detects c-kit bound BRII. As a control the lysates were blotted with anti c-kit and anti-HA antibody. To demonstrate the phosphorylation deficiency of c-kit KN the immuno-purified c-kit was detected with anti phospho-tyrosine antibody.

As demonstrated (Gilboa et al., 2000), BRII and BRI form ligand-independent complexes on the cell surface. Addition of ligand increases the percentage of hetero-oligomers. Therefore we wanted to address the question, whether BRIa, the type I BMP receptor, is binding to the BRII-LF/c-kit complex, thus forming a trimeric signalling complex. 293T cells were transfected with BRII-LF-HA alone or in combination with c-kit and BRIa-HA or both. After starvation, stimulation with the different ligands and immunoprecipitation of c-kit, the proteins were separated on SDS-PAGE and blotted. The membrane was incubated with anti-HA to detect BRII-LF and BRIa bound to c-kit simultaneously (Fig. 4.32, panel A).
To test whether BRIa alone can interact with c-kit we cotransfected both receptors, precipitated c-kit and probed the blot with anti-HA to detect bound BRIa (Fig. 4.32, panel B).


A
BRII-LF-HA + + + + + + + -
BRIa-HA - - + + + + + -
c-kit - - - + + + + -
20nM BMP2 - - - - + - + -
0,1 µg/ml SCF - - - - - + + -
BRII-HA
BRIa-HA
IP c-kit
WB HA
IP HA
BRII-HA
BRIa-HA
lysate
WB HA
c-kit
lysate
WB c-kit
1 2 3 4 5 6 7 8

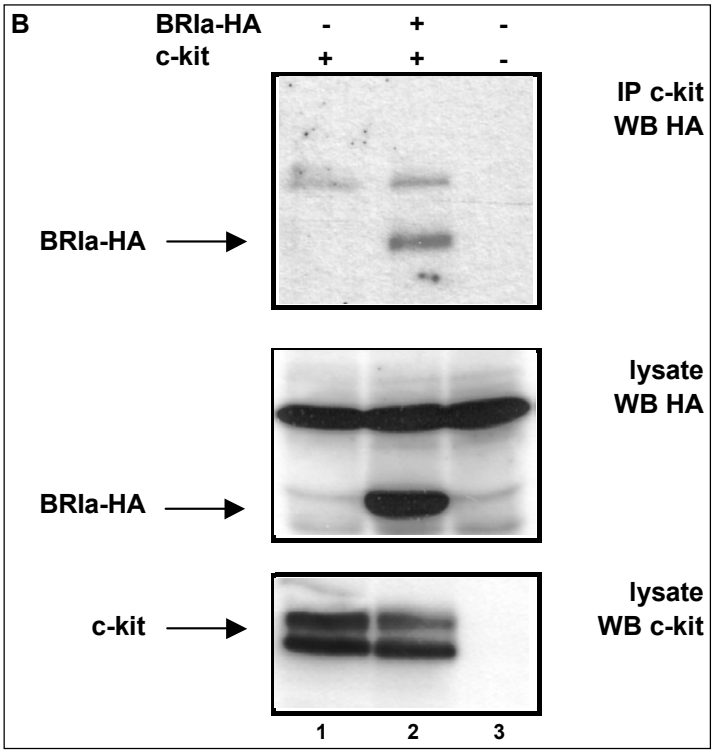


Fig 4.32 BRIa interacts with c-kit alone or in combination with BRII-LF
293T cells were transfected with the indicated receptor constructs. After lysis of the cells, the lysate was precipitated with anti c-kit. Probing the blot with anti-HA antibody detects c-kit bound BRII-LF and/or BRIa, respectively. As a control the lysates were blotted with anti c-kit and anti-HA antibody.

As depicted in Fig. 4.32 not only BRII-LF co-precipitates with c-kit, but BRIa as well. For the ternary-complex no ligand effect was observed (panel A, lanes 4-7). BRII-LF as well as BRIa could be precipitated with c-kit. To examine whether the interaction of c-kit with BRIa is mediated by the type II receptor, BRIa was cotransfected with c-kit (Fig. 4.32, panel B). Surprisingly, BRIa alone could interact with c-kit in a ligand independent manner (panel B, lane 2). Therefore we can conclude that we find on the cell surface complexes of BRII with c-kit (Fig. 4.29) as well as BRIa c-kit complexes (Fig. 4.32, panel B). There might be ternary ones composed of BRII, BRIa and c-kit (Fig. 4.4.6, panel A). The possible complexes are preformed complexes which are built in a ligand independent manner.

4.3.2.3 In vivo interaction between endogenous BRII-LF and c-kit in C2C12 cells

It is possible that the interaction between overexpressed BRII-LF and c-kit was driven by the large amount of proteins produced in the overexpression system. To address this question, it should be determined if the interaction occurred between endogenously expressed proteins in tissue cultured cells.

Initially, the appropriate antibodies for that purpose were tested (Fig. 4.33) to facilitate further examinations. Two different anti-c-kit antibodies were tested for immunoprecipitation, one home made from L. Rönnstrand (University of Malmö, Sweden) (LR, lane 1) and one

commercially available antiserum from Santa Cruz (sc, lane 2). Additionally, five BRII-LF recognising antisera were tested: BRII T18 and G17 from Santa Cruz (lanes 3-4), BRII 1805 and 1806 from orbigen (lanes 5-6) and home-made polyclonal rabbit SMN antiserum (LICR, Uppsala, Sweden) (lane 7). Western blotting of immunoprecipitations using these antibodies revealed that the c-kit antibody from Santa Cruz is precipitating endogenous c-kit in a more efficient way than the home made one (lanes 1-2). Moreover the capability of the anti BRII-LF antibodies to precipitate endogenous BRII-LF and together with it, associated c-kit, showed variance. Whereas the Santa Cruz antibodies and the home made SMN were inefficient (lanes 3-4, 7), both orbigen products coprecipitated c-kit (lanes 5-6). Therefore the following endogenous co-immunoprecipitations were performed using anti c-kit sc and anti BRII-LF 1805 from orbigen.

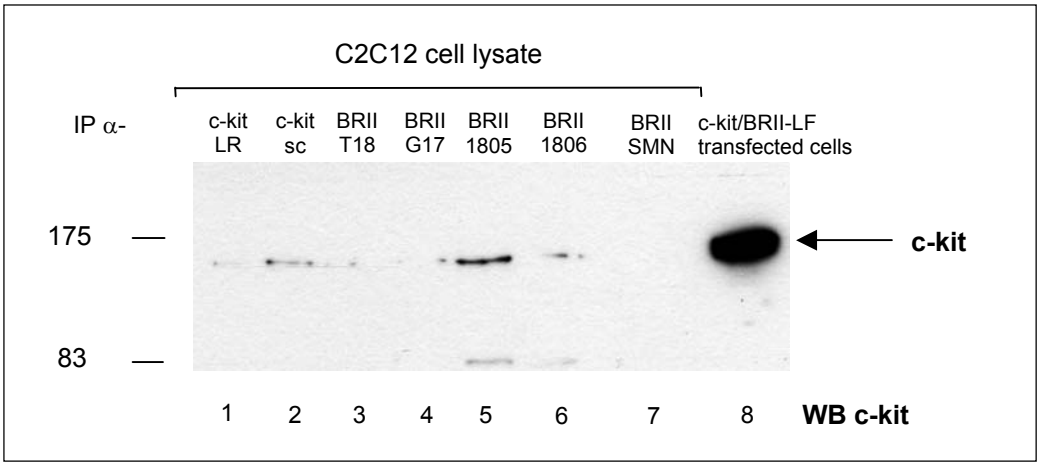


Fig 4.33 BRII-LF interacts with c-kit in C2C12 cells
$6x10^6$ C2C12 cells were lysed and the cleared lysate was immunoprecipitated with different antibodies recognising c-kit (lanes 1-2) or BRII (lanes 3-7). The immunoprecipitate was subjected to western blotting and detected with anti-c-kit antibodies (LR). c-Kit could be co-precipitated with the BRII-LF (lanes 5-6). As a control the precipitation of c-kit (lanes 1-2) is shown as well as the detection of c-kit in lysate from transfected 293T cells (lane 8).

To test ligand dependency of the proven endogenous interaction of BRII-LF and c-kit, immunoprecipitation of c-kit in stimulated and non-stimulated C2C12 cells was performed (Fig. 4.34). As controls for the immunoprecipitation anti-BRII 1805 antibody was used which precipitated BRII-LF and weakly co-precipitated c-kit (Fig. 4.33, lane 5, Fig. 4.34, lane 5).

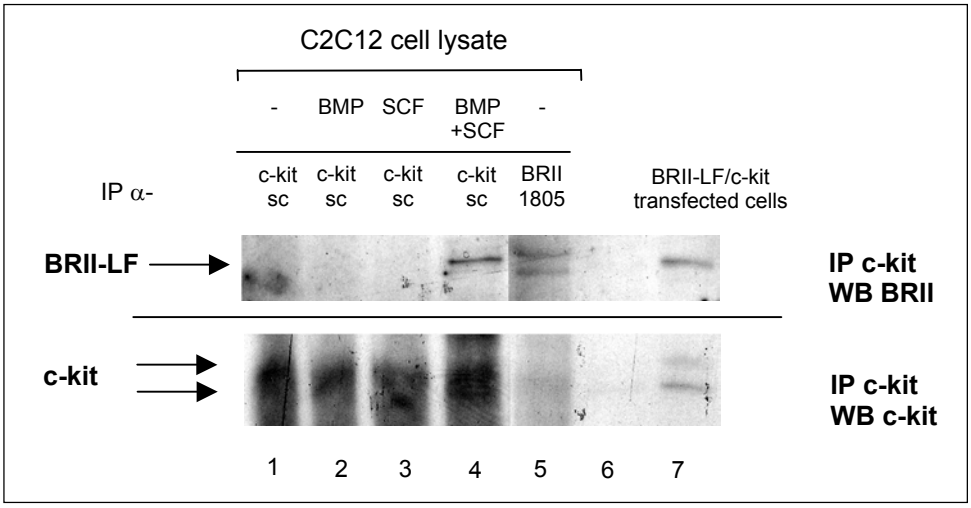


Fig 4.34 BRII-LF interacts with c-kit in C2C12 cells
$6x10^6$ C2C12 cells were starved and subsequently stimulated with 20nM BMP2 for 1h (lane 2), 0,1µg/ml SCF (lane 3), with both ligands (lane 4) or left untreated (lane 1). After lysis the cleared lysate was immunoprecipitated with antibodies recognising c-kit (lanes 1-4). The immunoprecipitate was subjected to western blotting and detected with anti-c-kit antibodies. As a control we precipitated with anti-BRII-LF antibody (1805) (lane 5).
Upper panel: westernblot probed with anti-BRII antibody (G17). The filter was stripped and reprobed with anti c-kit antibody (lower panel).

As demonstrated above, we could prove that endogenously expressed BRII-LF interacts with c-kit in C2C12 cells (Fig. 4.33). Stimulation with both ligands, strongly enhances the interaction, in contrast to the situation when the receptors are transiently overexpressed (compare Fig. 4.34, lane 4 with Fig. 4.33). As a control BRII-LF was precipitated with BRII antibodies and blotted with anti BRII and anti c-kit antibody (lane 5). Unfortunately the binding of c-kit to the c-kit antibodies sc could not be abrogated using the corresponding blocking peptide C-17 (data not shown).

4.3.3 Phosphorylation of BRII and c-kit of transfected and endogenous receptors

4.3.3.1 Phosphorylation of c-kit in transfected 293T cells

Having established that the BRII-LF interacts with c-kit, the influence of the receptors on each other should be examined. As described by Blume-Jensen, c-kit is highly serine phosphorylated in stimulated and in non stimulated PAE-cells (Blume-Jensen et al., 1995). The BRII-LF is a constitutively active serine/threonine kinase, as shown in the in vitro kinase assay, using the GST-fusion proteins (Fig. 4.19), leading to the conclusion that BRII-LF might phosphorylate c-kit as they are forming ligand-independent complexes. So the phosphorylation pattern of c-kit alone and co-transfected with BRII-LF should be determined. 293T cells were transfected with c-kit alone or in combination with BRII-LF and in vivo labelling with ^{32}P orthophosphate was performed. After starvation and stimulation with BMP2 or SCF and immunoprecipitation of c-kit, radioactive proteins were separated on a SDS-

PAGE and detected on a phosphoimager screen. c-Kit appears as a ^{32}P-labelled double band.

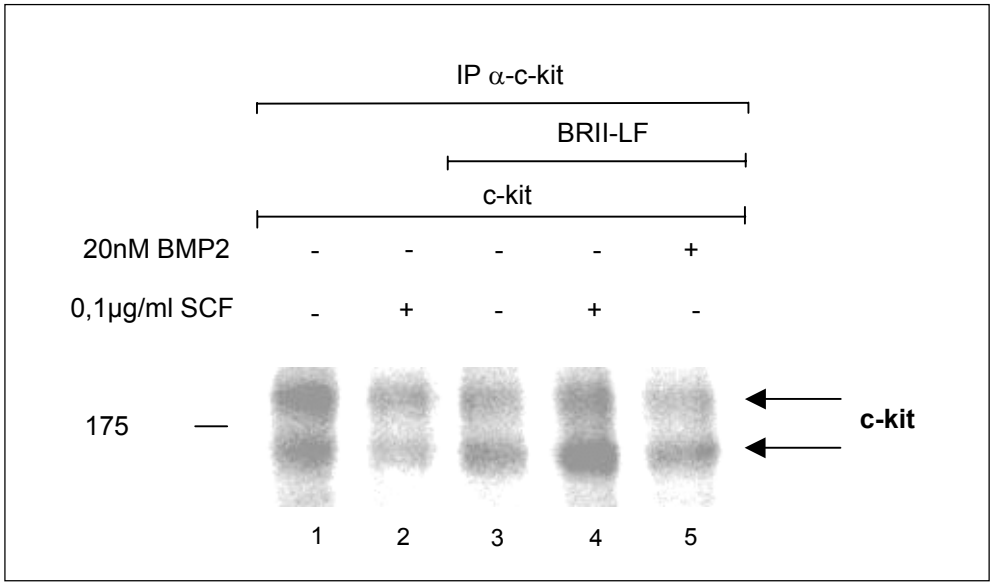


Fig 4.35 Phosphorylation of c-kit in transfected 293T cells
293T cells expressing c-kit (lanes 1-5) alone or together with BRII-LF (lanes 3-5), respectively were incubated with 1mCi/ml ^{32}P-orthophosphate, stimulated or not with 0,1µg/ml SCF (lanes 2, 4) or 20nM BMP2 (lane 5) for 1h and lysed in 1% Triton lysis buffer containing protease-and phosphatase inhibitors. The lysate was precipitated with anti c-kit antibody and protein A Sepharose and subjected to SDS-PAGE after extensive washes. ^{32}P marked c-kit is detected on the phosphoimager screen.

We did not see any significant changes in total phosphorylation of c-kit upon treatment of cells with SCF or BMP2, reflecting the high basal level of c-kit phosphorylation. Stimulation of c-kit with 0,1µg/ml SCF did not significantly enhance the phosphorylation observed on the ^{32}P-gel, although stimulation with SCF leads to receptor-dimerization, followed by auto-phosphorylation in trans on tyrosine residues (Ullrich and Schlessinger, 1990).

Transfection with BRII-LF or stimulation with BMP2 did not have any effect on the total phosphorylation of c-kit. But still the phosphorylation of some residues could potentially be affected. To address this question we performed phospho-peptide mapping of c-kit and c-kit with BRII-LF not stimulated or treated with different ligands (Fig. 4.36). The c-kit band from Fig. 4.35 was excised, trypsin digested and subjected to 2D phospho peptide mapping.

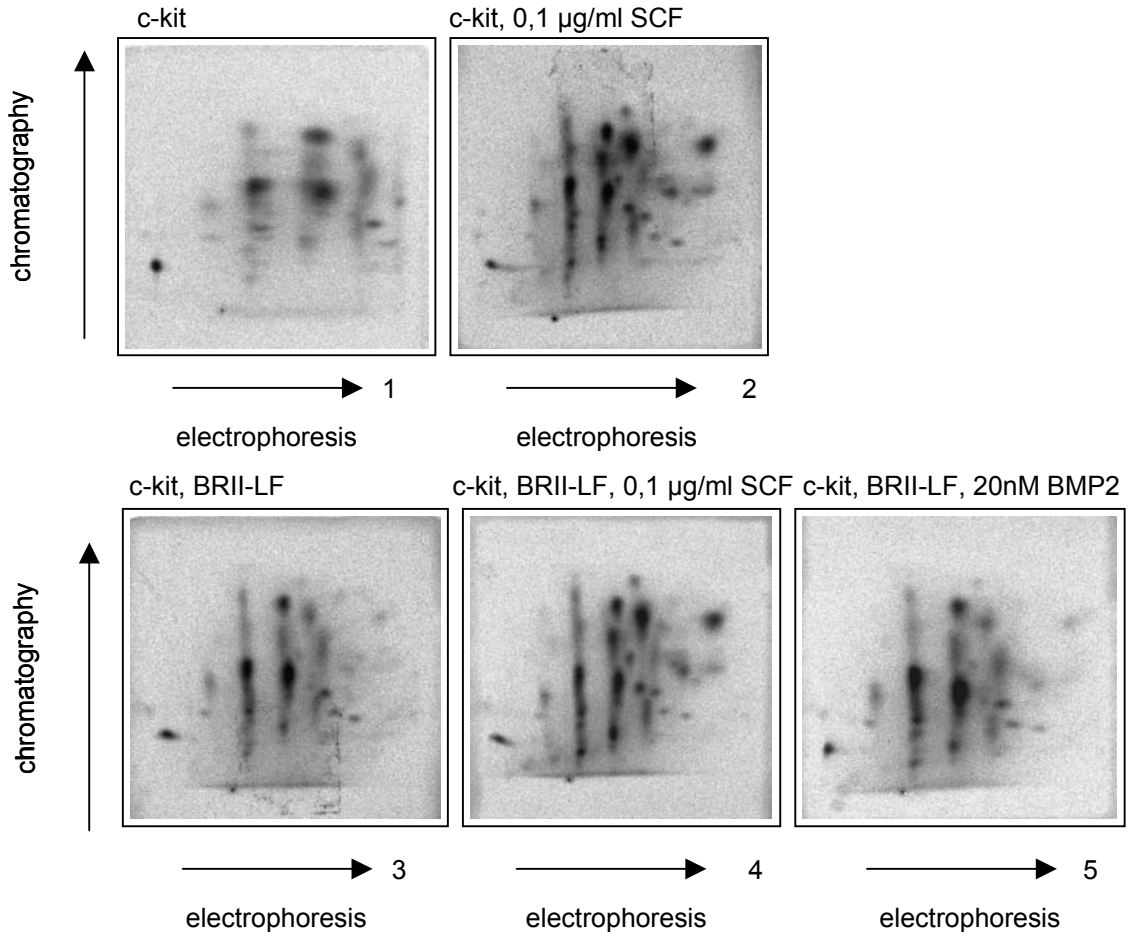


Fig 4.36a Phosphomaps of c-kit in transfected 293T cells
c-kit expressed in 293T cells was labelled with ^{32}P-orthophosphate under different conditions (Fig. 4.35). The marked receptor was isolated, digested with trypsin and subjected to two dimensional phospho peptide mapping. Maps1 and 2 show c-kit alone, maps 3-5 c-kit when BRII-LF is coexpressed.

Fig. 4.36 depicts the maps of c-kit under different conditions. Due to technical problems, map1 looks a bit fuzzy, but the high basal level of phosphorylation of non-stimulated c-kit can be observed. Stimulation with SCF (map2) enhanced the phosphorylation, resulting in phosphorylation on tyrosines, as it is known that SCF induces phosphorylation of c-kit on tyrosine residues, while the basal phosphorylation on serine and threonine residues is not considerably affected by the ligand (Blume-Jensen et al., 1995). Nevertheless a so far not characterized ligand-induced phosphorylation on a specific serine could be demonstrated. When BRII-LF was cotransfected (map3), c-kit showed the same phosphorylation pattern as in the non-stimulated state (map1). Stimulation with SCF resulted in the upregulation of tyrosine phosphorylation (map4), independent of BRII-LF (compare map2 and map4). BMP2 did not change the phosphorylation status at all (map5).

Therefore no influence of BRII-LF on the phosphorylation of c-kit could be observed.

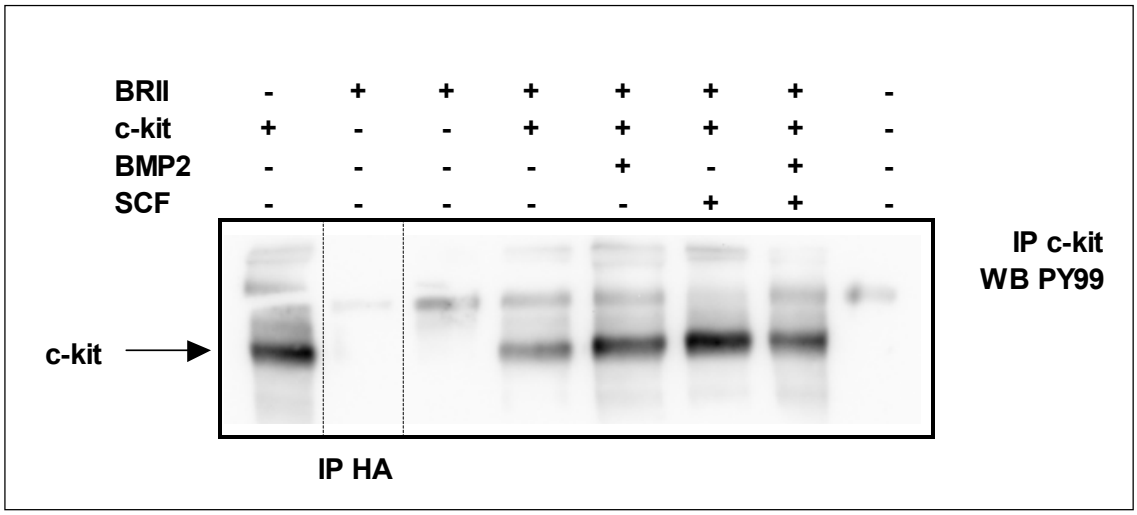


Fig 4.36b Phospho-tyrosine westernblot of c-kit in transfected 293T cells
c-kit expressed alone or together with BRII in 293T was precipitated with polyclonal rabbit antibodies anti c-kit and protein A Sepharose. Probing the blot with anti phospho-tyrosine antibody detects tyrosine phosphorylation of c-kit (lanes 1, 4-7).

Depending on the transfection efficiendy, c-kit exhibited a strong tyrosine phosphorylation. At a low receptor density SCF as well as BMP2 and SCF stimulation enhanced tyrosine phosphorylation (data not shown), whereas c-kit was heavily phosphorylated at tyrosine residues when expressed at high levels. Here, ligand did not induce a significant change. This is probably due to amount of receptors that are already ligand-independent homodimers on the cell surface (Fig. 4.36b).

4.3.3.2 Phosphorylation of BRII-LF in transfected COS7 cells

Knowing that not only BRII-LF but c-kit being constitutively active kinases, one important goal was to identify potential phosphorylation sites of c-kit on the BRII-LF. To address this question excessive in vivo labelling and phospho peptide mapping was performed.
Therefore 293T cells were transfected with BRII-LF alone or together with c-kit or c-kit KN, respectively. After starvation, cells were labelled with ^{32}P-orthophosphate, stimulated with different ligands and lysed. BRII-LF-HA was precipitated with HA-antibodies and the labelled protein was detected on a phosphoimager (Fig. 4.37).

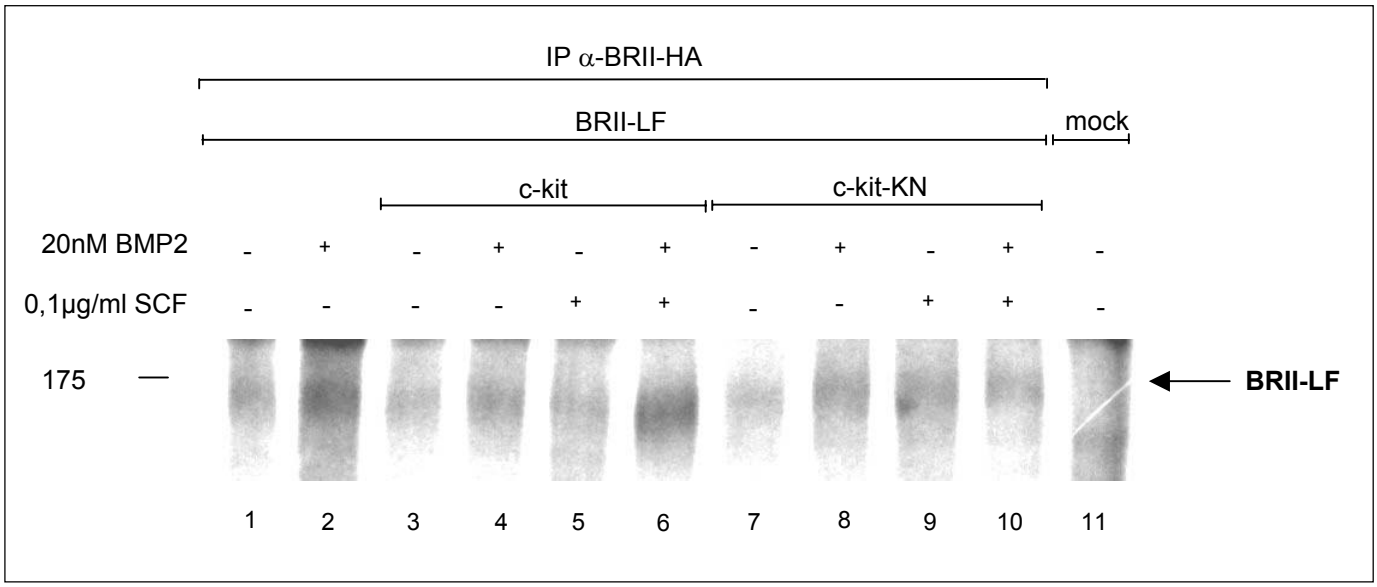


Fig 4.37 Phosphorylation of BRII-LF in transfected 293T cells
293T cells expressing BRII-LF (lanes 1-10) and c-kit (lanes 3-6) or c-kit KN (lanes 7-10), respectively were incubated with 1mCi/ml ^{32}P-orthophosphate for 4h, stimulated or not with 20nM BMP2 (lanes 2, 4, 8) or 0,1µg/ml SCF (lanes 5, 9) or both ligands (lanes 6, 10) for 1h and lysed in 1% Triton lysis buffer containing protease- and phosphatase inhibitors. The lysate was precipitated with anti HA antibody and protein A Sepharose and subjected to SDS-PAGE after extensive washes. ^{32}P marked BRII-LF-HA is detected on the phosphoimager screen.

The phosphorylation of the BRII-LF increased upon BMP2 stimulation (lane 2), cotransfection of c-kit did not elevate the background level as well as additional SCF stimulation did not (lanes 3, 5). Addition of BMP2 increased the phosphorylation level. Stronger stimulation was seen with both ligands, suggesting strong synergism (lanes 4, 6). When c-kit KN was cotransfected instead of c-kit the all-over phosphorylation picture was nearly the same, only the phosphorylation in the presence of both ligands was slightly reduced.

Phosphopeptide mapping of the BRII-LF from Fig. 4.37 showed that addition of BMP2 increased total phosphorylation of BRII-LF but did not enhance the phosphorylation of a specific spot (Fig. 4.38, maps 1-2). Cotransfection of c-kit did not result in appearance of a new spot as well (maps 3-4). When stimulated with both ligands, three spots on the BRII-LF were changed, whereas the most striking was a spot on the upper right side of the map which was poorly present when BRII-LF was stimulated with BMP2 or cotransfected with c-kit and stimulated with BMP2 (map6, Fig. 4.39). The presence of both receptors and ligands strongly enhanced the phosphorylation of that spot. The spot is marked in Fig. 4.39 with a red circle.

Besides that, cotransfection of c-kit KN led to a significantly weaker all over phosphorylation, but no specific spot was influenced. Even the spot on the upper right side of the map was still present, but weaker.

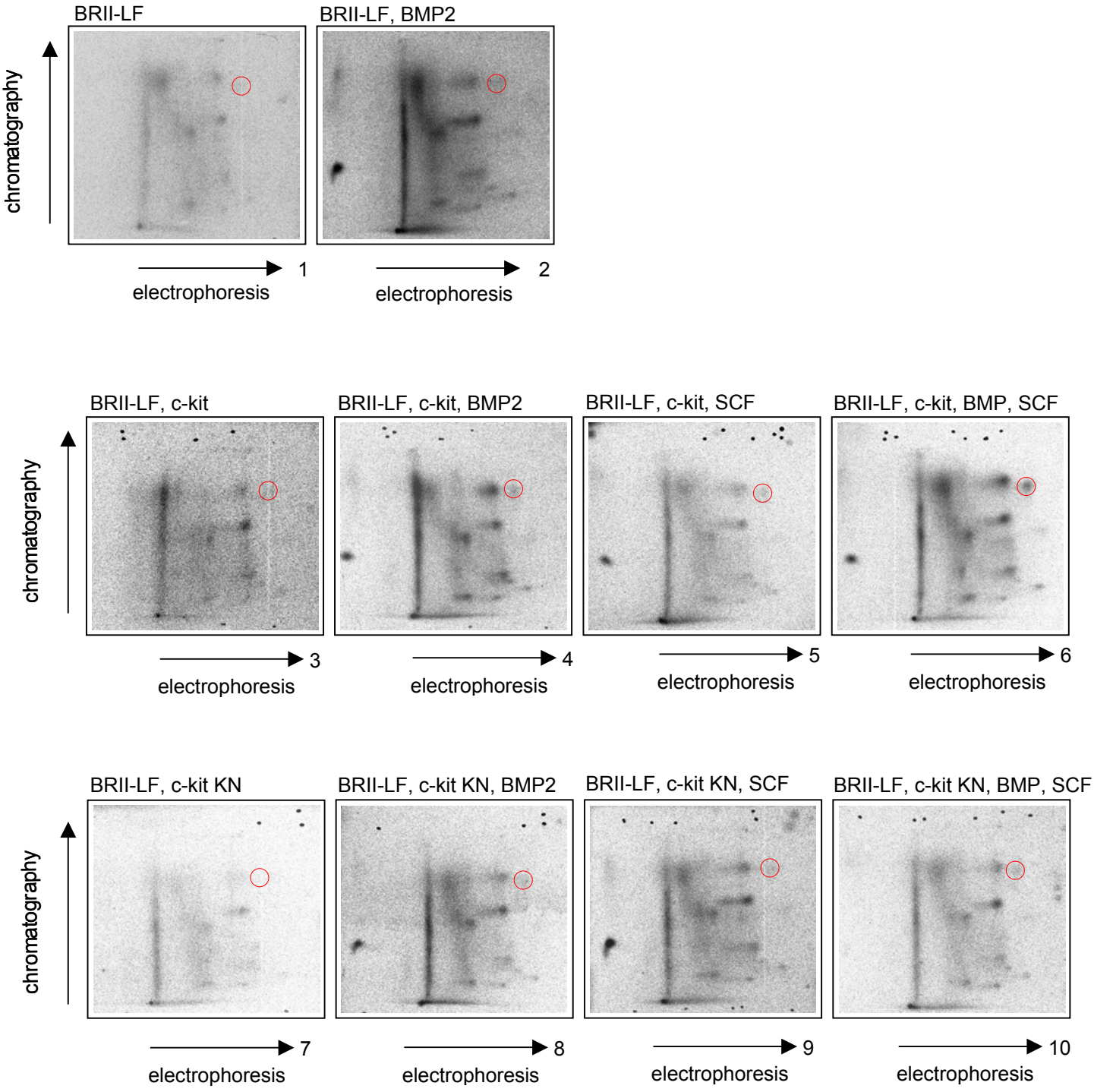


Fig 4.38 Phosphomaps of BRII-LF in transfected 293T cells
BRII-LF expressed in 293T cells was labelled with ^{32}P-orthophosphate under different conditions (Fig. 4.37). The marked receptor was isolated, digested with trypsin and subjected to two dimensional phospho peptide mapping. Maps1 and 2 show BRII-LF plus minus BMP2, maps 3-6 the influence of c-kit on BRII-LF phosphorylation and maps 7-10 the phosphorylation of BRII-LF when c-kit KN is co-transfected.

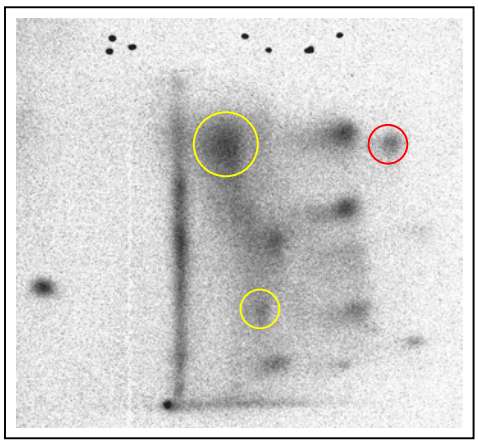


Fig 4.39 Phosphomap of BRII-LF cotransfected with c-kit and stimulated with BMP2 and SCF
Map of BRII-LF when c-kit is cotransfected and the receptors are stimulated with 20nM BMP2 and 0,1µg/ml SCF. Three new or differently migrating phospho peptides can be observed (coloured circles), whereas the most striking change was for the peptide marked in red which was much stronger phosphorylated than in any other condition.

Edman degradation of the spot marked with the red circle, demonstrated that this peptide was phosphorylated at position four but no clear amino acid analysis could be performed. Weak signals pointed towards serine phosphorylation but further confirmation is required. So we chose some candidate peptides in BRII-LF and mutated them. First results from new mappings indicated that S757 is phosphorylated upon expression of c-kit. This effect is unexpected because c-kit is a tyrosine kinase, leading to the conclusion that c-kit either facilitates auto-phosphorylation of the BRII-LF or it recruits a serine kinase into the complex or c-kit has dual kinase activity. But the dependence on kinase activity (see Fig. 4.38) hints towards direct phosphorylation.

4.3.3.3 Endogenous phosphorylation of BRII-LF in C2C12 cells

Phosphorylation of BRII or c-kit upon incubation with ligands, BMP or SCF, reflects activation of the receptors. As observed for transiently transfected receptors, BMP2 stimulation enhances BRII phosphorylation and stimulation with both ligands even enhances this effect (Fig. 4.37). To confirm these results under more physiological conditions, we performed in vivo phosphorylation experiments in C2C12 cells, trying to examine ligand influence on the receptor phosphorylation pattern.

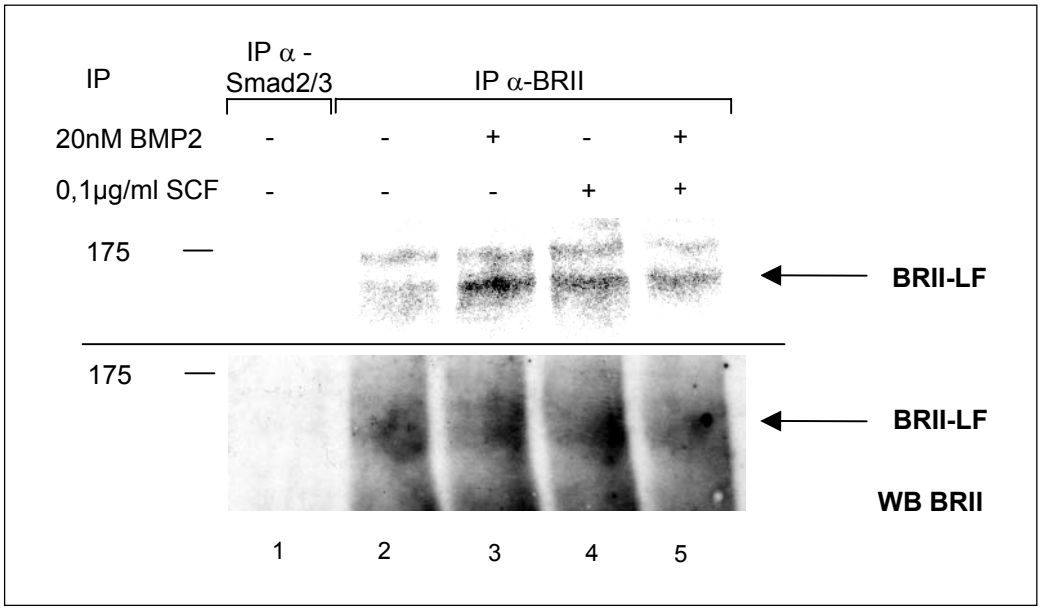


Fig 4.40 Phosphorylation of BRII-LF in non transfected C2C12 cells
C2C12 cells expressing endogenous BRII-LF and c-kit were incubated with 1mCi/ml ^{32}P-orthophosphate, stimulated or not with 0,1µg/ml SCF (lane 3) or 20nM BMP2 (lane 2) or both ligands together (lane 4) for 1h and lysed in 1% Triton lysis buffer containing protease- and phosphatase inhibitors. The lysate was precipitated with a mixture of anti BRII antibodies (NRR and SMN) and protein A Sepharose and subjected to SDS-PAGE after extensive washes. ^{32}P marked BRII-LF is detected on the phosphoimager screen. To demonstrate specificity, immunoprecipitation with anti Smad 2/3 was performed as a control.
The lower panel shows a westernblot anti BRII (G17) of a parallel immunoprecipitation as expression control.

As depicted above (Fig. 4.40), we found that BMP2 induced phosphorylation of endogenous BRII in orthophosphate-labelled C2C12 cells (lane 3), and co-stimulation with SCF inhibited the effect of BMP2. SCF alone showed some enhancement of total BRII phosphorylation (lane 4). The control westernblot of an experiment performed in parallel without 32P labelling demonstrates equal receptor precipitation, thus showing that the synergism of both ligands is not seen for endogenous receptors. Due to the low amount of receptors and weak phosphorylation mapping of the endogenous phosphorylation was not possible. Still, both ligands can induce the phosphorylation of these three specific spots, as seen for transfected receptors (Fig. 4.39, spots within circles). To examine the in vivo situation, only biological read outs can be used.

4.3.4 The influence of SCF and c-kit on BMP2 mediated signal transduction – cellular read outs

As endogenous BRII-LF and c-kit were found to associate, it was examined if this interaction and the phosphorylation of BRII via c-kit affected their known activities. To asses this, several experiments were performed as reportergene assays for c-kit and BRII-LF,

westernblots, proliferation and differentiation experiments, as well as characterization of the onset of gene expression using real time PCR. The focus examining the cellular readouts was on the BMP-pathway because on the one hand only BRII-phosphorylation is influenced by c-kit and not vice versa and on the other hand preliminary tests checking the influence of BMP2 upon c-kit signal transduction did not show any effect (data not shown).
Moreover we tried to identify the site in BRII-LF which is phosphorylated by c-kit. After mutation of that site we were exploring the role of the regulatory phosphorylation of c-kit on BRII-LF in in vivo systems.

4.3.4.1 Effects of SCF and c-kit on Smad-dependent reporter constructs

Given that c-kit is phosphorylating BRII-LF when cells were stimulated with SCF and BMP2, we further tested whether this synergism can influence BMP2 mediated signal transduction. For this purpose, C2C12 cells were transiently cotransfected with the renilla control reporter (pRLTK) under the control of the thymidine kinase promotor and pSBE-luc, a luciferase gene under the control of a Smad-binding element.

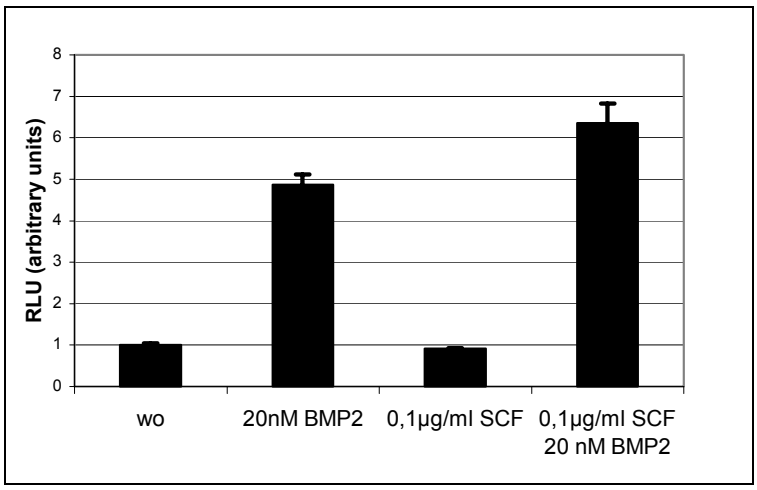


Fig 4.41 Smad-reportergene assay in transfected C2C12 cells
C2C12 cells were cotransfected with pSBE-Luc and pRLTK prior to starvation and stimulation with 20nM BMP2, 0,1µg/ml SCF or both.

The experiment depicted in Fig. 4.41 shows, that BMP2 shows a significant increase of transcriptional activity on the pSBE-reporter. SCF, in contrast, is not activating the Smad-pathway, whereas stimulation with both ligands simultaneously enhances the signal. So we can observe a synergistic effect on the pSBE-promoter. Similar synergistic effects were not observed testing the following reporters: pI3300OG1, p6OSE2-luc, pII147OG2, pII2900OG2 (all four osteogenin reporters), 12xGCCG, p3TP-lux, 15xGCCG and Id1. The majority of these reporters were BMP2 responsive, but no synergism of BMP2 and SCF was observed.

The next goal was to examine the effect of cotransfected receptors on SBE mediated signal transduction.

It is already known that transfection with BRII-SF or BRII-LF enhances the transcriptional response on a BMP responsive element, not only the signal in stimulated cells, but also the signal in non stimulated cells is highly elevated.

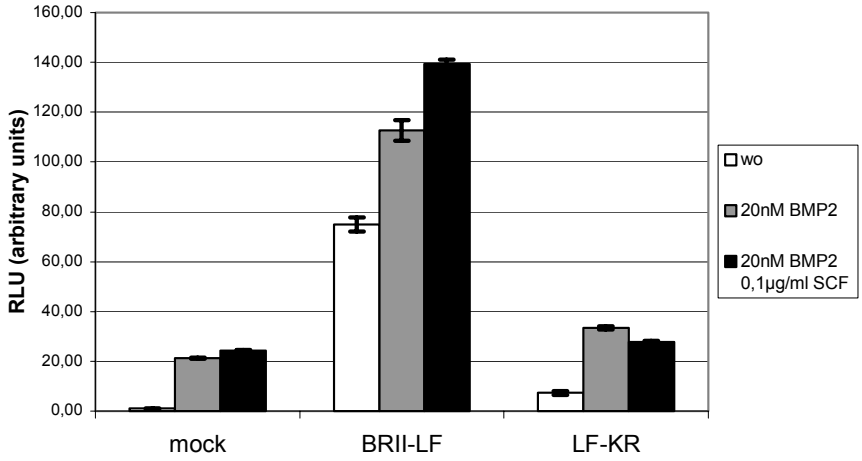


Fig 4.42 Smad-reportergene assay in transfected C2C12 cells
C2C12 cells were transfected with pSBE-luc and as reference pRLTK as well as with empty vector (mock), with BRII-LF or the kinase dead mutant and either left untreated or stimulated with 20nM BMP2, 0,1µg/ml SCF or both for 24h. Luciferase activity was measured using SBE as a reporter and RLTK to normalise the signals.

Even though cells transfected with BRII-LF showed an elevated level of luciferase activity already in the absence of ligands, the signal could be enhanced by stimulation with BMP or both ligands together. Still the synergistic effect can be observed. Ectopic expression of BRII-LF-KR did barely enhance BMP2 mediated signal transduction and abrogate the BMP2/SCF synergism completely.

To asses the importance of functional c-kit receptors for SCF mediated effects on BMP2 signal transduction, the influence of the receptor construct and its functionally inactive mutant were tested. Here, it should be examined which effects c-kit or c-kit mutants would evoke on the Smad mediated transcriptional response.

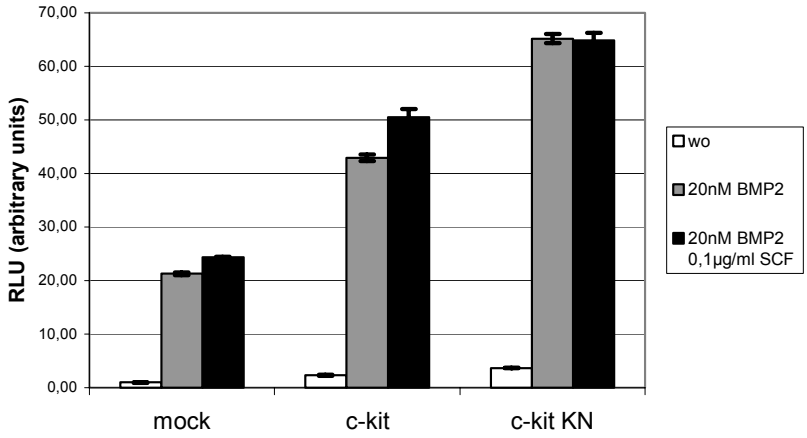


Fig 4.43 Smad-reportergene assay in transfected C2C12 cells
C2C12 cells were transfected with pSBE-luc and as reference pRLTK as well as with empty vector (mock), with c-kit or the kinase dead mutant and either left untreated or stimulated with 20nM BMP2, 0,1µg/ml SCF or both for 24h. Luciferase activity was measured using SBE as a reporter and RLTK to normalise the signals.

Transfection of c-kit significantly increased the BMP2 mediated signal as well as the signal during costimulation. Moreover, transfection with c-kit KN enhanced the stimulation mediated by c-kit. The synergistic effect induced by simultaneous stimulation with BMP2 and SCF, however, could not be observed in the presence of c-kit KN. Nevertheless we can conclude that the kinase of c-kit is not necessary for BMP2 mediated signal transduction. c-Kit strongly supports BMP2 induced transcriptional activity.

4.3.4.2 Effects of SCF on BMP2 induced Smad 1/5/8 phosphorylation

Next, we wanted to verify the synergistic effects observed by analyzing Smad1/5 phosphorylation.

Smad-dependent signalling is by far a key pathway initiated downstream of activated BMP receptors. Measuring the transcriptional activity using luciferase reporter genes we could show a synergistic effect of BMP2 and SCF on Smad induced transcription. As Smads have to be phosphorylated and thus activated by the receptors and only then can form complexes with the common mediator Smad4, we expected to see the synergistic effect on Smad1/5 phosphorylation as well.

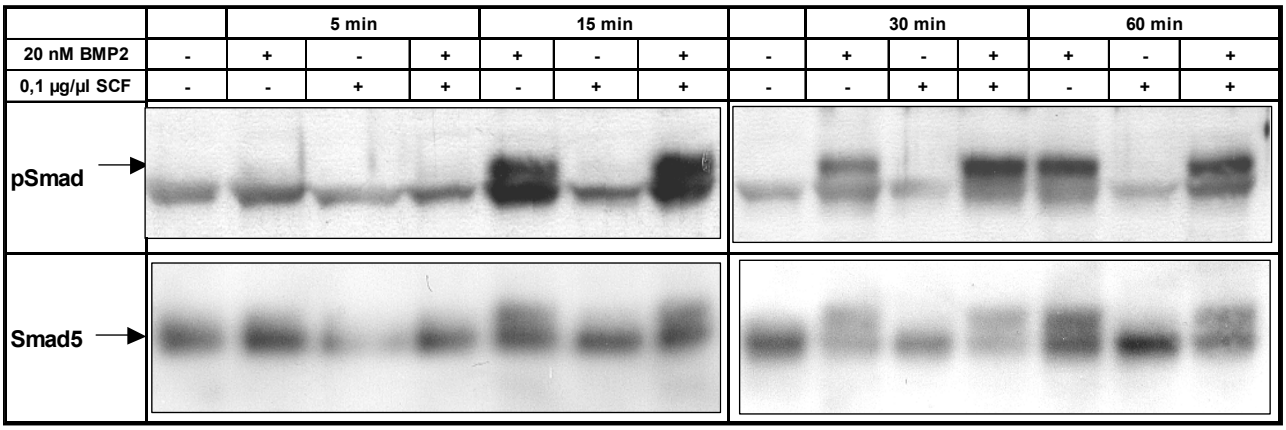


Fig 4.44 Smad-phosphorylation in BMP2 and SCF stimulated C2C12 cells
$1x10^5$ C2C12 cells were plated on a 6-well plate and grown over night. The following day, cells were starved for 24h and subsequently stimulated with 20nM BMP2, 0,1µg/ml SCF or a combination of both for the indicated time points. The cells were harvested in TNE lysis buffer and equal amounts of protein were subjected to gel electrophoresis and western blotting. Similar results were obtained within two independent experiments

Using an antibody recognizing C-terminal of Smad1/5/8 phosphorylated serine residues, we found that simultaneous treatment of the mouse pluripotent mesenchymal precursor cell line C2C12 cells with BMP2 and SCF or stimulation with BMP2 alone led to C-terminal phosphorylation of the BMP-specific Smad1/5; significant activation of Smad1/5 was observed already after 15 minutes, with a peak at 30 minutes. Smad1/5 activation by BMP2 alone reached a maximum at 30 minutes, and the level of activation was lower as compared to simultaneous BMP2 and SCF treatment. The same synergistic effect could be observed after 60 minutes of ligand treatment (Fig 4.44). Blotting of the whole cell lysates with antibodies to endogenous Smad1 or Smad5 (Santa Cruz) showed that C2C12 cells expressed predominantly Smad5, and Smad1 was undetectable (Fig. 4.44).

4.3.4.3 Effects of BMP and SCF on other Smad-dependent pathways

Smads, as signal transducers for BMP go to the nucleus and start transcription by binding to specific elements on the DNA, alone or complexed with other transcription factors. For TGF-β, many target genes are characterized, whereas for BMP2 only few target genes are known. Among them three Id proteins (inhibitor of differentiation) have been described as dominant negative regulators of basic helix-loop-helix transcription factors (Hollnagel et al., 1999). Id1 was shown to be a BMP2 responsive target gene (Lopez-Rovira et al., 2002). Recently, the Id1 promoter was very well characterized to be activated in a Smad-dependent manner, bearing two BMP-responsive regions (Korchynskyi and ten Dijke, 2002). Both of these regions contain classical Smad binding elements (SBEs) or a GGCGCC palindromic sequence flanked by two CAGC and two CGCC motifs, which are known to bind Smad1/5 as

well. Besides, the Id1 promoter contains binding regions for other transcription factors (Korchynskyi and ten Dijke, 2002).

Id1 was chosen being a prominent BMP2 target, even though reporter gene experiments using the Id1 promoter did not show any BMP2/SCF synergism (data not shown). To examine whether BMP2 and SCF show synergistic effects on the induction of the Id1 gene, we performed real time PCR with Id1 specific oligonucleotides.

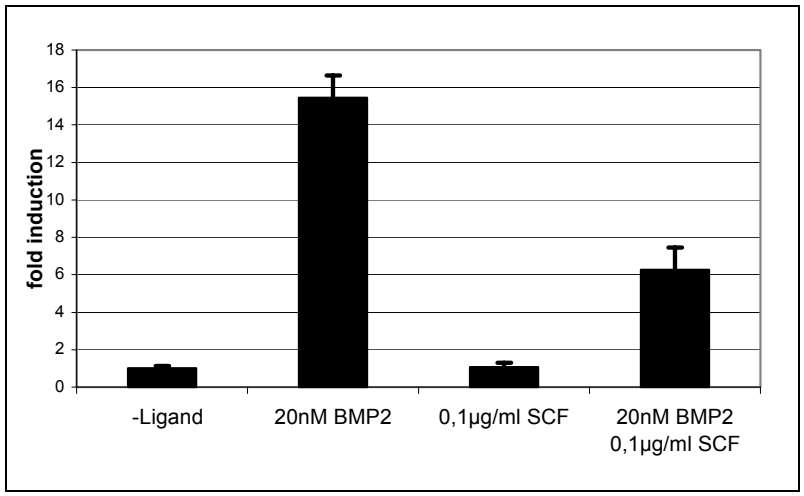


Fig 4.45 Id1 induction in stimulated C2C12 cells
$1x10^5$ C2C12 cells were plated on a 6-well dish, starved for 24h and either left untreated or stimulated with 20nM BMP2, 0,1µg/ml SCF or both for 20h. RNA was extracted and equal amounts of RNA were subjected to real time PCR using Id1 specific primers. The determination was in duplicates.

As demonstrated here, BMP2 was inducing Id1 transcription up to 16 fold, whereas SCF did not show any effect. Simultaneous treatment of C2C12 cells with BMP2 and SCF resulted in a significantly weaker induction of the Id1 transcription (up to 7 fold). As this result is in contrast to the results obtained in the reporter gene assay using pSBE or Id1, this effect might be due to the difference between artificial and natural promoters. In contrast to the exclusively Smad-dependent read outs, the more complex Id1 regulation is negatively influenced by stimulation with SCF in addition to BMP2. Thus we can conclude that SCF is on the one hand promoting Smad mediated transcriptional activity, on the other hand suppressing Smad-induced transcription, most likely by affecting inhibiting transcription factors.

4.3.4.4 Effects of SCF and c-kit on Alkaline phosphatase production

The next goal was to explore a functional effect of the cooperation of BMP2 and SCF on cell proliferation and differentiation.

To demonstrate that the interaction between BRII-LF and c-kit has biological significance, we studied the effect of SCF on the production of Alkaline Phosphatase (ALP), a marker gene for osteoblast differentiation (Takuwa et al., 1991). For this, three different approaches were

chosen. C2C12 cells were plated and after attaching over night starved and subsequently stimulated with 50nM BMP2, 0,1µg/ml SCF or both ligands together for three days. Stimulated cells were examined for ALP activity, doing histochemical analysis or enzymatic measurements.(Fig. 4.46 and 47).

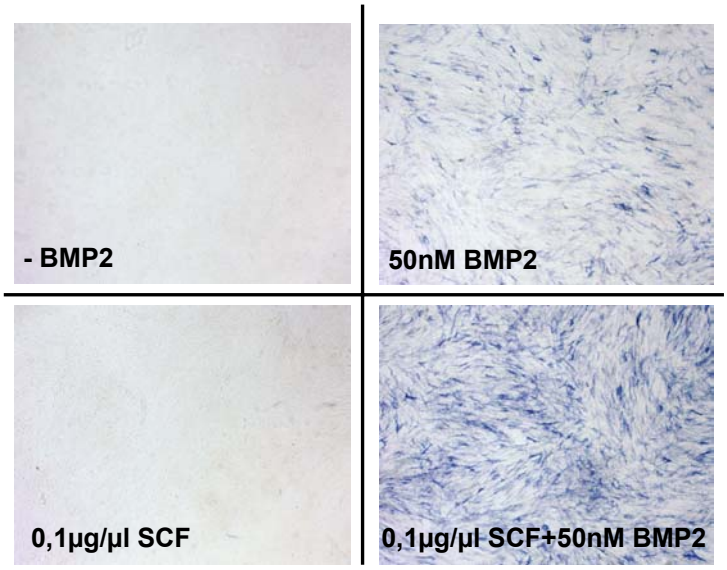


Fig 4.46a Alkaline phosphatase stain in C2C12 cells
$5x10^5$ C2C12 cells were seeded on a 6-well plate, after attaching over night, starved for 5h in DMEM with 0,5% FCS and subsequently stimulated with 50nM BMP2 or 0,1µg/ml SCF or both ligands for 72h. After washing with PBS, the cells were fixed and stained with Naphtol AS-MX phosphate and fast blue BB salt.

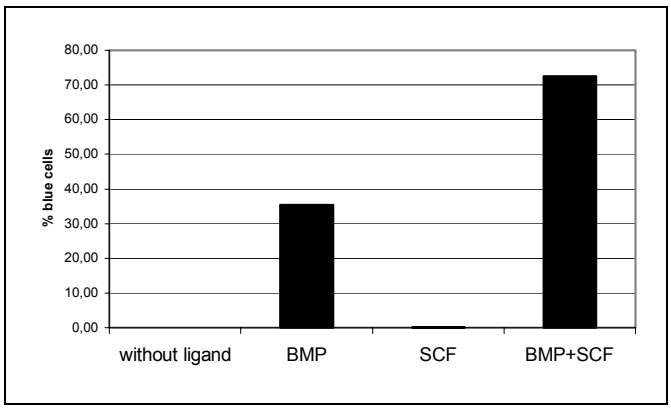


Fig 4.46b Quantification of the ALP stain in 4.46a
The percentage of blue cells in the stained area was quantified using metamorph offline, Visitron,

Incubation of cells with BMP2 and SCF resulted in enhanced ALP expression as compared to BMP2 alone, as evaluated by staining of cells (Fig. 4.46). The same result was obtained by measurement of ALP activity (Fig. 4.47). This enhancement was observed already after 24h of cell treatment and sustained until 72h of treatment (data not shown).

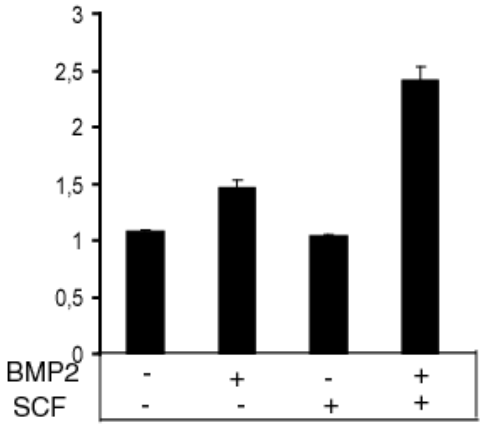


Fig 4.47 Enzymatic measurement of Alkaline phosphatase in C2C12 cells
$1{,}5x10^4$ C2C12 cells were seeded on a 96-well. Ater attaching over night, cells were starved for 5h in DMEM with 0,5% FCS and subsequently stimulated with 50nM BMP2 or 0,1µg/ml SCF or both ligands for 72h. After washing with PBS, the cells were lysed and the activity of Alkaline Phosphatase (in RLU) was determined using pNPP as a substrate (experiment by Mariya Yakymovych, LICR, Uppsala, Sweden).

To confirm the data obtained by staining differentiated C2C12 cells for ALP, we measured ALP activity in parallel, using pNPP as a substrate (Fig. 4.47). Here, BMP2 and SCF showed the same synergism as observed in the staining experiment.
Another approach to support our data obtained so far was using real time PCR with ALP specific primers. On the level of gene expression the greatest synergism of both ligands could be observed 20h after stimulation (data not shown).

Stimulation of differentiation of osteoblasts is often accompanied by inhibition of cell proliferation. We found that BMP2 as well as SCF inhibited proliferation of C2C12 cells, as evaluated by a MTS test and cell number counting. However, we could not observe a significant modulation of the BMP2-dependent inhibition of cell proliferation by SCF or vice versa (data not shown).

4.3.4.5 Effects of SCF and c-kit on the induction of Runx2/Cbfa1

The Runx family of transcription factors is composed of three members, Runx1, Runx2 and Runx3, which play important roles during normal development. Runx2, also known as Cbfa1, is a common target of TGF-β1 and BMP2 and was identified to play an essential role in osteoblast differentiation (Lee et al., 1999). It has been described that Cbfa1 is induced by Smad1, but not in a direct manner, whereas it seems that junB functions as an upstream activator of Cbfa1. Furthermore, not only the Smad pathway but also the MAPK pathway is involved in the induction of Cbfa1 (Lee et al., 2002).
To address the question of Cbfa1 upregulation in response to BMP2 or SCF we performed real time PCR experiments as well as immunoprecipitations and western blotting of endogenous Cbfa1 in C2C12 cells.

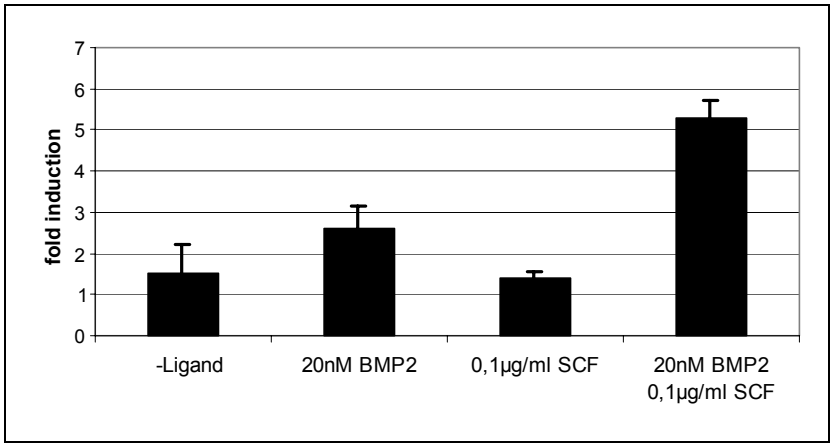


Fig 4.48 Real time PCR to determine the RNA for Cbfa1 in C2C12 cells
1x10^5 C2C12 cells were plated on a 6-well plate and after attaching over night starved for 24h with DMEM supplemented with 0,5% FCS. Subsequently they were stimulated with 20nM BMP2, 0,1µg/ml SCF or a combination of both ligands for 20h. The cells were harvested and equal amounts of cDNA were subjected to real time PCR using specific primers for Cbfa1. The experiment was performed in twice with different primer pairs, giving identical results (done by Nadine Hemmrich).

The results with the Cbfa1 gene showed a synergism of BMP2 and SCF. Here, 20h of BMP2 stimulation induces Cbfa1 transcription only marginal, SCF has no effect, whereas both ligands together induce the Cbfa1 gene up to 5 fold. To confirm these results, immunoprecipitations with endogenous Cbfa1 were performed. Surprisingly, Cbfa1 was shown to be induced not only by BMP2, but also by SCF alone and in a synergistic manner by BMP2 and SCF together, though on protein level (Fig. 4.49).

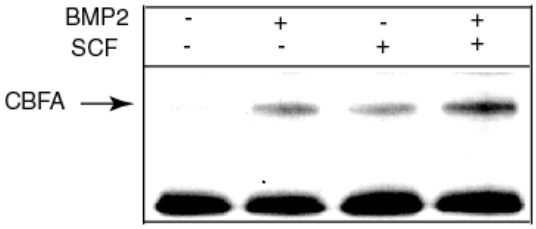


Fig 4.49 Immunoprecipitation of endogenous Cbfa1 in C2C12 cells
1x10^6 C2C12 cells were plated on a 10cm dish and after attaching over night starved for 24h with DMEM supplemented with 0,5% FCS. Subsequently they were stimulated with 20nM BMP2, 0,1µg/ml SCF or a combination of both ligands for 1h. The cells were harvested in lysis buffer and immunoprecipitation using Cbfa1 specific antibodies was performed. The precipitate was subsequently blotted and detected with anti Cbfa1.

4.3.4.6 Effects of BMP2 and SCF on the induction of Smad-independent pathways

The implication of ERK phosphorylation in BMP2 or SCF mediated signal transduction is described (Lou et al., 2000), (Okuda et al., 1992) and SCF and BMPs were found to activate Erk kinase in various cells.

Looking at ERK phosphorylation upon BMP2 or SCF stimulation or both we could observe a stimulation in waves. We found that treatment of cells with BMP2 and SCF together delayed

the first peak of Erk activation observed upon treatment of cells with BMP2 or SCF alone (Fig 4.50). The effect of simultaneous incubation of cells with BMP2 and SCF on the second phase of Erk activation (30min) was even more pronounced; when SCF or BMP2 strongly stimulated Erk, simultaneous treatment with BMP2 and SCF led to activation of Erk on the level lower than with the treatment with each of the ligands alone (Fig. 4.51a). However, after 60 minutes of incubation, cells treated with BMP2 and SCF still had a significant level of Erk activation, while cells treated with BMP2 or SCF alone showed only minimal Erk phosphorylation (Fig. 4.50a).

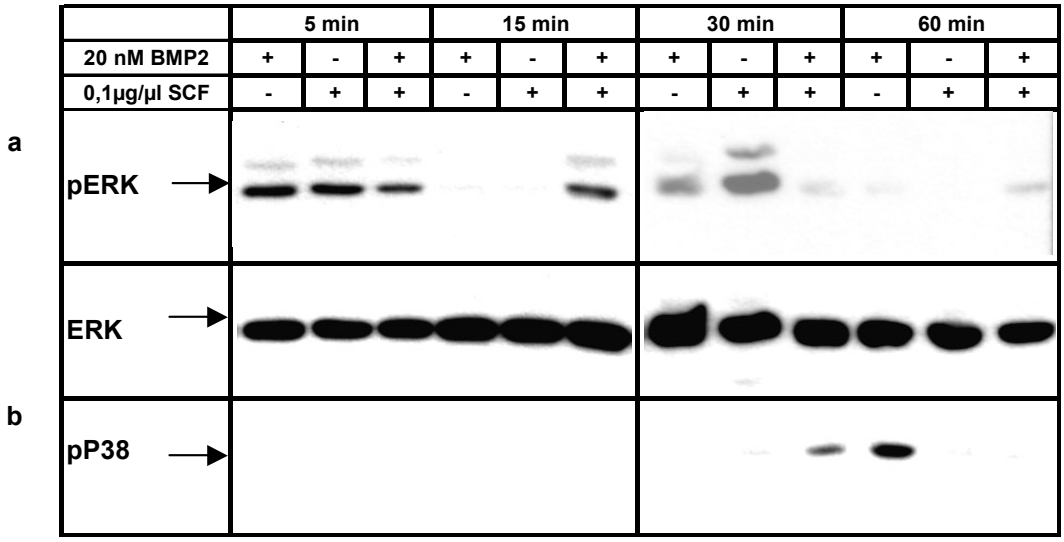


Fig 4.50a and b BMP2 and SCF dependent ERK1/2 and p38 phosphorylation, time course
$1x10^5$ C2C12 cells were plated on a 6-well plate and after attaching over night starved for 24h with DMEM supplemented with 0,5% FCS. Subsequently they were stimulated with 20nM BMP2, 0,1µg/ml SCF or a combination of both ligands for the indicated time points. The cells were harvested and western blotting using pERK or pP38 antibodies was performed. ERK was detected to demonstrate equal loading.

Besides ERK phosphorylation, the phosphorylation of p38, another MAPK family member, plays an important role in BMP2 mediated signal transduction (Gallea et al., 2001).

For the Phospho-p38 still a phosphorylation in waves could be observed but with other oscillation: SCF alone did not induce any p38 phosphorylation at all. BMP2 induced p38 phosphorylation after 60 min of stimulation whereas both ligands together induced p38 phosphorylation only after 30 min of stimulation, followed by a rapid dephosphorylation (Fig. 4.50b).

Whereas we see a forward-shift in case of p38 phosphorylation we can observe a backward-shift in case of ERK1/2 phosphorylation (Fig. 4.51).

It is not clear at this point, how these observations can be connected to physiological data.

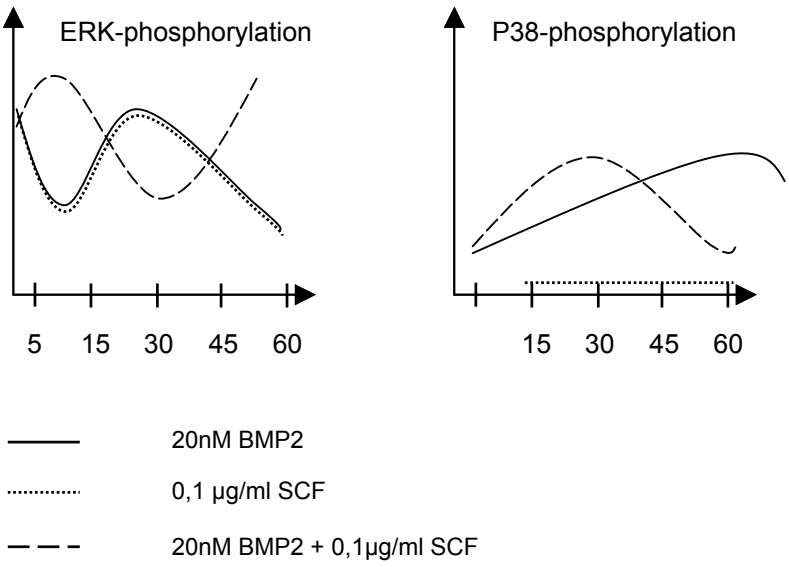


Fig 4.51 Scheme of BMP2 and SCF induced ERK1/2 and p38 phosphorylation
This figure depicts the time dependent ERK or p38 phosphorylation. As observed, BMP2 (solid line) and SCF (dotted line) have a similar kinetics in phosphorylating ERK, whereas a combination of both factors (dashed line) shows a time shift. In case of p38, SCF alone (dotted line) is not able to induce p38 phosphorylation whereas SCF in addition to BMP2 shifts the response to an earlier time point (dashed line).

We could show that C2C12 cells have BMP receptors as well as c-kit. Signal transduction after ligand binding results in phosphorylation of intracellular signal transducers and the onset of various pathways. As depicted here, BMP2 leads to the onset of the Smad-, the ERK- and the p38-pathway. Stimulation with SCF shows no Smad- or p38-activation, only significant activation of ERK was recorded. Co-stimulation with BMP2 and SCF modulates all three pathways mentioned with more pronounced, dramatic effects on the ERK- and the p38-phosphorylation with shifting of the speed of the cellular response.

4.4 BRII Phosphorylation (Hassel et al., submitted)

Phosphorylation is the most common and important mechanism of immediate and reversible regulation of protein function. Protein phosphorylation and dephosphorylation function together in signal transduction pathways to induce rapid changes in response to external stimuli as growth factors, hormones and neurotransmitters. Many polypeptide growth factors stimulate cytoplasmic phosphorylation upon binding to their receptors. Thereupon, phosphorylation of receptors, followed by phosphorylation of cytoplasmic protein kinases (such as raf, MEK and MAPK) is initiated. Cell cycle is regulated by phosphorylation of cyclin-dependent protein-kinases. Some intracellular proteins are mono-or biphosphorylated, but most of the proteins (receptors) are highly phosphorylated and serve as docking sites for many different signal transducers. Tyrosine kinase receptors are generally not tyrosine phosphorylated in the monomeric state (Ullrich and Schlessinger, 1990). Upon ligand addition they form dimers followed by transphosphorylation by the activated receptor-kinases. BMP and TGFβ-receptors are serine-threonine kinases. The TGFβ type II receptor is described to be a constitutively active kinase in vitro, despite the fact that it could not be proven in vivo (Wrana et al., 1994), (Luo and Lodish, 1997). TβRII transphosphorylates TβRI upon ligand binding (Souchelnytskyi et al., 1996), (Wrana et al., 1994) and thus activates the receptor for the phosphorylation of target proteins, the Smads.
BMP receptors can form homo-and heterooligomers even in the absence of ligand (Gilboa et al., 2000). The aim of this project is to examine the phosphorylation of and by the BRII.

4.4.1 Maps of BRII in transfected COS7 cells

To determine the phosphorylation status of the BRII two dimensional phosphopeptide maps were performed. In order to generate large amounts of BRII-LF we decided to transiently transfect COS7 cells with the BRII-LF and to examine the phosphorylation status of that protein in non-stimulated and BMP2 stimulated cells.

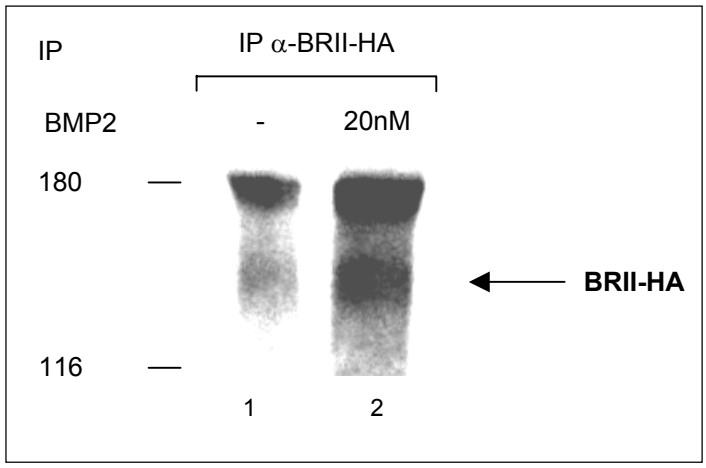


Fig. 4.52 Phosphorylation of BRII in COS7-cells
COS7 cells were transfected with the BRII-LF, labelled with orthophosphate ($^{32}P_i$) for 4h and either left untreated (lane 1) or stimulated with 20nM BMP2 for 1h (lane 2). The receptor was immunoprecipitated using anti-HA antibody and visualised by a phosphoimager.

Fig. 4.52 shows the ability of BMP2 to enhance BRII-LF phosphorylation.

To investigate which amino acids are phosphorylated 2D phospho-peptide mapping was performed.

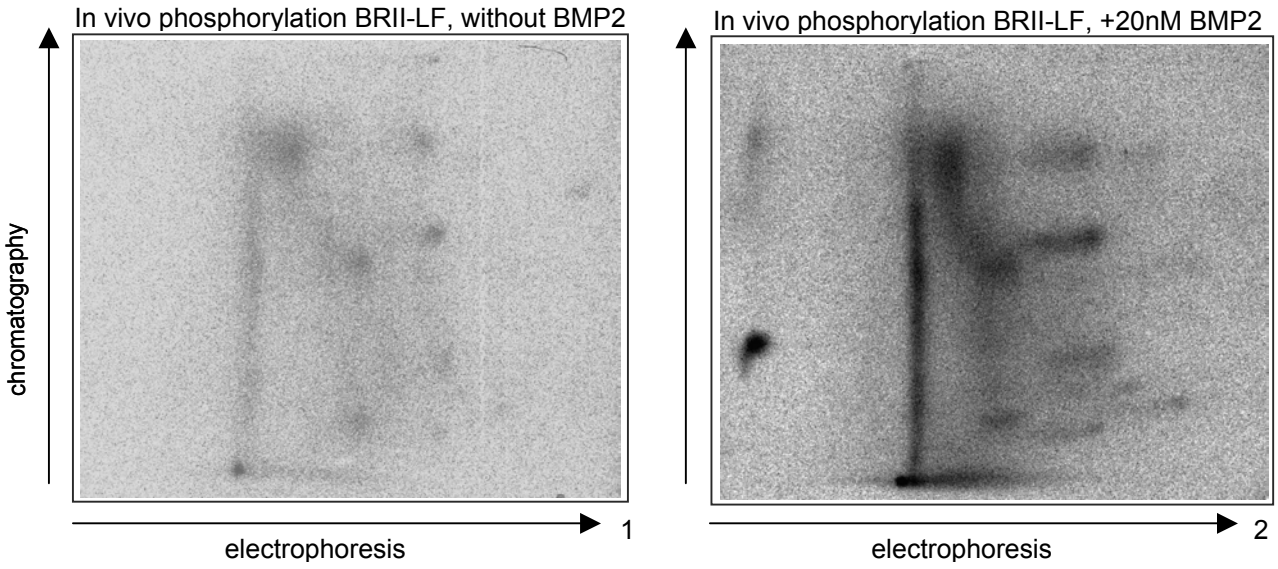


Fig. 4.53 Phosphopeptide maps of the BRII in COS7-cells
The receptors from Fig. 4.52 were extracted form the gel, digested with trypsin and subjected to 2D-phosphopeptide mapping. Map1 shows the BRII-phosphorylation without ligand stimulation, map2 the BRII-phosphorylation after addition of 20nM BMP2.

As depicted in Fig. 4.53 the all over phosphorylation of BRII-LF is increased after BMP2-stimulation (map2). Comparing map1 and map2 there is no clear up-regulation of a defined spot, all spots are stronger in the presence of ligand.

4.4.2 Phosphorylation of BRII in non-transfected C2C12 cells

In order to analyse phosphorylation under more physiological conditions and to avoid phosphorylation events due to overexpression, the BRII-LF was isolated from C2C12 cells, a murine mesenchymal precursor cell line (Katagiri et al., 1994) which differentiates upon BMP2 stimulation to osteoblasts.

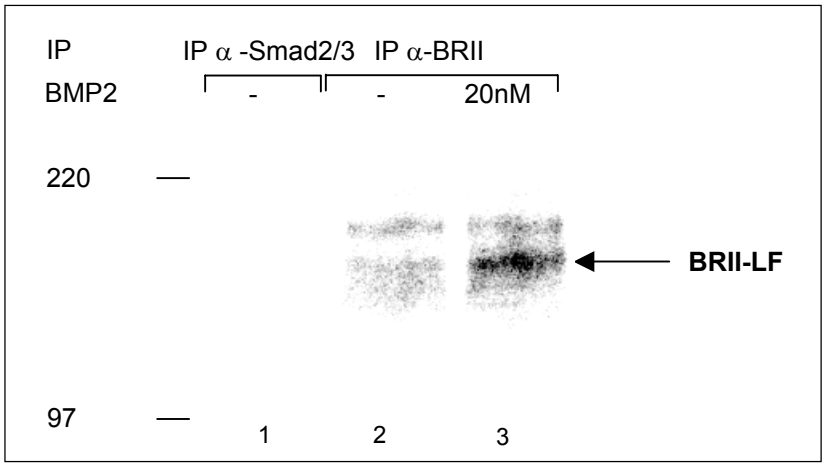


Fig. 4.54 Phosphorylation of BRII in C2C12-cells
C2C12 cells were plated on a 10cm dish, starved for 2 hours, labelled with orthophosphate for 4h and left non-stimulated (lanes 1-2) or stimulated with 20nM BMP2 for 1h (lane 3). The receptor was immunoprecipitated using anti-BRII (NRR, VMN) antibody (lanes 2-3) and visualised by autoradiography. Lane 1 shows a control-immunoprecipitation using anti-Smad2/3 antibody.

As depicted here the BRII-LF phosphorylation is increased after BMP2 stimulation (lanes 2-3), as it was seen for the transfected receptor (Fig. 4.52). Unfortunately the phosphorylation of the endogenous receptor is too weak, 2D maps did not give detectable spots.

4.4.3 BRII-phosphorylation after in vitro kinase assay

To be able to distinguish between spots originating from auto-phosphorylation and spots originating from trans-phosphorylation by other kinases an in vitro kinase assay of isolated receptors was performed. 293T cells were transfected with either BRII-LF or BRII-SF, therefore spots of tail-peptides could be identified on the maps.

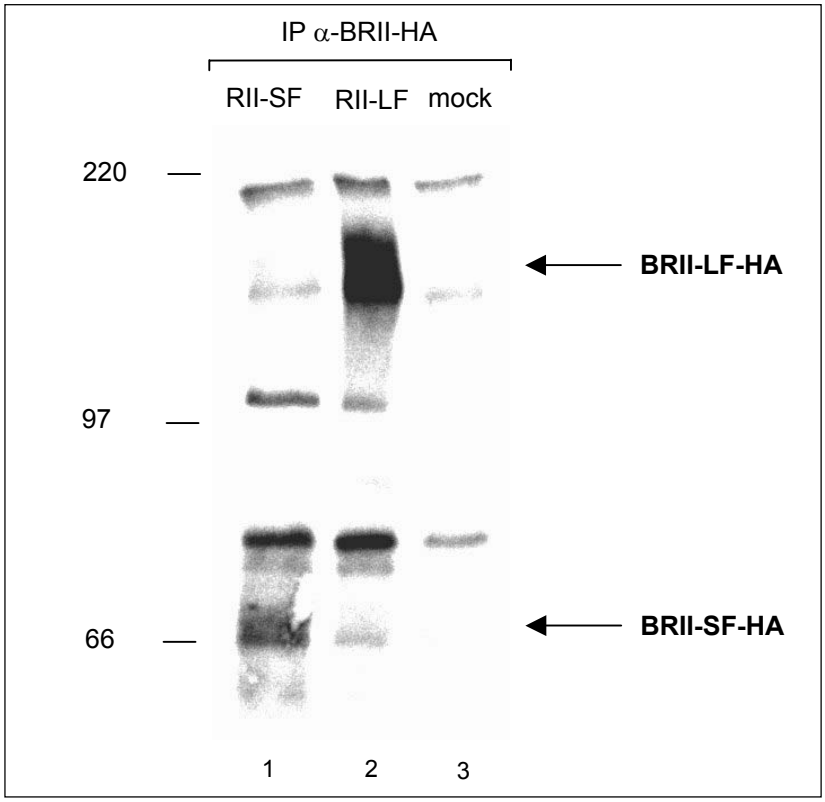


Fig. 4.55a In vitro kinase assay of BRII-SF and -LF
293T cells were transfected with pCDNA3 (lane 3), the BRII-SF (lane 1) or the BRII-LF (lane 2), respectively; the receptor was immunoprecipitated using anti-HA antibody and an in vitro kinase assay was performed. The phosphorylated receptor is visualised by a phosphoimager.

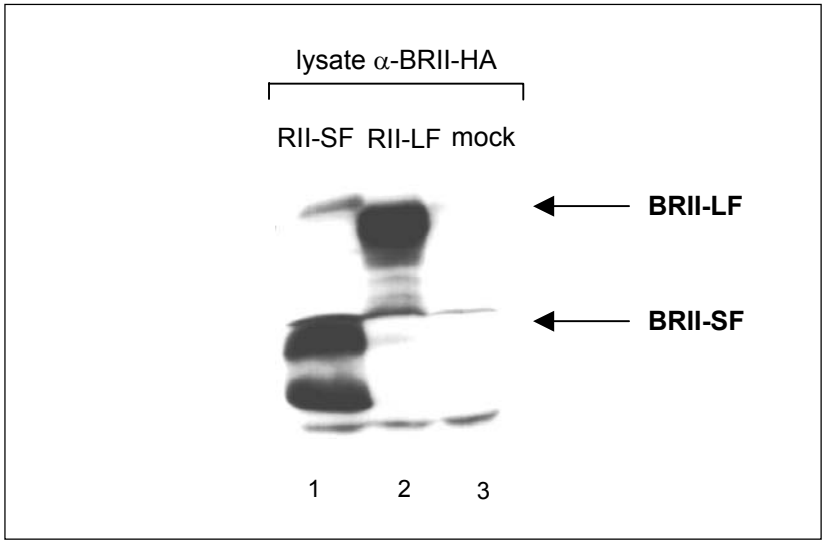


Fig. 4.55b Expression control of BRII-SF and BRII-LF for the in vitro kinase assay
293T cells were transfected with pCDNA3 (lane 3), the BRII-SF-HA (lane 1) or the BRII-LF-HA (lane 2), respectively. The lysate was blotted anti-HA to detect receptor expression.

As described for TβRII, the BRII is an active auto-phosphorylating kinase (Wrana et al., 1994). The BRII-LF seems to be even more potent in auto-phosphorylation than the BRII-SF. To elucidate the complete phosphorylation pattern of the in vitro phosphorylated protein, phosphopeptide maps were generated (Fig. 4.56).

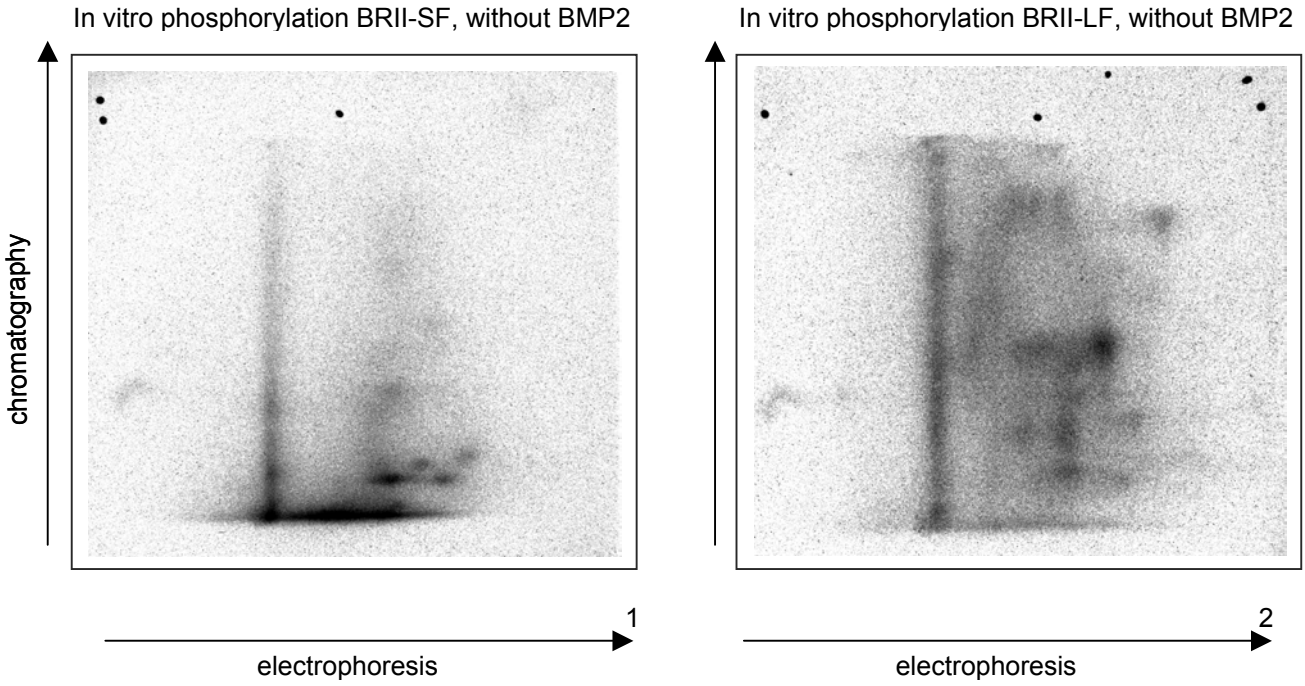


Fig. 4.56 Phosphopeptide maps of BRII-SF and –LF in vitro
The receptors from Fig. 4.56 were extracted, digested with trypsin and subjected to 2D-phosphopeptide mapping. Map1 shows the BRII-SF-phosphorylation, map2 the BRII-LF-phosphorylation.

BRII-SF is weaker phosphorylated as seen in Fig 4.56. This is also confirmed by 2D-maps (Fig. 4.57). In addition, in the in vivo phosphorylation experiment BRII-SF gave no detectable band compared to BRII-LF (data not shown). The phosphorylation of BRII-SF and BRII-LF in vivo is different as well.
This suggests that most auto-phosphorylation sites are located in the BRII-tail.

4.4.4 Analysis of the phosphopeptide maps

4.4.4.1 Comparison of the results from in vivo phosphorylation and in vitro kinase assays

Comparing the maps of in vitro kinase assays and in vivo phosphorylation studies conclusions can be drawn on receptor phosphorylation. The receptor phosphorylation is due to auto kinase activity or due to the involvement of other kinases.

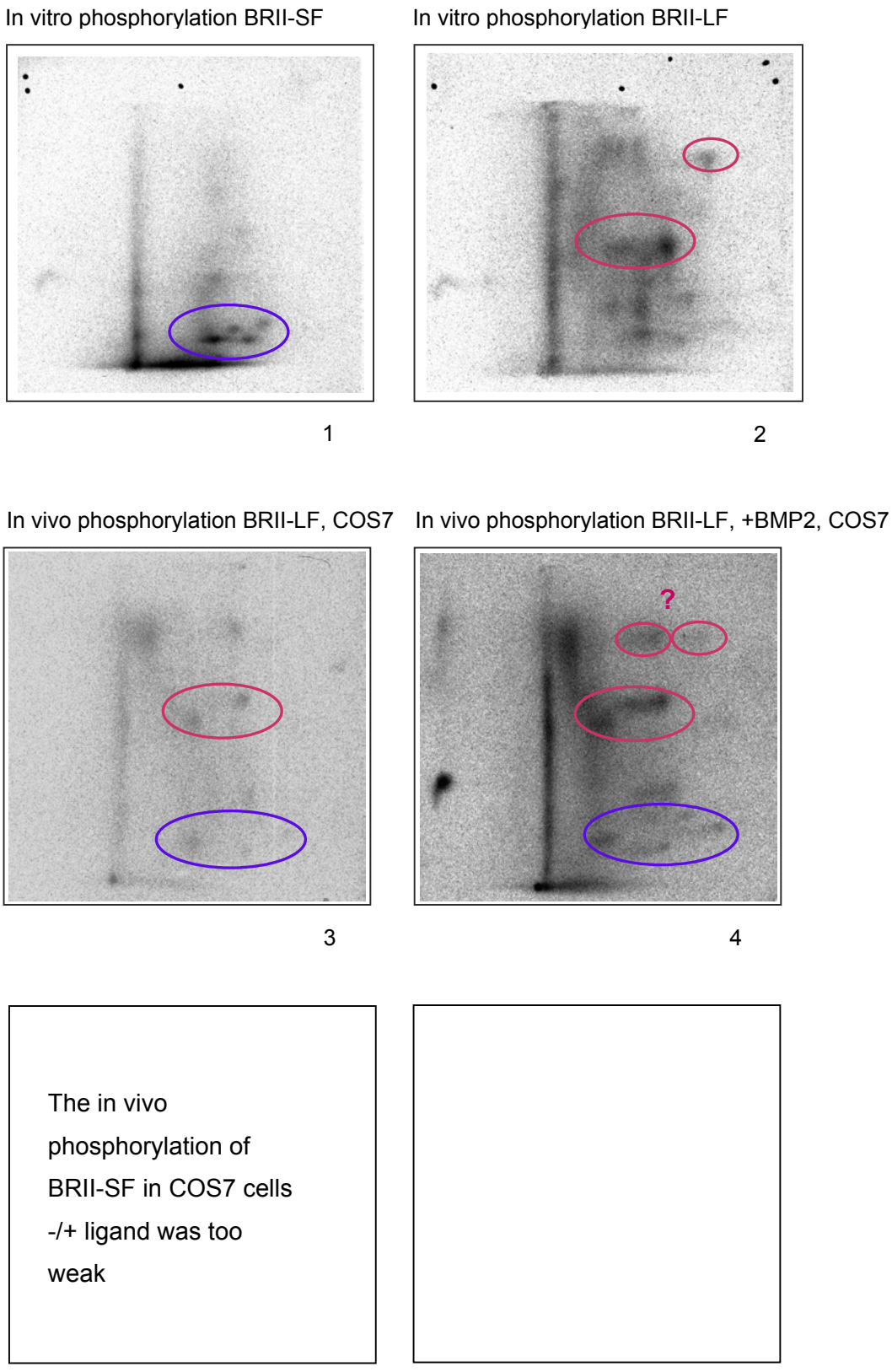


Fig. 4.57 Comparison of the phosphopeptide maps in vitro and in vivo
Comparison of different phospho peptide maps generated from transfected cells. Map1 depicts BRII-SF phosphorylation after in vitro kinase assay, Map2 shows BRII-LF phosphorylation after in vitro kinase assay and Map3 shows BRII-LF after in vivo labelling in COS7 without ligand, map4 depicts BRII-LF in COS7 cells stimulated with 20nM BMP2 for one hour. No maps could be generated from BRII-SF in COS7 cells or in C2C12 cells (endogenous receptors) due to weak phosphorylation.

Fig 4.57 demonstrates the differences between in vitro kinase assays and in vivo phosphorylation as well as the difference between BRII-LF and –SF, respectively. As depicted above (map1), the BRII-SF shows only few spots in an in vitro kinase assay, leading to the conclusion that the peptides marked in blue are phosphorylated kinase peptides. On map2 the BRII-LF in vitro kinase assay is demonstrated. In comparison to the left BRII-SF panel, more spots can be detected. Thus, the tail serves as acceptor for multiple phosphorylation events. The red marked peptides are phosphorylated BRII-LF (or tail) peptides. Map3 depicts BRII-LF phosphorylation in COS7 cells after in vivo labelling, map4 shows BRII-LF after in vivo labelling and subsequent BMP2 stimulation.

Comparison between the BRII-LF in vivo phosphorylation (maps 3-4) and the two in vitro kinase assays (maps1-2) demonstrates, that some in vivo phosphorylated BRII-LF peptides are in the kinase domain (blue circle), and some are either BRII-tail peptides or only phosphorylated in the BRII-LF (red circles). For the upper spot on the very right position (map2) it is not clear which is the corresponding one on map4, therefore it is marked with a question mark. However, some differences between BRII-LF in vitro and in vivo phosphorylation have to be noted, pointing out that physiologic conditions change receptor behaviour and phosphorylation status. This finding is in contrast to previous publications (Luo and Lodish, 1997).

4.4.4.2 Analysis of the 2D phosphopeptide map of BRII-LF in vivo labelling

In order to investigate the phosphorylation sites in BRII-LF two dimensional phospho peptide mapping was performed. Analysing the BRII-LF after expression in COS7 cells and stimulation with BMP2, resulted in 12 clearly detectable spots. Spots1, 2, 4 and 6 are the major BRII-LF spots (Fig. 4.58).

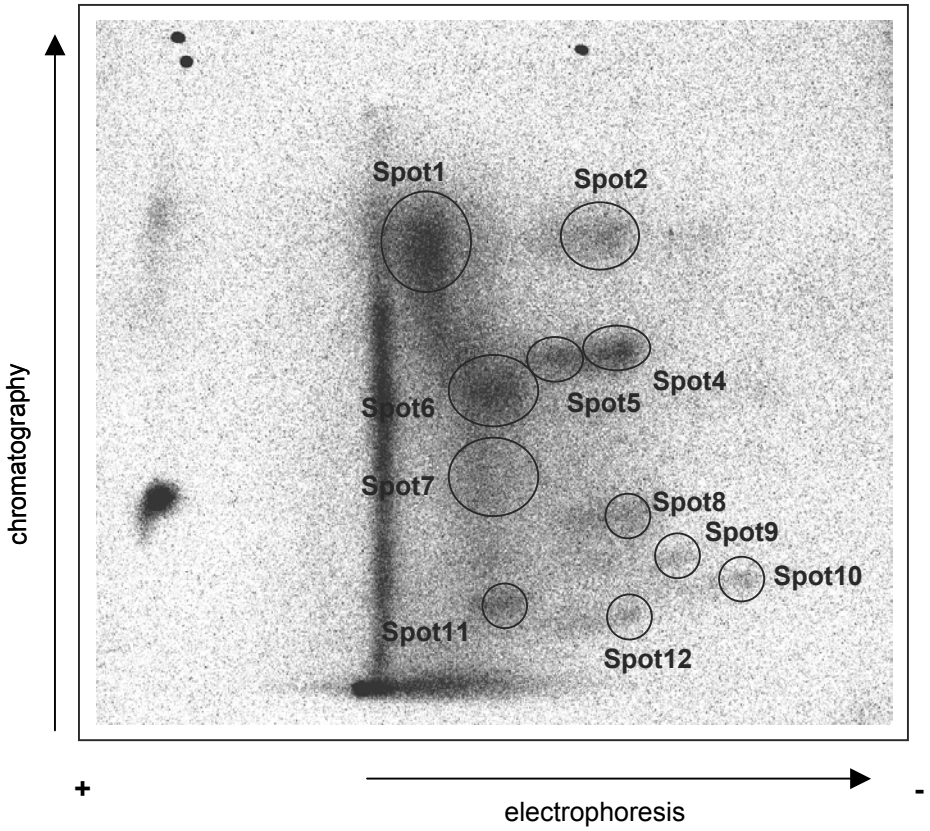
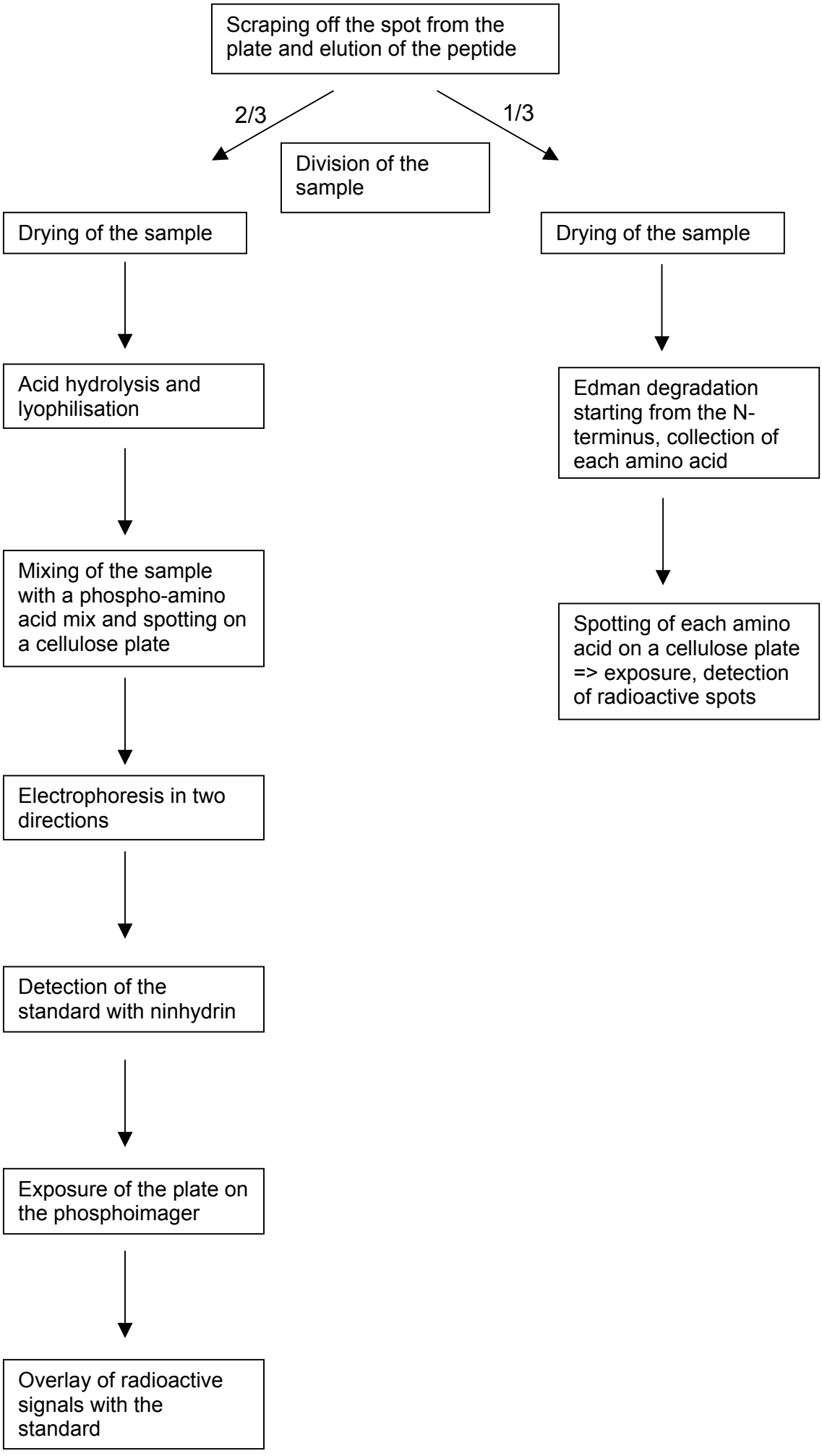


Fig. 4.58 Phosphopeptide map of the BRII in vivo
From the Map of BRII-LF plus BMP2 (Fig. 4.54) the marked spots were isolated and subjected to phospho-amino acid-analysis and Edman-degradation.

To identify the radioactively labelled peptides they were eluted from the plate and subjected partially to phospho amino acid analysis and Edman degradation, respectively (Christer Wernstedt, LICR, Uppsala).


Scraping off the spot from the plate and elution of the peptide
2/3
1/3
Division of the sample
Drying of the sample
Drying of the sample
Acid hydrolysis and lyophilisation
Edman degradation starting from the N-terminus, collection of each amino acid
Mixing of the sample with a phospho-amino acid mix and spotting on a cellulose plate
Spotting of each amino acid on a cellulose plate => exposure, detection of radioactive spots
Electrophoresis in two directions
Detection of the standard with ninhydrin
Exposure of the plate on the phosphoimager
Overlay of radioactive signals with the standard

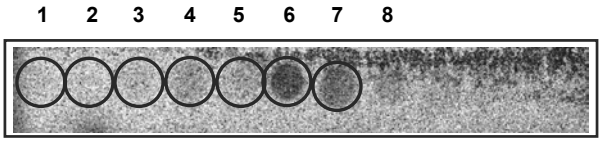


Fig. 4.59 Edman degradation of Spot1
From the Map of BRII-LF plus BMP2 (Fig. 4.58) spot 1 was isolated and subjected to Edman degradation.

The Edman degradation of Spot1 shows a very weak radioactive signal in the 4th circle, i.e. the 4th amino acid and a strong signal for the 6th and 7th amino acid. These amino acids seem to be phosphorylated. Because the first "real" phosphorylated amino acid is no.6, the phosphorylation signal decreases during the degradation. This amino acid shows the classical "tailing" pattern as well. The degradation process is never complete and some amino acids are eluted with delay, resulting in a second, weaker radioactive spot. We can conclude that this peptide is phosphorylated at position6.

Phospho amino acid analysis is performed to determine the phosphorylated amino acid (see methods 3.4.22.3). The peptide is hydrolysed with acid, mixed with a standard and subjected to two kinds of electrophoresis at different pHs. The negatively charged phosphorylated amino acids migrate with different velocities to the positive pole, resulting in a two dimensional pattern.

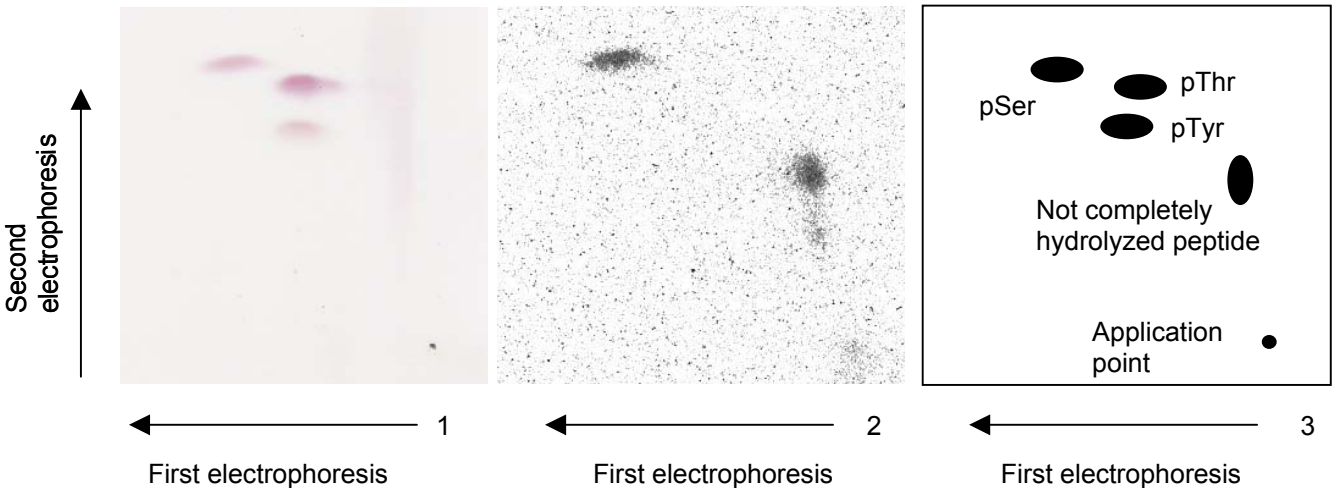


Fig. 4.60 Phospho amino acid analysis of Spot1
From the Map of BRII-LF plus BMP2 (Fig. 4.58) spot 1 was isolated and subjected to phospho amino acid-analysis. Picture1 shows the ninhydrin stain of the amino acid standards, picture2 the scan of the phosphoimager. As seen by overlay, the radioactively labelled amino acid is serine. Picture3 shows a schematic draft of the interpretation of the experiment.

Phospho amino acid analysis of Spot1 results in a strong spot being phospho-serine, according to the overlay with the standard.

After these two assays we know that the peptide from spot1 is phosphorylated at position6. The phosphorylated amino acid is a serine.

Spot	Phosphorylated amino acid	Position of the phosphorylation
Spot1	pSer	pos(4)/6
Spot2	pSer	pos6
Spot4	pSer	pos3
Spot5	pSer/pTyr	pos3/6
Spot6	pSer/pTyr	pos6/>8
Spot7	-	pos3
Spot8	pSer	pos3
Spot9	-	pos2
Spot10	pSer	pos2
Spot11	pSer	pos3
Spot12	pSer	pos4

Tab.4.6 Identification of the BRII-peptides
With the help of phospho-amino acid-analysis and Edman-degradation the phosphorylated amino acids and their position in the BRII- peptide could be identified.

Table 4.6 is summarising the results of Edman degradation and phospho amino acid analysis. Some spots could not clearly be identified as the phosphorylation signal was often very weak. The results presented here are representative for three independent experiments. Even though the BRII-LF, when compared to other serine/threonine kinase receptors, is not heavily phosphorylated the majority of the peptides could be analysed. As expected, most of the peptides are serine-phosphorylated, only few phosphorylated tyrosines could be detected. To identify the candidate peptides, the BRII-LF was virtually digested with trypsin and the resulting peptides were examined for the presence of a phosphorylatable amino acid in the right position, as seen by Edman degradation followed by calculation of the mobility during electrophoresis and chromatography (Table 4.9).
Mobility plot5, predicts the mobility of phosphopeptides and shows their migration pattern: http://acrux.igh.cnrs.fr/gs/mobility5.html;
The NetPhos program from the center for biological sequence analysis, predicts potential phosphorylation sites for different proteins: http://www.cbs.dtu.dk/services/NetPhos.

Calculation of the electrophoretic and chromatographic mobility

To determine phosphorylatable peptides a list of the predicted tryptic peptides has to be made, assuming a limited digest of trypsin which cleaves after lysine and arginine. It has to be kept in mind that partial digestion takes place when there are multiple arginine or lysine residues in tandem. Arg/Lys-Pro is not cleaved at all. In addition, Arg-Asp/Glu or Lys-Asp/Glu is not efficiently cleaved as well as Arg/Lys-X-pSer/pThr. No problems occur in cleaving Arg-pSer/pThr.
The mobility in the electrophoresis m_r is determined by the charge (e) to mass (M) ratio. To simplify one can calculate m_r=e/M. The net charge on an amino acid at a particular pH can

be calculated by summing the charges of the constituent amino acid side chain together with the charges on the α-NH2 and α-COOH groups, which are listed in table 4.7. The mass of every amino acid is listed in table 4.8.

Amino acid	**Charge at pH1,9**
N-terminal NH_2 group	+1
C-terminal COOH group	Neutral
Arg	+1
Asp	Neutral
Cys-SO_3H	~-1
His	+1
Glu	Neutral
Lys	+1
p-Ser	-1
p-Thr	-1
p-Tyr	-1

Tab.4.7 Expected charges on amino acids in peptides at pH1,9
Cys-SO_3H is the product of performic acid oxidation which is usually performed prior to tryptic digestion. Cysteine itself has no charge.

The mobility of a peptide in the chromatography depends on the hydrophobicity of the peptide. An estimate can be obtained by summing up all the hydrophobicities of all the amino acids in the peptide. The relative mobility of the amino acids in isobutyric buffer is listed in table 4.8 To calculate the mobility of a peptide the relative mobility values of all the amino acids are summed and the total is divided by the number of amino acids in the peptide. This value is not a very accurate determination of the mobility, because the amino acids in the peptide lack charges on their α-NH2 and α-COOH groups, which would significantly contribute to the mobility values. Therefore the calculated values tend to be lower and more bunched together than they really are.

amino acid	one letter code	molecular weight	R_f in isobutyric buffer
Ala	A	89	0,61
Arg	R	174	0,69
Asn	N	132	0,44
Asp	D	133	0,46
Cys-SO3H	C	121	0,23
Glu	E	147	0,53
Gln	Q	146	0,51

amino acid	one letter code	molecular weight	R_f in isobutyric buffer
Gly	G	75	0,5
His	H	155	0,67
Ile	I	131	0,92
Leu	L	131	0,95
Lys	K	146	0,58
Met-Sulfone	M	149	0,59
Phe	F	165	0,93
Pro	P	115	0,73
Ser	S	105	0,48
p-Ser		185	0,35
Thr	T	119	0,57
p-Thr		199	0,43
Trp	W	204	0,72
Tyr	Y	181	0,72
p-Tyr		261	0,53
Val	V	117	0,82

Tab.4.8 Molecular weight and mobilities of amino acids during chromatography

Coming back to our example: Spot1 is serine phosphorylated at position 6. Looking through the list of tryptic peptides we find three candidate peptides, all located in the BRII-tail: NHSVN**S**HAATTQYANGTVLSGQTTNIVTHR,
LNINS**S**PDEHEPLIR, AQRPN**S**LDLSATNVLDGSSIQIGESTQDGK and two peptides assuming a phosphorylation at position four, which is only possible if the trypsin did not cut properly (misscut).

calculation for LNINS**S**PDEHEPLIR :

$$m_r = \frac{+1-1+0+0+0+1+0}{2*131+2*132+2*131+105+185+2*115+133+2*147+155+174} = 0{,}0004844$$

$$R_f = \frac{2*0{,}95+2*0{,}44+2*0{,}92+0{,}48+0{,}35+2*0{,}73+0{,}46+2*0{,}53+0{,}67+0{,}69}{15} = 0{,}65$$

according to that the m_r and R_f values of all potential peptides were calculated.

The faster peptides migrate to the right position, the bigger is m_r and the more the peptide is on the right end of the plate. The bigger R_f is, the more the peptide is on top of the plate. The peptide of spot1 is poorly migrating during electrophoresis but well during chromatography.

Looking through all the candidate peptides (table 4.9) LNINS**S**PDEHEPLIR is the most likely one because of its calculated properties.

position	aminoacid	peptide	m_r *1000	R_f
2	pSer	G**S**LDERPVAVK	1,400	0,64
		N**S**INYER	0,924	0,58
		E**S**SDENLMEHSLK	1,103	0,58
		N**S**TK EPR	1,718 (1,965)	0,45 (0,53)
		S**S**TAVYLAEGGTATTMVSK	0,439	0,57
3	pSer	VF**S**FANR	0,974	0,6814
		YL**S**LHTSDWVSSCR	1,017	0,6
		EN**S**LAVR	1,026	0,627
		SV**S**PTVNPMSTAMQNER	0,451	0,5852
		NL**S**HNR R	2,2 (2,77)	0,59 (0,604)
		NI**S**SEHSMSSTPLTIGEK	0,869	0,58
		IP**S**PETSVTSLSTNTTTTNT.....	0.000	0,57
		ES**S**DENLMEHSLK	1,103	0,58
		QF**S**GPDPLSSTSSSLLYPLIK	0,374	0,66
		FG**S**K	2,037	0,59
		NH**S**VNSHAATTQYANGTVLSGQTTNIVTHR	1,058	0,57
		TN**S**NNNNSNPCSEQDVLAQGVPSTAADPGPSKPR R	0,242 (0,464)	0,54
		SG**S**GEK	1,364	0,49
4	pSer	LAH**S**VTR	2,062	0,65
		DLN**S**R	1,325	0,58
		DCE**S**ALK	0,000	0,53
		NI**SS**EHSMSSTPLTIGEK	0,869	0,58
		RPT**S**LPLNTK	1,463	0,63
		TPY**S**LK	1,140	0,63
		WRP**S**TWVISTESLDCEVNNNGSNR	0,307	0,57
		AVH**S**K	2,890	0,61
	misscut	N**R**N**S**INYER	1,441	0,58
6	pSer	NHSVN**S**HAATTQYANGTVLSGQTTNIVTHR	1,058	0,57
		LNINS**S**PDEHEPLIR	0,484	0,65
		AQRPN**S**LDLSATNVLDGSSIQIGESTQDGK	0,540	0,59

3/6	pSer/pTyr	NH**S**VN**S**HAATTQ**Y**ANGTVLSGQTTNIVTHR	0,508	0,56

6, >8	pSer/pTyr	NHSVN**S**HAATTQ**Y**ANGTVLSGQTTNIVTHR	0,777	0,57
		AVHSK**S**STAV**Y**LAEGGTATTMVSK	0,693	0,58
		SSTAV**Y**LAEGGTATTMV**S**K	0,000	0,56

Tab.4.9 Potential candidate peptides for BRII-LF phosphorylation sites
The tryptic BRII-LF peptides were listed according to their potential phosphorylation sites. Column1 shows the phosphorylation sites, column2 the phosphorylated amino acids, in column3 the tryptic peptides with phosphorylatable amino acids in the right position are listed; grey background indicates tail peptides. Potential migration during electrophoresis is listed in column4 and in column5 the potential migration during electrophoresis. In brackets the behaviour in case of alternative cleavage sites is shown.

As shown in Table 4.9 for most of the peptides many candidate-peptides could be identified. For a peptide that is phosphorylated at serine at position 4 there are nine different possibilities, three kinase peptides and six tail-peptides, one of which would assume a misscut of the enzyme. As we find spot12 in the middle of x-axis the map, the migration during electrophoresis is intermediate, but as it is quite at the bottom (y-axis) the mobility during chromatography is not very high. Therefore, the peptide DLNSR is the candidate with the highest probability (m_r = 1,325; R_f = 0,58), which would fit to the results from the comparison of the map that this spot is supposed to be a kinase peptide. This prediction has to be confirmed by mutation of that side followed by phosphopeptide mapping to confirm the identification.

The only peptide which could be clearly identified is the NH**S**VN**S**HAATTQ**Y**ANGTVLSGQTTNIVTHR, containing three phosphorylation sites (S815, S818 and Y825), which seem to be all phosphorylated. At this peptide mono-phosphorylation of S815 (spot4), bi-phosphorylation at S818 and Y825 (spot6) and triple-phosphorylation (spot5) can be observed. S815A seems to be also a target for auto-phosphorylation in the in vitro-kinase assay, as seen in Fig 4.58, spots in the red circle.

Still, many peptides have not been identified yet. For this, we chose a MALDI-based approach to elucidate the BRII phosphorylation. Further studies are in cooperation with Dr. Ulf Hellman from the Ludwig Institute for Cancer research, Uppsala, Sweden.

4.4.4.3 Tyrosine phosphorylation sites in BRII

In the BRII, only one phosphorylated tyrosine could be identified so far (Y825). As tyrosine phosphorylation is described to be difficult to detect with phospho peptide mapping, it is very likely that we missed some tyrosine phosphorylation sites. A more easy method to detect phosphorylated tyrosines is with the use of phospho-specific antibodies. The drawback of this method is the problem to localise the phosphorylation site. As seen in phospho-tyrosine westernblots, there is a phosphorylated tyrosine in the kinase domain as well, which could not be mapped so far (data not shown). The kinase for both of these phosphorylation events (Y825 and Y in the kinase domain) is not known so far.

BRII-LF is tyrosine-phosphorylated in the absence of ligand (Y825 and diploma thesis S. Hassel), and cotransfection of BRIa and stimulation with BMP2 for 3h enhanced that signal. Confirmation of this assumed back-phosphorylation caused by BRIa was not possible so far, often due to technical problems. As c-kit, a tyrosine kinase, was shown to be a member of the BRII-LF, BRIa complex (Fig. 4.32), the phosphorylation could originate either from a dual-kinase activity of BRIa or from the phosphorylating via c-kit. In both cases the tyrosine phosphorylation and its impact on the regulation of signal transduction still is interesting and worth to have a closer look at.

4.4.5 Intramolecular phosphorylation of BRII

In studies examining the regulation of specific biochemical events by reversible phosphorylation, assaying the protein kinases themselves can often lead to significant progress in understanding the mechanistic details of the system.

Studies with many protein serine and tyrosine kinases demonstrated both intra– and intermolecular mechanisms of auto-phosphorylation. An intra-molecular mechanism involves phosphorylation of the same peptide chain, while an inter-molecular mechanism involves the kinase domain phosphorylating amino acid residues on another peptide chain of the same type. As preceding experiments point to a high phosphorylation of the BRII-tail, it has to be examined whether this phosphorylation originates from a cis-acting phosphorylation event or whether the BRII kinase can phosphorylate the tail in trans, i.e. from the other BRII-LF in the preformed homo-oligomer. In this experiment, bacterially expressed BRII-tail is added to an in vitro kinase assay of BRII-SF or BRII-LF. If the kinase can act in trans then the bacterially expressed protein is phosphorylated. The tail does not show any auto-phosphorylation as depicted in Fig. 4.19 (chapter interacting proteins, characterisation of pull-down proteins).

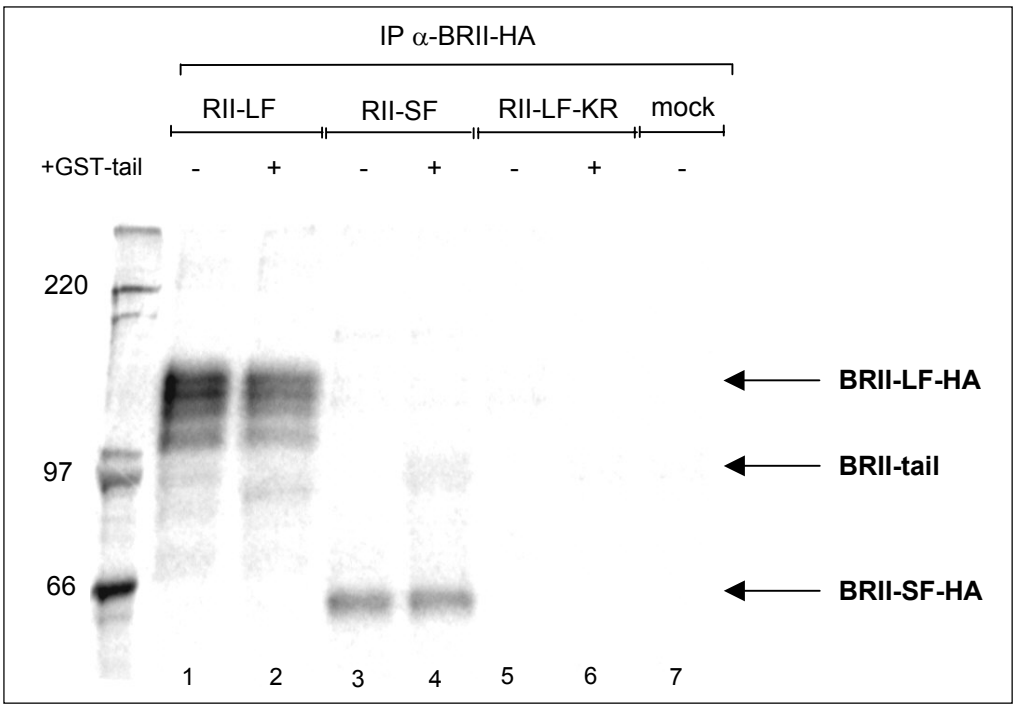


Fig 4.61a In vitro kinase assay to examine trans-phosphorylation of BRII to the tail
293T cells were transfected with BRII-LF (lanes 1-2), BRII-SF (lanes 3-4) and BRII-KR (lanes 5-6) or pcDNA3 (lane 7), the receptor was immunoprecipitated using anti-HA antibody. To some samples GST-BRII-tail coupled on Glutathion-sepharose was added (lanes 2, 4, 6) and an in vitro kinase assay was performed. The phosphorylated receptors and the in vitro phosphorylated tail is visualised by autoradiography.

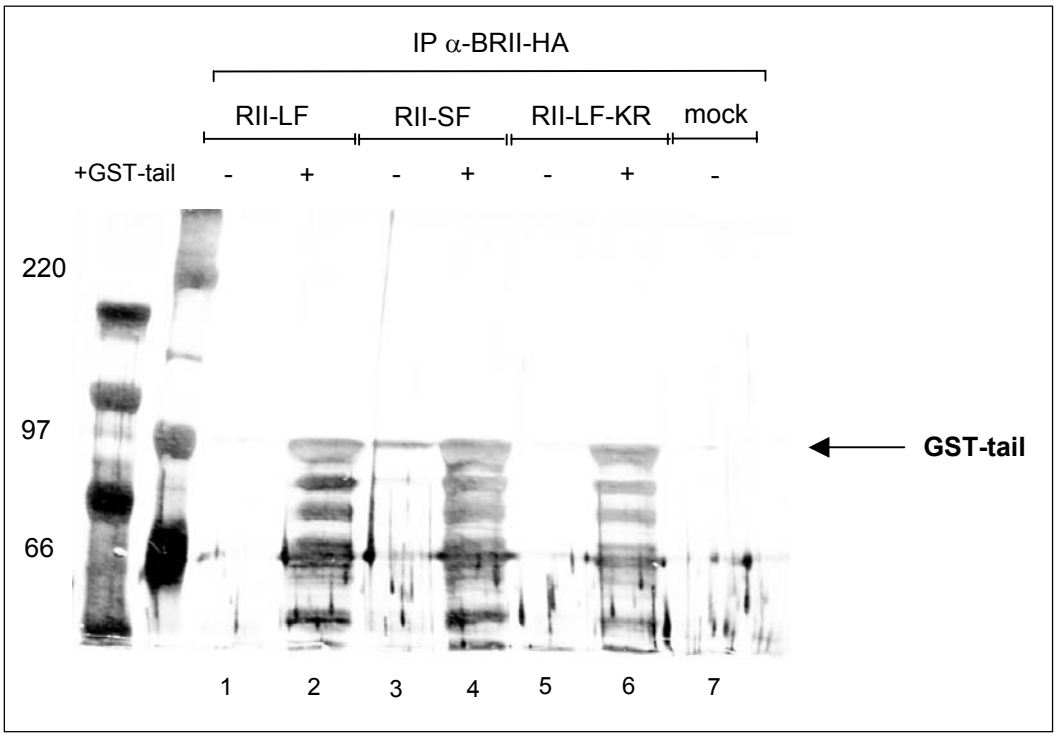


Fig 4.61b Silverstain of the in vitro kinase assay to examine trans-phosphorylation of BRII to the tail
293T cells were transfected with BRII-LF (lanes 1-2), BRII-SF (lanes 3-4) and BRII-KR (lanes 5-6) or pcDNA3 (lane 7), the receptor was immunoprecipitated using anti-HA antibody. To some samples GST-BRII-tail coupled on Glutathion-sepharose was added (lanes 2, 4, 6) and an in vitro kinase assay was performed. The GST-tail is visualised on the silver stained gel

As depicted in Fig 4.61, both BRII splice variants are able to phosphorylate the recombinant BRII-tail (lanes 2 and 4), the BRII-LF slightly stronger than the BRII-SF. In contrast, the BRII-LF-KR is not able to do so (lanes 5-6). Thus, this result indicates that the BRII is able to phosphorylate the BRII-tail in trans in an inter-molecular mechanism.
As a control, purified GST could not be phosphorylated by the different BMP receptor constructs (data not shown).

Whereas the first experiments were performed with bacterially expressed BMP receptors, the following one is examining the situation for transiently overexpressed receptors to mimic the natural situation. We chose cotransfection of BRII-LF with BRII-SF-KR to avoid auto-phosphorylation of BRII-SF and vice versa. If the BRII can phosphorylate itself in trans, i.e. from the other BRII-LF in the preformed homo-oligomer the BRII-K230R shows a signal in the in vitro kinase assay.

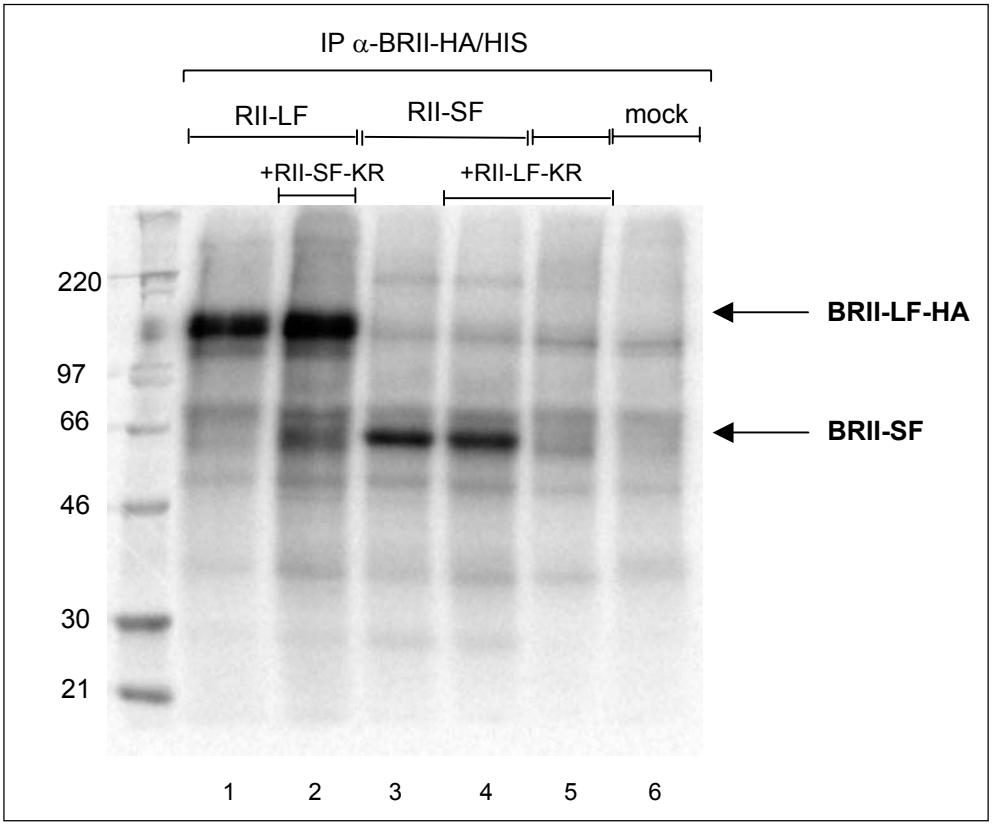


Fig 4.62 In vitro kinase assay to examine trans-phosphorylation of BRII
293T cells were transfected with BRII-LF (lanes 1-2), BRII-SF (lanes 3-4) and BRII-LF-KR (lanes 4-5) or pcDNA3 (lane 7). To some transfections BRII-SF-K230R-HIS (lane 2) was added. The HA- and His-tagged receptors were purified by immunoprecipitation and a kinase assay was performed. The phosphorylated receptors are visualised on an autoradiogramm.

In Fig. 4.62 we see that BRII-LF can phosphorylate the BRII-SF-KR in trans (lane 2). In contrast, BRII-SF is only weakly able to phosphorylate BRII-LF-KR (lane 4). As a control the auto-phosphorylation of the constructs is examined, BRII-LF as well as BRII-SF are able to phosphorylate itself.

BRII-LF can phosphorylate the GST-tail (Fig. 4.62) as well as BRII-SF in trans (Fig. 4.62).

In contrast to the experiment using GST-tail BRII-SF is only weakly able to phosphorylate the BRII-LF when transiently overexpressed. This might be due to the different confirmation of the tail alone compared with the BRII-LF structure or due to differences between bacterially and eucaryotic expressed proteins.

4.4.6 Analysis of BRII-Mutants

To analyse the BRII-LF phosphorylation and the influence of certain phosphorylation on signal transduction, BRII-LF mutants were generated. Thus, some potential candidate peptides were selected and mutated. Potentially phosphorylated serines and threonines were exchanged to alanine and potentially phosphorylated tyrosines to phenylalanine to obtain structurally intact receptors. The expression level of all the mutants, tested in COS7 and 293T cells was equal (data not shown).

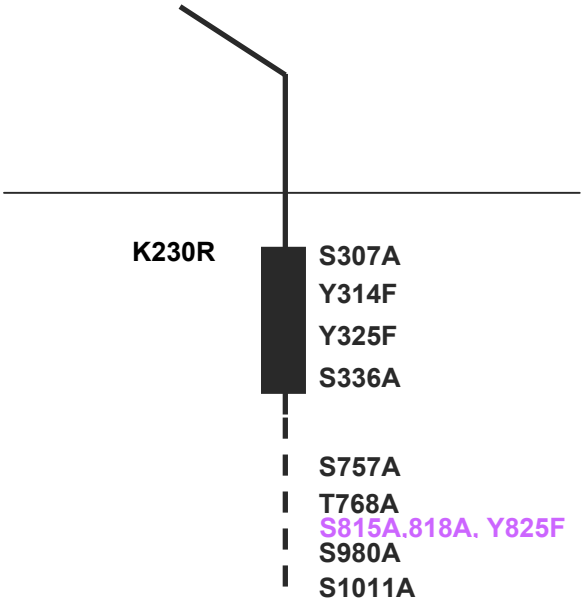


Fig. 4.63 Scheme of the mutants in the BRII-LF
Point mutations in BRII-LF shown on the right site have been generated to test phosphorylation events. The positions marked in violet are already identified targets for phosphorylation. K230R, is the negative control as this mutant was shown to be kinase-dead due to its defect in ATP-binding.

4.4.6.1 In vitro kinase assay of the mutants

The mutants depicted in Fig 4.63 were first tested for the ability to auto-phosphorylate.

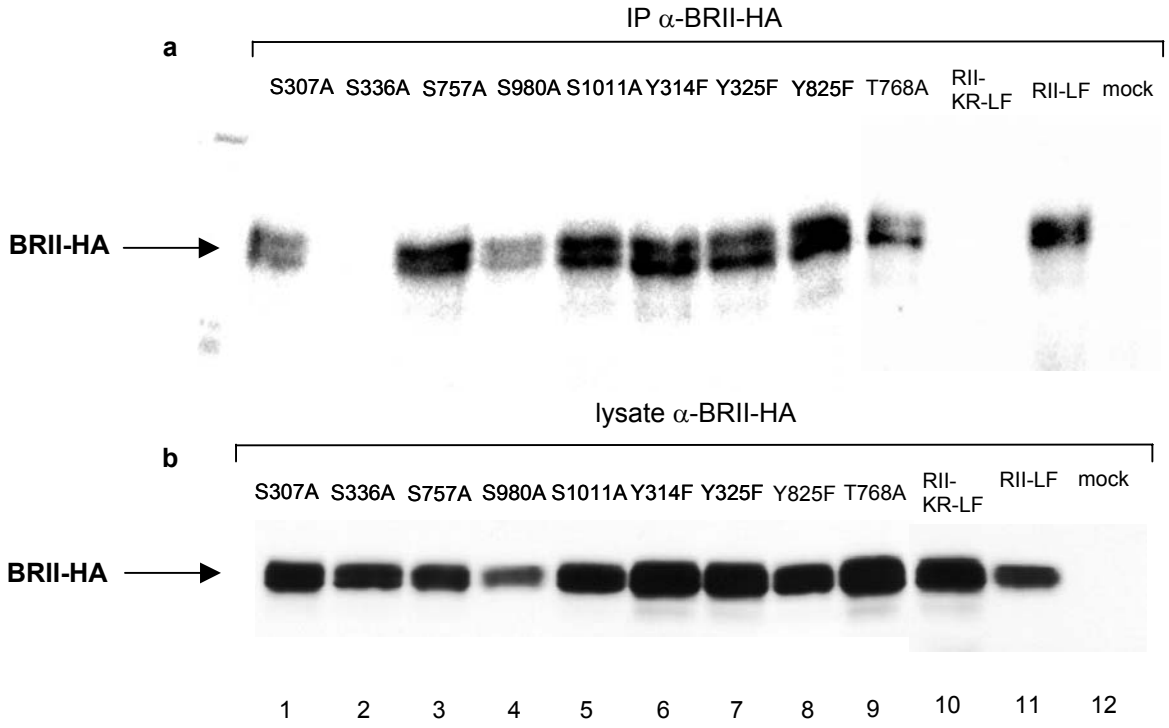


Fig. 4.64a In vitro kinase assay of the BRII-LF point mutations
293T cells were transfected with the BRII-LF mutants (lanes 1-10) and the wildtype variant (lane 11), the receptor was immunoprecipitated using anti-HA antibody and an in vitro kinase assay was performed. The phosphorylated receptor is visualised by autoradiography.

Fig. 4.64b Expression control of the BRII-LF point mutations
293T cells were transfected and the lysate was subjected to SDS-PAGE. The expressed receptors were detected by westernblotting using anti-HA antibody.

Wildtype and mutant BRII-LF were isolated from 293T cells by immunoprecipitation and labelled in vitro by auto phosphorylation (Fig. 4.64). By comparing the intensities of the radioactive bands the kinase activity of the receptor variants can be judged. As expected, the BRII-LF is auto-phosphorylating (lane 11) whereas the K230R is not (lane 10). The Y314F mutant shows a weaker signal which might be due to a lower expression level. However, the S336A mutant, which shows equal expression compared to wildtype and K230R, completely abrogates phosphorylation (lane 2). The other mutants do not show significant differences compared to the wildtype receptor. The weaker signal for S980A is due to a lower expression level, whereas all the other receptors are equally expressed (Fig. 4.64).

To investigate whether the BRII-LF point mutations have the same potential to transmit signals like the wild type they were tested in two functional assays, a reporter gene and the alkaline phosphatase assay.

4.4.6.2 Reportergene assay for BRII-mutants

In C2C12 cells the different receptor mutants were tested for their ability to induce transcription from the SBE-luciferase reporter in response to BMP2.

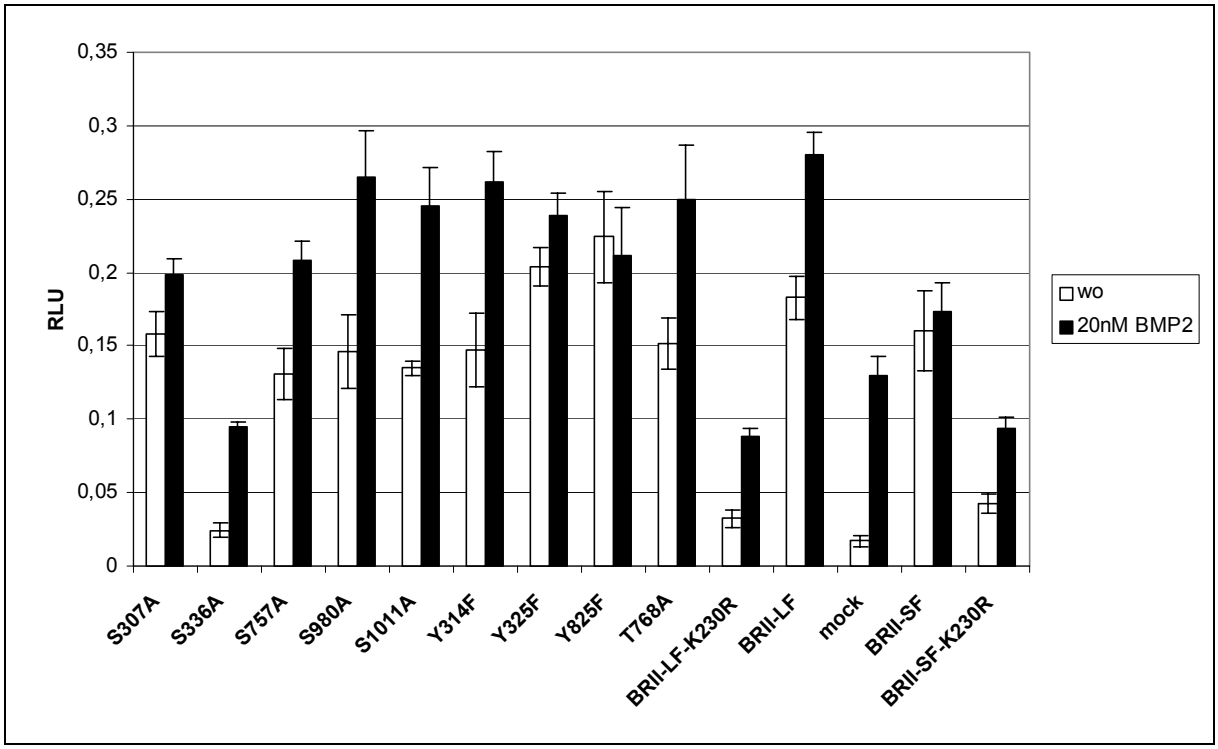


Fig. 4.65 Reportergene assay
C2C12 cells were transfected with the BRII-LF receptor variants and stimulated with 20nM BMP2 for 24h. Luciferase activity was measured using SBE as a reporter and RLTK to normalise the signals. This experiment is representative for three independent experiments.

As depicted in Fig. 4.65 the BRII-LF is enhancing the signal compared to mock transfected cells, a similar result is obtained by transfecting the BRII-SF. Both kinase dead variants (K230R) inhibit the signal due to their inability to bind ATP. Whereas most of the other mutants show no significant difference compared to wildtype the mutant Y325F as well as the mutant Y825F show high transcription levels even in the absence of ligands and no or low response to ligand. Surprisingly, the mutant S336A is completely inhibiting the activation of the reporter gene. As previously shown BRII-LF-K230R and S336A can not phosphorylate themselves. Both mutants block the signal in the reportergene assay, so it can be concluded that (auto-) kinase activity of the BRII is important for the onset of the Smad-pathway.

4.4.6.3 Alkaline phosphatase measurement

Besides the activation of the Smad-pathway, BMP2 can activate the ALP pathway which is shown to be Smad-independent (Nohe et al., 2002). To investigate whether this pathway is influenced by the various BRII-LF mutants, we measured ALP activity in transiently transfected C2C12 cells.

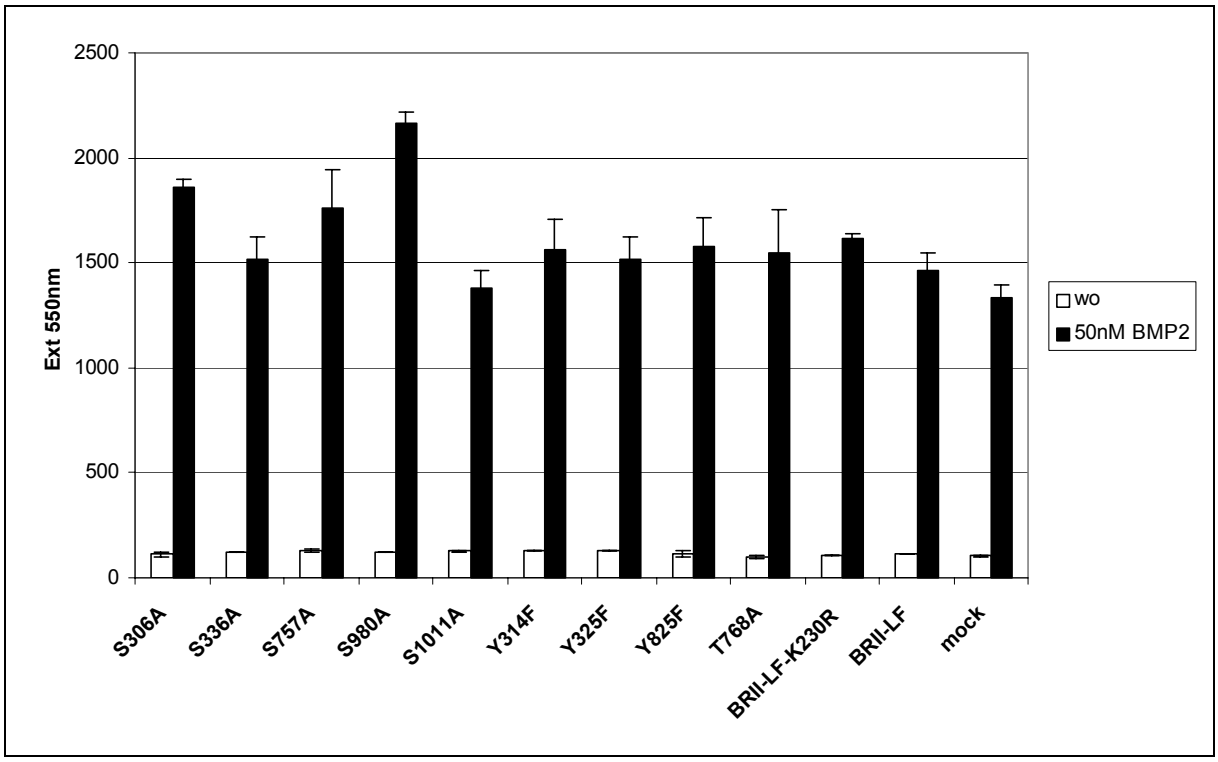


Fig. 4.66 Alkaline phosphatase activity measurement
C2C12 cells were seeded in a 96-well plate and transfected with the BRII-LF receptor variants. The following day cells were stimulated with 20nM BMP2 for 72h. Alkaline phosphatase activity was measured using pNPP as a substrate.

As described previously (Katagiri et al., 1994), ALP activity is enhanced upon stimulation with BMP2 in C2C12 cells. Transfection with BRII-LF slightly enhances this effect. Transfection with the BRII-LF mutant S1011A shows no stimulating effect, whereas all other mutants induce ALP production. Surprisingly, the two kinase defect mutants S336A and BRII-LF-K230R, do not show any inhibiting effect. Since both mutants are inhibiting the Smad-pathway as shown in Fig 4.66, the data presented here further support our previous finding that the ALP pathway is Smad-independent. Furthermore it suggests that kinase activity of BRII is not needed for the induction of ALP.

4.4.6.4 Surface expression of BRII-mutants

As demonstrated above, the two BRII-LF mutants BRII-K230R and BRII-LF S336A do inhibit auto-phosphorylation and Smad-dependent signalling. The mutant BRII-LF-K230R was designed according to other kinase-deficient variants, which do not bind ATP. The reason for the inhibiting effect of S336A is still speculative. One reason could be that the mutant is not expressed on the cell surface. To test this, C2C12 cells were transiently transfected with BRII-LF-HA as a control and the two mutants, BRII-LF-K230R-HA and BRII-LF-HA S336A. The cells were subjected to copatching, that means antibody treatment, which leads to clustering of the receptors to patches, followed by fixation. The stained receptors were detected by immunofluorescence (experiment done by Simone Schmitt) .

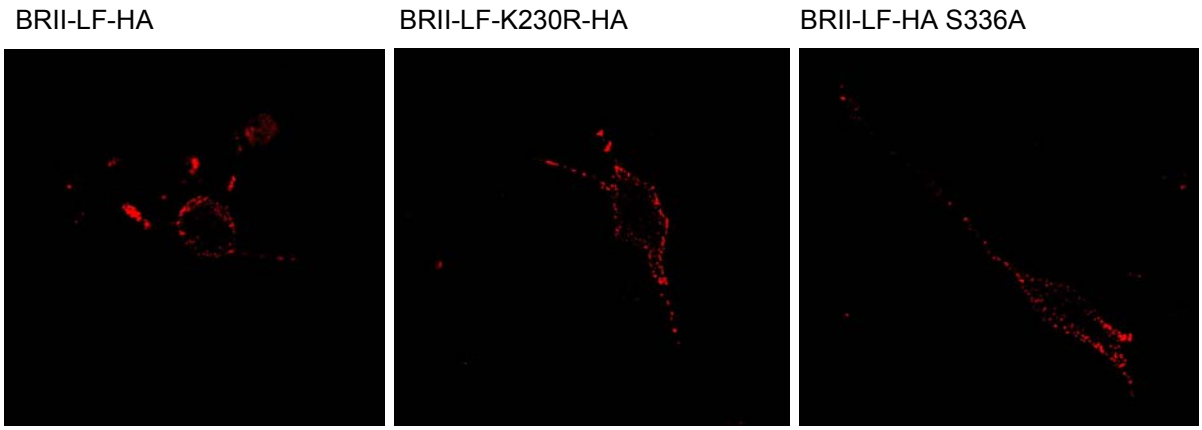


Fig. 4.67 Immunofluorescence of BMP receptors to test surface expression
C2C12 cells were seeded on coverslip chmanbers and transfected with the BRII-LF receptor variants. The following day cells were incubated with anti-HA followed by incubation with the secondary antibody and fixation. Receptors on the cell surface were detected by immunofluorescence microscopy.

As depicted in Fig 4.67 all three BMP type II receptors are expressed on the cell surface. Here, only surface-proteins were stained, as the antibodies could not enter the non permeabilized membranes. Thus, BRII-LF-HA S336A is inhibiting the Smad-pathway despite normal surface expression. The protein is not retained in the endoplasmatic reticulum or in the golgi apparatus.

5. Discussion

The BMP receptor mediated signal transduction is not deeply explored. Therefore our aim was to address different aspects of BMP mediated signal transduction with main focus on BRII and its regulation. As the aspects of this work address different topics, they will be discussed separately in the following.

5.1 BRII - receptor oligomerization studies (Nohe et al., 2002), (Hassel et al., 2003)

Physical proximity of proteins and furthermore their interaction controls cellular processes. Interactions of receptors lead to phosphorylation events that trigger subsequent signal transduction. In most cases, ligand induces the formation of oligomers. For a small subgroup of receptors ligand-independent homo- or hetero-oligomerization is described. The Epo receptor, the EGF receptor as well as the TGF-β receptors belong to that group (Constantinescu et al., 2001), (Martin-Fernandez et al., 2002), (Henis et al., 1994). Surprisingly, serine/threonine (*e.g.* TGF-β R) as well as tyrosine kinase receptors (*e.g.* EpoR, EGFR) can be found with this specific property. Due to the different activation mechanisms between these groups, the mechanisms to keep the receptors silent, vary as well. Whereas the EGF receptor is N-glycosylated at a specific site to suppress spontaneous ligand-independent oligomerization (Tsuda et al., 2000), TβRI is associated with an inhibitory protein, FKBP12, that keeps it in an inactive state (Wang et al., 1996b). The Epo receptor is shown to exhibit different, active and inactive orientations of the transmembrane and cytosolic domains (Seubert et al., 2003). All the receptors mentioned undergo conformational changes to induce signal transduction.

5.1.1 BMP receptor oligomerization

As close relatives to the BMP receptors, the TGF-β receptors, have been shown to form ligand-independent preformed homo- as well as hetero-oligomeric complexes, even though to a very low extent, our interest was to examine the oligomerization pattern of the BMP receptors (Gilboa et al., 1998), (Wells et al., 1999).

Our lab compared the TGF-β and the BMP receptor system and demonstrated that the BMP receptor oligomerization pattern shows a higher degree of flexibility (Gilboa et al., 2000). For BMP receptors there exist preformed homo- and heteromeric receptor complexes. Ligand addition increases the percentage of BRI homooligomers, as well as heterooligomers of BRI and BRII. The ligand-induced homo-oligomerization is specific for BRI, whereas the latter could be observed for the TGF-β receptors as well, but in a lower percentage. In the absence

of ligand monomers as well as homomers and heterodimers can be observed (Gilboa et al., 1998).

Thus, we could show that on the cell surface there are preformed complexes of BRI and BRII and homomers, which could be recruited by ligand-binding into hetero-oligomeric signalling complexes.

The fist aim was to determine the oligomerization domain within BRII. To address this and further questions truncation mutants of BRII were created (TC1-TC8). The respective stop-codons were introduced after a proline-tyrosine rich sequence. As it could be demonstrated, the hetero-oligomerization requires the kinase domain of BRII. TC1, the very short BRII variant lacking the whole intracellular domain, could not be recruited in a ligand-independent fashion (Fig. 4.3 and 4.4). Addition of ligand induced the formation of hetero-oligomers containing all truncation variants of BRII (Fig. 4.5). Thus, TC1 is a tool to discriminate between ligand-induced and ligand-independent complex formation.

With the help of the truncation TC1 and the kinase-deficient BRII-SF-KR the signal transduction between ligand-induced and preformed complexes should be investigated.

5.1.2 Influences of the different BMP receptor complexes on signal transduction

Using cells stably expressing the truncation variants or being transiently transfected, some characterized BMP dependent signal transduction pathways were examined. As it has been demonstrated before, BMP2 induces Smad1/5/8 as well as p38 phosphorylation in signalling cell lines. Upon BMP2 stimulation the osteoblastic marker gene Alkaline phosphatase is upregulated as well.

We could show that TC1, without a kinase domain, expected to act in a dominant-negative fashion, was unable to block Smad1/5/8 phosphorylation as well as pSmad1/5/8 induced reportergene activity (Fig. 4.7 and 4.8). In contrast, p38 phosphorylation was completely abolished (Fig. 4.9 and 4.13). Moreover the BMP2 mediated induction of ALP was completely blocked by TC1 (Fig. 4.10 and 4.14). BRII-SF-KR was inhibiting both pathways, p38 and pSmad1/5/8 significantly, demonstrating that the kinase domain is essential for both pathways.

Not being recruited in preformed complexes, TC1 does not block the Smad-pathway. Whereas BRII-SF-KR, that can be recruited in both complex types, is blocking both, Smad- and p38 pathway. Finally we concluded that the p38 seems to be Smad-independent as TC1 did completely block it. Other data obtained with delta Smad 4 not blocking ALP do support the theory.

One drawback of that new model is the lack of knowledge upon receptor composition. Still, the number of BMP type I and type II in the preformed as well as the ligand induced complexes could not be determined. Experiments with BMP muteins, who exhibit defects in

either BRII or BRI binding give hints that for ALP production two BRI and one BRII molecule are necessary. For preformed complexes and induction of Smad-signalling, ligand bound to BRII as well as BRI dimer is necessary (Knaus and Sebald, 2001). As the onset of MAPk pathways is mediated by other mechanisms than the Smad-pathway we postulate different proteins being associated with BRI or BRII, here named Protein A, B and C (see Fig. 5.1). These proteins could either facilitate complex formation or inhibit complex formation by binding to one partner and preventing binding of the other.

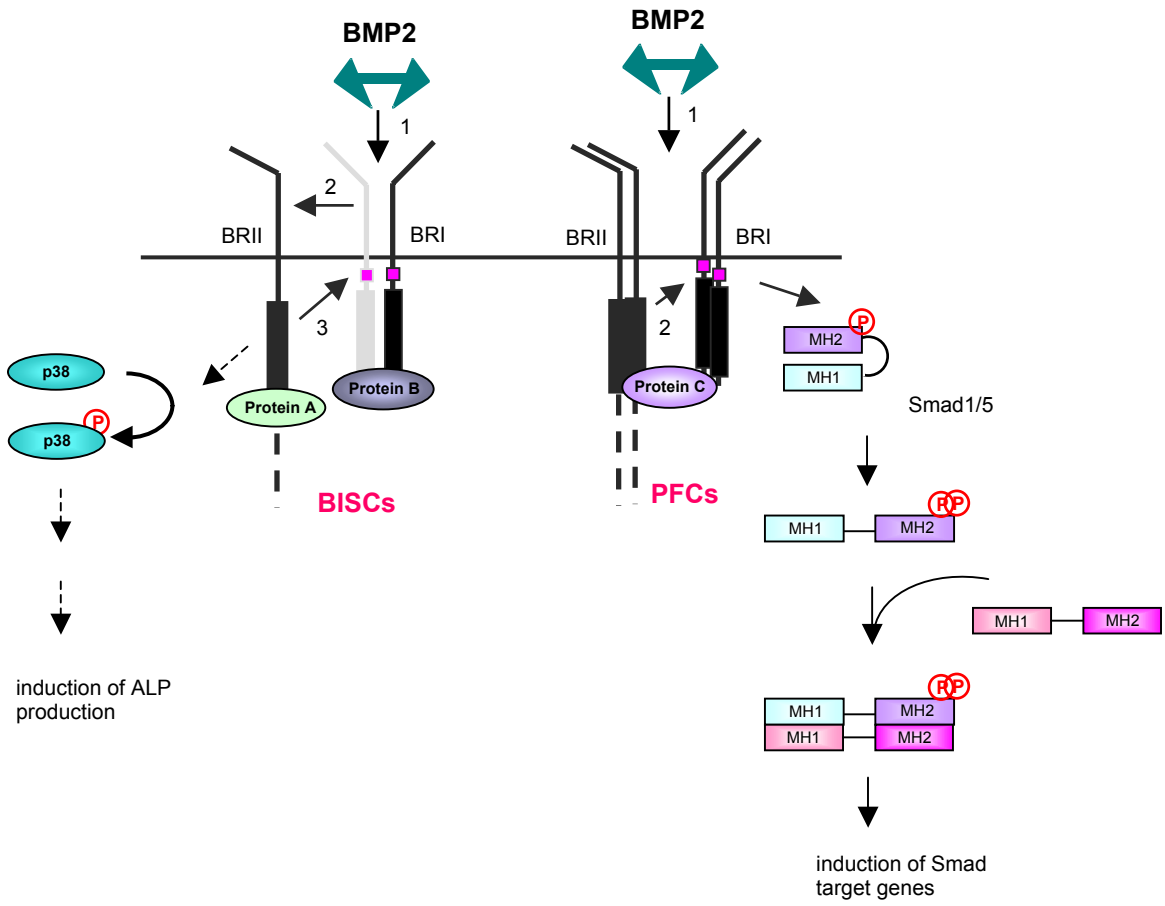


Fig. 5.1 Different BMP receptor complexes induce different signalling pathways
BMP2 can either bind to preformed complexes (PFC) on the cell surface and induce Smad-dependent signalling or it binds to the high affinity receptor, BRI. Subsequently BRII, the low affinity receptor is recruited and these BMP induced signalling complexes (BISCs) promote the p38 pathway which finally leads to ALP induction.

5.1.3 BMP receptor arrangement

ICS (image correlation spectroscopy) is a method to determine the close proximity of two stained proteins. Here the cluster density of BMP receptors was determined (Anja Nohe). It could be shown that the mode of clustering for BRII is stable, whereas the clusters of BRIa are rearranged in the presence of BRII. We conclude that the BRIa when rearranging is

forming preformed complexes with BRII. These model is supported by the observation that the signalling of constitutively active BRIa-Q233D can be enhanced by cotransfection of BRII (Fig. 4.15). Thus, the BRIa-Q233D is recruited, mediated by BRII, into the regions of efficient Smad-dependent signalling.
Therefore it is of great importance to determine BMP receptor assembly and clustering and the cellular compartments where these events, including signalling, occur.

5.1.4 The Pros and cons of Smad-independent pathways

In 1999 Iwasaki and coworkers discovered in PC12 cells, that BMP2 induces pathways alternative to the Smad1/5/8 phosphorylation, namely p38 phosphorylation (Iwasaki et al., 1999). Gallea et al. showed that this is the case for C2C12 cells as well (Gallea et al., 2001). Moreover it was demonstrated that BMP-stimulation leads to subsequent activation of ERK, PKC, PI3K and JNK, probably cell-type dependent (Gallea et al., 2001), (Palcy and Goltzman, 1999), (Lou et al., 2000), (Guicheux et al., 2003), (Vinals et al., 2002). The mechanisms that lead to the activation of these pathways are still under investigation. As it has been shown there exist two different types of p38 signalling in cells, a long term and a short term. We showed the short term p38 pathway to be Smad independent and to be involved in ALP signalling (Nohe et al., 2002).
As the ALP staining is a long term readout with 72 hours of stimulation it is very difficult to determine which factors influence the production of that marker gene of osteoblast differentiation.
ALP production induced by BMP2 requires newly protein synthesis, whereas mediated by Wnt3a it does not. In this case, canonical β-catenin signalling seems to be involved (Rawadi et al., 2003). It could be that BMP2 is inducing a second factor, maybe Wnt3a, which in turn, regulates ALP production and is regulated via alternative pathways (Van Bezooijen et al., 2004). But still the ways how BMP2 transmits the signals for ALP induction have to be discussed.
There are several publications supporting the involvement of p38 in the regulation of ALP. Using specific inhibitors, p38 was shown to be essential in BMP2 mediated up-regulation of type I collagen, fibronectin, osteopontin, osteocalcin, and alkaline phosphatase activity, whereas ERK mediated BMP-2 stimulation of fibronectin and osteopontin (Lai and Cheng, 2002). Moreover, an intact ERK cascade has impact on ALP production as well (Gallea et al., 2001). Overexpression of the p38-activating MAPK, MKK3 did not induce ALP or OC, indicating that p38 activation is necessary but not sufficient for the acquisition of the osteoblast phenotype or that the activation of p38 happens via another mechanism (Gallea et al., 2001). Others suggest ALP activation only via p38, without involvement of ERK (Suzuki et al., 1999), (Suzuki et al., 2002). Prostaglandins are shown to be regulators for bone

function and resorption. Kaktia and coworkers claim that prostaglandin E1 (PGE(1)) stimulates p38 MAP kinase through the activation of PKA, resulting in the enhancement of ALP activity. Stimulation with ephinephrine or prostaglandin E1 is sufficient for ALP induction, no BMPs are needed (Kakita et al., 2004).
These experiments support Smad-independent onset of ALP production and involvement of p38 in that pathway. But most of the experiments were done using inhibitors, either chemical (MEK inhibitor PD98059, p38 inhibitor SB203580) or on the protein level (BMP inhibitor Noggin, Wnt inhibitor dickkopf 1). Because the specificity of these inhibitors is not very high, as we could see in our own experiments and even solvents can influence signalling, it is difficult to draw final conclusions.

On the other hand, there are several publications supporting the model of Smad-dependency of ALP production. Fujii and coworkers demonstrated induction of ALP via constitutively active Alk1, Alk2, Alk3 and Alk6. Infection of Smad1 and Smad5 showed the same effects and I-Smads were shown to inhibit ALP (Fujii et al., 1999). Here it seems that the induction of ALP is mediated via BMP Smads and inhibited by Smad6 and Smad7. One major drawback is the adenoviral system they used. As we could observe with retroviral transduction, cells highly infected loose their normal behaviour and react in an uncontrolled way. Therefore cells with low expression levels have to be chosen and taken for further experiments. Using adenoviral transduction the cells might react in an unspecific manner.
When investigating the liver/bone/kidney ALP promoter, Tyzlanowski et al., found E2-box sequences therein. These sequences were found to bind to SIP1, also known as ZEB-2, that is a known Smad1 binding partner and repressing transcription by recruiting the transcriptional repressor CtBP (Tylzanowski et al., 2001), (Postigo et al., 2003). SIP1 can repress ALP activity induced by constitutive active ALK2 /Smad1/Smad5. As ALP as long-term read-out is difficult to analyze the onset of ALP via ca. ALK2 hints towards Smad1/ involvement. The repression via SIP1, however, is not a very strong support. As SIP1 action is repressed by Smad1 binding SIP1 and inducing its displacement from DNA, the onset of the regulation of ALP in vivo can not be explained via this mechanism.
Growth factors as EGF and FGF have been shown to influcence Smad-dependent signalling in a negative manner. Via receptor tyrosine kinase activated ERK phosphorylates Smad1 at certain sites in the linker region, thus inhibiting transcriptional activation (Kretzschmar et al., 1997). Nakayama and colleagues could block the onset of the ALP pathway by stimulating cells with EGF and FGF-2, thus leading to Smad1 linker phosphorylation and subsequent ALP inhibition. Transduction with constitutively active MEK mimicked the ligand effect, while it was blocked by dominant negative Ras or treatment with a MEK1 inhibitor, PD098059 (Nakayama et al., 2003). But even the activity of Smad1, that was mutated at ERK

phosphorylation sites, was inhibited by ERK, pointing out that there are more levels of potential inhibition and control of Smads and MAPK pathways, maybe via ERK phosphorylation of transcription factors or coactivators.

Another publication shows the involvement of Smads and other factors in ALP production. During the osteoblastic differentiation of 2T3 cells the expression of a dominant negative PI3K abolished the induction of ALP. The actions of this lipid kinase are mediated by phosphorylation of Akt, which in turn has been shown to regulate nuclear translocation of Smad1 and Smad5. Thus, BMP2 seems to stimulate Smad1/5 and, in addition, activate PI3K. The PI3K induces Akt phosphorylating Smads which influences nuclear translocation of Smads and the trigger of ALP (Ghosh-Choudhury et al., 2002).

Complex processes often need regulation mediated and adjusted by several factors. Hata et al. showed, that Smad1/5 and p38 act concomitantly on the regulation of PPaRγ (proliferator-activating receptor gamma) during the adipogenesis of mesenchymal cells (Hata et al., 2003). In this case, activated Smad1/5 is responsible for the expression of PPaRγ, which in turn regulates adipogenic differentiation. Whereas p38 is supporting adipocytic differentiation downstream of PPaRγ and transcriptional activation of PPaRγ. Thus, BMP2 controls adipocytic differentiation by using two distinct signalling pathways that play differential roles and act together (Hata et al., 2003).

Maybe we observe similar regulation processes, probably at different levels in the regulation of ALP production. The ALP induction is a long term readout with 72 hours of stimulation, therefore it is very difficult to determine which factors influence the production of that marker gene during osteoblast differentiation. Thus it seems to be likely that BMP2 mediated pathways (p38 and maybe Smad1/5) induce transcription of a factor that subsequently induces ALP and signals itself in a MAPK or PI3K dependent manner.

As we believe in our data, we do not postulate the ALP pathway being a exclusively non-Smad-pathway. We see TC1 inhibiting both, ALP and p38, but no influence on Smad1/5/8 signalling. Thus, a participation of Smad1/5/8 in BISC signalling can not completely be excluded. As we could observe, the regulation or modulation of the ALP signal, anyway, seems to be mediated by p38.

When critically examining the results obtained in oligomerization and signalling experiments, it can be concluded, that PFCs are clearly Smad-dependent, but on the basis of our experiments, it can not be proven that signalling mediated via BISCs is Smad-independent. To solve this problem, a BRIa mutant was created with mutations in the L45 loop, that should be unable to bind and thus phosphorylate BMP-Smads, according to Itoh et al. (Itoh et al., 2003). Surprisingly that mutant exhibited a very strong induction of the Smad-pathway, as it was determined by reportergene experiments, and could not be used to dissect BISC signalling.

5.1.5 Smad-dependent and independent pathways in disease

As is has been demonstrated, *BMPR2* mutations induce primary pulmonary hypertension (Lane et al., 2000), (Deng et al., 2000), (Thomson et al., 2000). The cause of their pathogenicity is still unclear. Among the 41 mutations identified so far, the majority can be found in the kinase domain, but 9 are situated in the tail domain inducing frame shifts or stop codons, four more point mutations (Waite and Eng, 2003). The severity of the disease can not be connected to the location of the mutation. As BRII is the receptor that initiates BMP receptor signal transduction, pathogenic mutations might influence signalling. Mutations in the extracellular domains affecting cysteines are shown to block Smad-dependent signalling. Moreover transmembrane and intracellular kinase mutants showed the same effect. For tail mutations that effect could not be observed. All mutants investigated show upregulation of the p38 pathway and enhanced proliferation (Rudarakanchana et al., 2002). Nishihara and colleagues could show that the inhibition of the Smad-pathway was due to a defect in transport of the receptor to the plasma membrane. The tested tail mutations were behaving as wildtype (Nishihara et al., 2002). Here, the mutations were not tested for influences on the p38 pathway.

Still the question is open why PPH mutants induce diseases even if their signalling seems to be normal.

On the one hand BRII interacting proteins, like Tctex-1 or others, who have not been identified yet, might regulate cellular responses, cell shape and differentiation. On the other hand, as described above, not all the mutants were tested for the induction of known BMP pathways. Thus, the mutations, even though they are very different, might result in inhibition of the same pathway. Interestingly, Nishihara described BRII mutations negatively affecting the Smad-pathway, whereas tail mutations were shown to be normal. This is another proof of the existence of Smad-dependent and Smad-independent signalling side by side.

5.2 BRII associated proteins (Hassel et al., 2004)

Up to now only few BMP receptor interacting proteins are described. It has been shown that BRAM1 and XIAP interact directly with BRIa. Whereas the function of BRAM1 has not been investigated yet, XIAP and associated TAB1/TAK link BMP signalling towards MAPK activation (Kurozumi et al., 1998), (Yamaguchi et al., 1999), (Yamaguchi et al., 1995, Shibuya, 1996 #219), (Shibuya et al., 1998).
Recently LIMK1 was identified as a BRII interacting protein (Foletta et al., 2003). Interaction between LIMK1 and BRII inhibits the ability of LIMK1 to phosphorylate cofilin, an actin-depolymerizing factor, and addition of BMP4 reduced this effect (Foletta et al., 2003). Thus BRII is linked to the regulation of actin dynamics. Tctex-1, a light chain of the motor complex dynein, interacts with the cytoplasmic domain of BRII and is a target for BRII mediated phosphorylation (Machado et al., 2003). It links BRII towards protein transport along microtubuli, that might facilitate receptor – Smad interaction (Machado et al., 2003).

The detailed molecular functions of a protein remain unclear since the molecular networks involving it have not been elucidated. Therefore it is important to identify novel interaction partners to approach the complex BMP2 signalling network. Newly identified proteins link the BMP receptor complex to diverse cellular processes such as DNA repair, signalling and metabolic processes.

5.2.1 Identification of BRII interaction partners

To identify BRII interacting proteins, GST pulldowns with GST-BRII-SF, GST-BRII-LF and GST-BRII-tail were performed (see Fig. 4.19). Associated proteins were fished from the murine mesenchymal cell line C2C12, that expresses BRII-SF as well as BRII-LF and phosphorylates the signal transducers Smad1/5/8 as well as p38 and ERK upon BMP2 stimulation. Interacting proteins were identified with mass spectrometry. The Z-Value is an indicator for probability of identification. If the Z-Value is higher than 2,19 (marked in green) the identification does not have to be confirmed by westernblotting to be accepted. Using these criteria, Tubulin β5, arylsulfatase A, sulfotransferase estrogen preferring and otoconin-90 were identified as BRII-SF interactors. For BRII-LF only few interacting proteins could be found, none of them with a high probability. But with BRII-tail integrin linked kinase (ILK) and interleukin 2 receptor β (IL2Rβ) precursor were precipitated and clearly identified (Tab. 4.1-4.3).

5.2.2 Groups of interaction partners

The majority of all BRII interacting proteins is involved in signal transduction, few proteins are so far unknown or can not be related to the BMP pathway. A relatively small percentage are structural and metabolic proteins as well as proteins involved in transcriptional regulation. In addition to signal transduction, thus, BRII is involved in other cellular processes as regulation of cellular stability, membrane shape and metabolism (Tab. 4.4, Fig. 4.21).

5.2.3 Functions of interaction partners

As we have identified more than 50 proteins interacting with the BRII, the focus here will be on some proteins that may influence BRII mediated cellular processes and play important roles to better understand BMP2 pathways.

Despite the fact that the conditions for the pulldown were relatively harsh and thought to precipitate intracellular proteins, four transmembrane proteins were pulled down. Among them was BRII-SF, which was fished with the BRII-SF. As it has been demonstrated, BRII as well as TβRII is forming ligand-independent homooligomers on the cell surface, the presence of BRII-SF in that screen is a proof of the theory (Henis et al., 1994), (Gilboa et al., 1998).

5.2.3.1 Smad-independent signalling

The BRII-SF interactor PKCβ, that represents a family of phospholipid-dependent serine/threonine kinases, has been shown to be involved in signalling in C3HT101/2 cells, which is another and more primitive mesenchymal cell line (Suda et al., 1999), (Thorsen et al., 2003). Moreover it regulates osteoclast formation and function, potentially via the ERK-pathway (Lee et al., 2003b). It has been shown that PKC signalling is required for BMP-dependent induction of apoptosis via Casp-9, Casp-3, Casp-6 and Casp–7 (Hay et al., 2001) (Hay et al., 2004) and for BMP2-dependent upregulation of junB and c-fos mRNAs and subsequent, AP-1 mediated αI collagen-expression (Palcy and Goltzman, 1999), (Palcy et al., 2000). With the confirmed interaction of BRII-SF and PKCβ the gap between BMP2 signalling and induction of caspase activity and apoptosis can be closed and deeper insight in Smad-independent pathways resulting in cell fate decisions is gained.

Besides BRII-SF two other transmembrane receptors were found associated with BRII, the tyrosine kinase receptors IL2Rβ and c-kit. Signalling via these receptors is mediated by JAK-Stat, MAPK and PI3K pathways. The crosstalk between BMP and tyrosine kinase signalling could occur on receptor-level as well as on the level of the respective signal transducers. For c-kit influence on BRII phosphorylation could be determined and both receptors are shown to influence Smad-dependent and for c-kit Smad-independent signalling. Hereby, cellular responses are modulated.

Moreover, PI3Kp55γ, an alternative regulatory subunit of PI3K is associated with BRII-SF. Originally it was found to bind to and regulate tyrosine kinases, thus regulating MAPK pathways and PKB pathways (Bondeva et al., 1998). Found associated with BRII, PI3Kp55γ could regulate the receptor itself or mediate further downstream signalling.
JIP1 (JNK interacting protein-1) is acting as a MAPK aggregating module, interacting with MAPKKK and JNK, complexing MLK, MKK7 and JNK and inhibiting JNK by cytoplasmic retention (Mooser et al., 1999), (Yasuda et al., 1999), (Willoughby et al., 2003). Besides interaction with signal promoting molecules, as PKCβ, BRII binds to signal blocking molecules like JIP1 as well. On the one hand, BRII may be promoting MAPK pathways by interacting with MAPKKK8 and MOS, on the other hand it might exert inhibiting functions via JIP1, thus leading to a controlled balance of signal transduction.

5.2.3.2 BRII associated phosphatases

Phosphorylation is a simple mechanism to change protein activity. Protein phosphatases that catalyze the dephosphorylation of proteins, are classified into two major functional groups, protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases (PPs). One of the BRII-SF associated proteins is protein tyrosine phosphatase receptor type O (PTPRO), which exhibits a de-phosphorylating activity directed against tyrosines (Pixley et al., 1995). As there are data supporting specific tyrosine phosphorylation in the BRII-kinase domain (see chapter 4.4 and 5.4), PTPRO might regulate specifically the phosphorylation here. Besides, protein phosphatase 2A, subunit Bβ (PP2A Bβ) was precipitated with BRII-tail. PP2A is one of the major cellular serine/threonine phosphatases. Protein phosphatases are involved in regulating kinase activity. The interaction with both types, one serine/threonine and one tyrosine kinase might be important for the regulation of BRII phosphorylation, which was shown to be on serines as well as tyrosines.

5.2.3.3 Chaperones

With cdc37 and crystallin αB two molecular chaperones were found associated with BRII. Cdc37 was identified stabilizing Cdc4, together with Hsp90 and moreover it is found to associate with the IKK complex, that plays important roles in the signal-dependent activation of NFκB (Stepanova et al., 1996), (Chen et al., 2002). Cdc37 is a molecular chaperone, linked with the activity of several other kinases, including oncogenic v-Src, casein kinase II, MPS-1 kinase, and sevenless (Kimura et al., 1997). Another chaperone is crystallin αB, that is associated with BRII-SF. It belongs to the family of small heat shock proteins and prevents denaturation of partially unfolded proteins (Bullard et al., 2004). The presence of two molecular chaperones could either stabilize the correct folding of BRII or function as adapter

complexing various proteins with BRII, in case of cdc37, CK2 could be recruited via that complex-stabilizing protein.

5.2.3.4 Receptor localization and internalization

Neurobeachin is a protein containing a high-affinity binding site for the type II regulatory subunit of protein kinase A (PKA RII), that is linked to localization of cAMP-dependent protein kinase activity (Su et al., 2004). Moreover it contains a BEACH domain and might be, as other BEACH domain containing proteins, involved in protein sorting. Neurobeachin is potentially involved in post-Golgi membrane traffic, one of its functions being to recruit protein kinase A to the membranes with which it associates (Wang et al., 2000). Thus, Neurobeachin might transport BRII to the cell surface or link PKA to the receptor, supporting Smad-independent signalling.

IL2Rβ is not a kinase itself, but it acts via other associated molecules. Moreover it internalizes via a novel, clathrin-independent pathway, that might be via detergent-resistant membrane domains and requires dynamin, pointing towards rafts (Lamaze et al., 2001). The theories about IL2Rβ located in rafts are controversal. Marmor observes IL2Rα to be constitutively associated with rafts and the IL-2-mediated heterotrimerization of IL-2R chains is shown to occur in soluble-membrane fractions, where IL2Rβ and γ reside (Marmor and Julius, 2001). Matko and colleagues, however, observe IL2Rβ and γ to be constitutively associated with rafts and ligand recruits IL2Rα into it (Matko et al., 2002). Moreover, there is another hint that at least a subpopulation of BRII is associated with rafts (A. Hartung, personal communication). As it was demonstrated, another BRII interaction partner, PKCβ, is important for recruitment of the I kappa B kinase complex into lipid rafts (Su et al., 2002).

Eps15 and Eps15R are essential and constitutive components of clathrin-coated pits and are required for clathrin-mediated endocytosis (Poupon et al., 2002). It has been shown, that Eps15 and Eps15R are targets for mono-ubiquitination that is involved in protein trafficking. Mono-ubiquitinated Eps15(R) binds to mono-ubiquitinated targets itself and may function as adaptor between ubiquitinated membrane cargo and either the clathrin coat or other endocytic scaffolds (Klapisz et al., 2002), (Polo et al., 2002). As there are hints for BRII-recruitment into rafts (via IL2Rβ) or into clathrin-coated pits (via Eps15R), investigation of BRII localization is of high importance, also regarding the subcellular distribution of preformed versus ligand induced complexes. In addition, the interaction with Eps15R hints towards BRII-ubiquitination and degradation. The potential E3 ligases for the BMP receptors are Smurfs or XIAP, as IAPs have been shown to be involved in protein ubiquitination and degradation (Park et al., 2004).

5.2.3.5 Transcriptional responses

CtBP (C-terminal binding protein) is recruited to DNA by transcription factors that contain a PXDLS motif. Due to its interaction with adenovirus E1A protein it is implicated during oncogenic transformation. CtBP forms a large complex on DNA, containing among others HDAC1 and HDAC2, which is involved in gene targeting and histone modifications (Shi et al., 2003). Recently it has been shown that Smad6 is recruiting CtBP to the Id1 promoter to suppress BMP-mediated signalling (Postigo et al., 2003). Moreover Brinker (Brk), a transcriptional repressor protein encoded by a Dpp (BMP2 homologue of Drosophila) target gene recruits the corepressors Groucho or CtBP for repressing some Dpp-responsive genes (Hasson et al., 2001). Thus, CtBP is involved in BMP2 induced repression of target genes. The interaction of CtBP with the kinase domain of BRII-SF in the absence of ligand might retain it in the cytoplasm. Upon ligand stimulation or other stimuli CtBP might be released from the receptor and act in the nucleus. The interaction of BRII-SF and CtBP does not require nuclear localization of one interaction partner, as CtBP has been found in the golgi (Nardini et al., 2003).

Essential roles of LIM homeodomain proteins in cell fate determination during development have been proven. The LIM homeobox transcription factor 1α (Lmx1a) binds A/T-rich sequences in promoters and stimulates transcription. As it was recently demonstrated, BMP signalling from epidermal ectoderm is necessary and sufficient for inducing Lmx1a and other co-factors that also regulate the extent of roof plate induction in mice. Chizhikov and coworkers could show, that Lmx1a controls multiple aspects of dorsal midline patterning and hence, is a major mediator of early BMP signalling in the developing spinal cord (Chizhikov and Millen, 2004). Even if it is unlikely that a transcription factor is interacting with BRII-tail in vivo, due to different subcellular localization, Lmx1a could be precipitated with BRII-tail because of its two LIM domains. As it has been demonstrated, LIM domains do specifically interact with the BRII-tail (Foletta et al., 2003). Another possibility could be cytoplasmic cleavage of BRII-tail followed by subsequent nuclear translocation. Here, Lmx1a and BRII-tail could possibly interact as well.

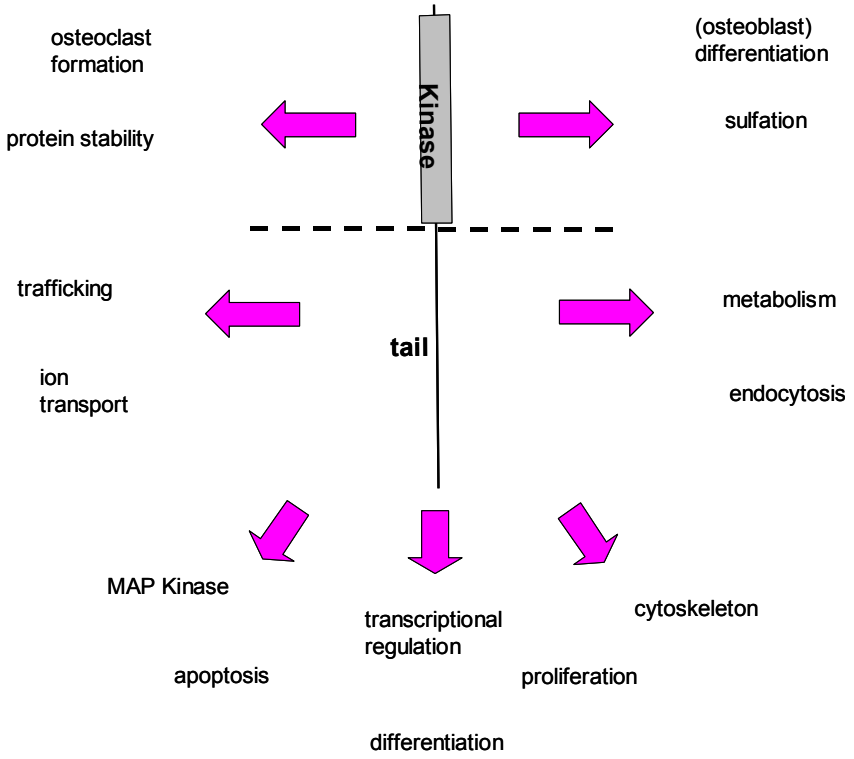


Fig. 5.2 Cellular processes influenced by BRII interactors
Proteins interacting with the BRII-SF, have been found to be involved in processes depicted above the dashed line. The BRII-tail-interactors regulate processes, depicted below the dashed line on both sides of the receptor. The whole receptor or BRII-SF and BRII-tail are involved in the processes listed below the receptor.

Taken together, proteins that do coprecipitate with the BRII are involved in the regulation of a variety of cellular processes. Due to the amount not all proteins can be reviewed. Fig. 5.2 is summarizing and simplifying the mechanisms BRII seems to take part.
Proteins interacting with the BRII-SF have been found to be involved in protein stability, sulfation of proteins and osteoclast formation as well as (osteoblast) differentiation (depicted above the dashed line). The BRII-tail-interactors, below the dashed line on both sides of the receptor, are involved in receptor trafficking, ion transport, metabolic processes and endocytosis. The whole receptor or BRII-SF as well as tail are involved in the processes listed below the receptor.

With this method we can conclude that certain proteins interact with specific parts of the receptor, e.g. with the BRII-tail. Thus, BRII-mutants (PPH-mutants) or BRII-SF should not exhibit certain properties in relation to endocytosis, trafficking, ion transport or metabolism. On the other hand, BRII-SF was shown to participate in regulation of protein stability, sulfation of proteins, osteoclast formation and (osteoblast) differentiation. The BRII-tail might sterically hinder the association of some BRII-SF interactors. With the help of the specific

BRII-SF interactors and their functions differences between BRII-SF and BRII-LF can be discovered.

5.2.4 Confirmation of BRII interactors

So far, the interaction of associated proteins with BRII could be confirmed by coimmunoprecipitation for ILK, IL2Rβ, cGK1β and cdc37 (Diploma Thesis Raphaela Schwappacher) as well as for Eps15R (Christina Sieber). The impact of some of these interactions on BMP signalling is mentioned in the Diploma thesis of R. Schwappacher and will be discussed further.

Here, investigations were focused on c-kit interaction with BRII, which is influencing BMP2 mediated signalling and c-kit signalling is synergizing with BMP2 on the ALP pathway (see Chapter 4.3 and 5.3).

Moreover for PKCβ and for CtBP the interaction with BRII could be confirmed. using GST pulldown and subsequent western blotting (Fig. 4.23). In vivo as well as in vitro, PKCβ is interacting with the kinase domain, present in BRII-SF and BRII-LF, confirming the pull down with BRII-SF. The same holds for CtBP, that interacts with the BRII kinase domain but not with the tail. To further explore PKCβ mediated signalling, the PKCβ specific inhibitor LY333531 could be used. For CtBP involvement reporter gene assays using the Id1 promoter might help to find answers.

5.2.5 Interacting proteins BRII versus TGF-β receptors

So far only three BRII interacting proteins have been described: LIMK1, Tctex-1 and BRIa in the preformed complex (Foletta et al., 2003), (Machado et al., 2003), (Gilboa et al., 2000). In the search for BRII interactors using GST-pulldowns none of the interactors could be confirmed. That might be due to the fact that LIMK1 and Tctex-1 have been identified with the help of a yeast-two hybrid screen. The difference here can be explained by the use of a different screening and selection method. In the GST-pulldown, more than 100 spots were excised and only 52 could be determined as a certain amount of protein was required for MALDI TOF MS. Thus, it is very likely that some proteins were missed. The absence of BRIa in our screen can be due to the omitted spots or due to the harsh conditions for that screen, not aiming for receptors.

As it has been shown, LIMK1 interacts with the BRII-tail via its LIM domain (Foletta et al., 2003), it is not surprising that here another LIM domain containing protein could be identified. The Lim homeobox transcription factor (Lmx1a) that precipitated with BRII-tail, contains two LIM domains, which most likely do interact. Lmx1a was described being only nuclear and it still has to be elucidated how the tail domain and Lmx1a could meet in vivo or whether the interaction was caused by nucleus lysis and the affinities of LIM domains for the tail region.

When comparing BRII associated proteins with TβRII associated proteins, only one analogy can be found. Both, TβRII as well as BRII, interact with a light chain of dynein. BRII is complexed with Tctex-1 and TβRII with mLC-1, belonging to another group of dynein light chains (Machado et al., 2003), (Tang et al., 2002). All our newly discovered interactors do not have an equivalent within the TGF-β receptor interactors. Functionally, Dab-2 and Eps15R, link receptor complexes towards clathrin-mediated endocytosis as both are components of clathrin-coated pits (Kowanetz et al., 2003), (Klapisz et al., 2002). The mechanisms of recruitment via Dab-2 are not discovered yet and thus can not be compared to Eps15-mediated endocytosis.

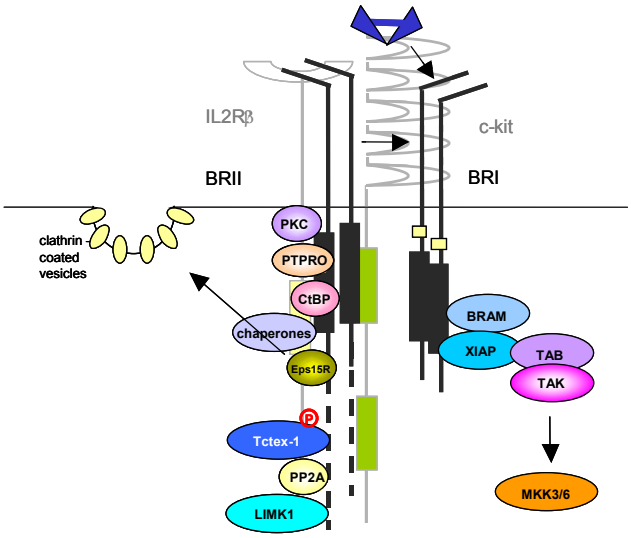


Fig. 5.3 Reduced scheme of BMP receptor interactors
To the picture of already characterized BMP receptor interactors some of the newly characterized interactors are added. The two interacting receptors are drawn in transparent colours, the transmembrane PTPRO is shown as cytoplasmic protein.

Despite the fact that some of the interactions still have to be confirmed and some of the interactors have to be investigated in more detail regarding impact on signalling, we could significantly contribute to the extension of the knowledge of BMP signalling.

5.3 c-Kit as a BRII-associated protein (Hassel et al., submitted)

SCF (Stem cell factor) is a growth factor with prominent, well described functions in haematopoiesis, generation of melanocytes and germ cells and, moreover, plays a role in development of Cajal cells and in learning processes in the hippocampus. SCF exists in a soluble and a membrane bound form. The receptor for that cytokine is c-kit, the cellular homologue of v-kit. The absence of SCF or c-kit is lethal in utero, as signalling mediated by this signal transduction cascade is crucial for haematopoiesis, pigmentation and reproduction (Linnekin, 1999).

5.3.1 Interaction of BRII and c-kit in vitro and in vivo

In a GST-pulldown experiment followed by MALDI analysis the tyrosine kinase receptor c-kit was found associated with BRII-tail. This interaction could be confirmed with GST-pulldown and subsequent westernblotting (Fig. 4.28). No interaction of c-kit was observed with bacterially expressed GST-BRII-SF or GST-BRII-LF. In contrast to that, c-kit did interact with BRII-LF when overexpressed in COS7 as well as in 293T cells. That might be due to the fact that the GST-BRII-LF exhibits another conformation than the BRII-LF in cells with intact transmembrane anchor, leading to a different conformation of the BRII-tail. Thus, the tail domain is exposed and interacts with c-kit. BRII-SF lacking the tail domain did not interact with c-kit, leading to the conclusion that the interaction domain is found in the tail. The interaction is enhanced by adding BMP2 (Fig. 4.29), but seems to be independent of c-kit kinase activity (Fig. 4.30). BRIa-HA was shown to interact with c-kit as well, but the composition (double complex or triple complex) of the signalling unit is not determined yet. Cotransfection with BRII-LF did not inhibit BRIa/c-kit complex formation (Fig. 4.32).
In non-transfected C2C12 cells BRII-LF and c-kit interact in a ligand-free system. Concomitant stimulation with both ligands strongly enhances the interaction, whereas stimulation with one ligand has no effect (Fig. 4.34). Testing the binding situation in vivo, i.e. in endogenously expressing cells, the involvement of BRIa was not tested.
Thus, BRII and c-kit are shown to interact in vitro as well as in vivo. This interaction is mediated by BRII-tail and both ligands enhance binding in vivo. In an overexpression system, BRIa interacts with c-kit as well.
Nevertheless it is very interesting to investigate the interaction of BRIa with c-kit. On the one hand a triple complex with BRII, BRIa and c-kit could be formed, on the other hand, c-kit is either interacting with BRII or with BRIa. Interaction with one or the other possible partner would lead to modulation of signalling. To prove the binding modes, double immunoprecipitations with BRII-Flag, BRIa-HA and c-kit could be performed. Immunofluorescence stainings would give hints on receptor distribution and complex formation with and without ligand and during internalization.

5.3.2 Trans-phosphorylation between BRII and c-kit

As all components are active kinases and in eukaryotes, phosphorylation is probably the most important regulatory event, BRII-LF and c-kit were tested for trans-phosphorylation. To determine sites of phosphorylation, 2-dimensional phosphopeptide mapping was performed. Whereas phospho-maps of c-kit showed no changes induced by BRII, BRII-LF phosphorylation was induced BMP2, and by c-kit when SCF and BMP2 were added. With c-kit KN the whole map was weaker, but no spot disappeared (Fig. 4.38). At all the maps, BMP addition increased total phosphorylation level (Fig. 4.38), but only cotransfection of c-kit and stimulation with both ligands showed upregulation of three distinct spots (Fig. 4.39). It could be shown that the phosphorylation of one peptide (upper left part of the map) is enhanced, depending on c-kit. Further analyses proofed phospho-serine at position 6. The peptide that is most strikingly phosphorylated in a c-kit dependent manner is, as we call it the “upper right spot” (Fig. 4.39). By phospho-amino acid analysis, it could be shown that this peptide has phospho-serine at position 4. Calculation of peptide migration (see Tab. 4.9) and analysis of BRII mutants (data not shown) point to S757 being the BRII site phosphorylated by c-kit. Although the phosphorylation of serines induced by c-kit, a tyrosine kinase is unexpected, c-kit could either recruit a serine-kinase that phosphorylates BRII or induce a conformational change that facilitates auto-phosphorylation of BRII. Moreover c-kit could exhibit dual-kinase activity and phosphorylate BRII-LF itself. With the phosphorylation event being weaker for c-kit KN the phosphorylation could be direct. But on the map of BRII auto-phosphorylation (Fig. 4.57), spots at both positions can be observed. This hints towards c-kit facilitating BRII auto-phosphorylation, even though the spots on the map are not a final proof as different peptides can exhibit the same migration properties. Observing the endogenous phosphorylation of BRII an increase upon BMP2 stimulation could be observed as well as an inhibition of total phosphorylation by costimulation with SCF. Surprisingly SCF alone enhanced BRII phosphorylation as well (Fig. 4.40).

Besides the physical interaction of BRII-LF and c-kit we demonstrate c-kit dependent phosphorylation events on BRII-LF, with S757 being the main phosphorylation target. So far, no interaction and in vivo phosphorylation of serine/threonine and tyrosine kinase receptors has been demonstrated.

5.3.3 Influences of BRII and c-kit on signal transduction

In order to gain a better understanding of the signal transduction activated by the interacting receptors and to test for synergistic effects, we used biological read out systems.

5.3.3.1 Smad-dependent pathways

As the influence of the c-kit/BRII interaction can be observed on BRII phosphorylation, the aim was to test Smad-dependent and independent pathways for signalling upon BMP2 and SCF stimulation. In a reportergene experiment using the pSBE-reporter, BMP enhanced Smad-signalling up to 5 fold, whereas both ligands increased that further more up to 6,5 fold (Fig. 4.41). Although that increase is not very strong, it was observed several times. Other BMP2 dependent reportergenes, not only measuring Smad-activation, did not show BMP2/SCF synergisms. Cotransfection of BRII-LF strongly enhanced the Smad-dependent signalling. It pushed the ligand-independent as well as the ligand-dependent signal (Fig. 4.42). Both ligands could further increase the BMP2 signalling from 110 fold up to 140 fold. In both cases, with or without BRII transfection, SCF is increasing the BMP2 signal by the factor 1,3 (Fig. 4.42). The signal for BRII-KR, although rather low, is not increased by SCF and no synergism can be observed (Fig. 4.42). Thus, c-kit can activate BRII-LF but the BRII-LF kinase is essential to act. If BRII is kinase deficient, it might be activated by c-kit at a certain level but it can not transmit the signal.

Interestingly, transfection of c-kit significantly enhanced BMP2 mediated signal transduction (Fig. 4.43). In this experiment, BMP2 induces signalling in the mock control up to 20 fold, and both ligands 25 fold. Transfection of c-kit induces 42 fold, and with both ligands 50 fold. c-kit KN is enhancing even more, but the synergistic effect can not be observed (Fig. 4.43).

c-Kit stimulates BMP2 mediated signalling, but neither cotransfection of BRII or c-kit is elevating the synergistic level compared to BMP2 stimulation alone (compare Fig. 4.42 and 4.43). In C2C12 cells c-kit seems to be a limiting factor, but in contrast to BRII it is not a signalling receptor without ligand addition, as no ligand independent SBE activation can be observed.

Similar effects as in the reportergene assays could be observed in westernblots to determine Smad1/5 phosphorylation. Concomitant stimulation with BMP2 and SCF enhances Smad1/5 phosphorylation slightly compared with the BMP2 level (Fig. 4.44). As the factor measured in the reportergene assay was 1,3 fold, it can be concluded that a similar difference can be observed regarding phosphorylation. Here are reportergene assays a more quantitative method to determine differences.

Moreover, other known Smad-dependent pathways should be examined. The induction of Id1 is one of the immediate early responses upon BMP2 stimulation, as Id1 mRNA upregulation could be observed after 1h of BMP2 treatment (Lopez-Rovira et al., 2002). The Id1 promoter-enhancer element that is responsible for BMP2 dependent gene induction is of 1,5kb in length (Lopez-Rovira et al., 2002). In that promoter region consensus sites for YY1,

Sp1, Egr-1, ATF/CREB, and four CAGAC boxes are located. Here, the GCCGNCGC (GC box), binding Smad 4 and the CAGAC boxes binding Smad1 and Smad4 are identified. Although all sites are bound with low affinity, multimerization of these boxes might enhance signalling (Lopez-Rovira et al., 2002). During the construction of the BRE-reporter Korchynskyi analysed the Id1 promoter, showing that the SBE sites are the major Smad binding sites. But in addition many more sequences can be found herein, that possibly bind to other transcription factors (Korchynskyi and ten Dijke, 2002). Thus, measuring Id1 activation, there are differences compared to the naked pSBE-reportergene.

But still it is surprising that BMP2 alone induces Id1 transcriptional activity 16 fold, whereas BMP2/SCF induces only 6 fold. Here SCF strongly inhibits BMP2 mediated signal transduction (Fig. 4.45). This might be due to the difference between the 1,5 kb Id1 promoter containing various transcription factor binding sites and the SBE-reporter. As c-kit is known to induce a variety of signalling pathways (see chapter 1.8), some factors induced might inhibit Id1 induction.

5.3.3.2 ALP pathway

As the induction of the ALP pathway as an early osteoblastic marker gene, is a long term response, the signal transduction leading to that pathway is still not explored. The discussion about the ALP induction are still controversially. It can not be stated whether the induction of ALP happens via Smad-dependent or Smad-independent pathways (compare Chapter 5.1.4). Therefore the topic is now treated separately.

As we could show using three different methods (ALP stain, enzymatic measurement and real time PCR), BMP2 is inducing ALP production, but additional stimulation with SCF is strongly enhancing BMP2 dependent ALP production. SCF itself has no influence on ALP production (Fig. 4.46 and 4.47). As we could previously show, p38 is regulating the pathway leading to ALP production (Nohe et al., 2002).

These synergism of BMP2 and SCF effects observed here might be caused by both factors showing transcriptional activity on the ALP promoter. Whereas the BMP2 induced factors are sufficient for ALP induction, the transcription factors induced by SCF are not able to induce transcription in the ALP pathway alone. Concomitant action of these factors enhances signalling drastically (Fig. 4.46 and 4.47). The factors induced by BMP2 are phosphorylated Smad1/5, pERK and p38, which seem to be involved in ALP regulation (Fujii et al., 1999), (Gallea et al., 2001), (Suzuki et al., 2002). Moreover both pathways could act one after the other, as it has been described for the involvement of Smad1/5 and p38 pathways during adipogenesis. Here, BMP2 controls adipocytic differentiation by using two distinct signalling pathways that play differential roles and act together (Hata et al., 2003).

Besides the postulated interaction of the two pathways on transcriptional bases, the crosstalk could occur in the cytoplasm. Activated kinases by one pathway might trans-phosphorylate adaptor-molecules or transcription factors of the other pathway. Thus, SCF might induce phosphorylation and inhibition of a negative regulator of the ALP pathway or phosphorylate and activate a positive regulator. So it could be explained why SCF alone has no influence on the ALP pathway, as well.

Growth factors as EGF and FGF, signalling via tyrosine kinase receptors, have been shown to influcence Smad-dependent signalling in a negative manner. SCF is signalling via c-kit, a tryrosine kinase receptor, related to PDGFR and EGFR (Szymkiewicz et al., 2002). All three receptors are known to induce the ERK-pathway. As it has been demonstrated, activated ERK is inhibiting Smad1/5 signalling via linker phosphorylation (Kretzschmar et al., 1997). Smad1/5 can be phosphorylated and modulated by the ERK pathway to inhibit ALP production. Furthermore activated ERK is shown to exhibit more levels of control of Smad-pathways, maybe via phosphorylation of transcription factors or cofactors (Nakayama et al., 2003).

The synergistic effect of BMP and SCF signalling on ALP production could be mediated by the onset of identical pathways, thus multiplicating signals.

5.3.3.3 Smad-independent pathways

Cbfa1 induction

Cbfa1 (core binding factor 1), also named Runx2, is identified to play important roles during osteoblast development. Besides being a prominent BMP target gene it is upregulated by TGF-β as well (Lee et al., 1999).

Whereas Cbfa1 mRNA was induced by BMP2 up to 2 fold, concomitant stimulation with BMP2 and SCF increased the mRNA level up to 4 fold, compared with the unstimulated control. SCF had no effect (Fig. 4.48). On protein level, Cbfa1 was induced by BMP2 or SCF and stronger, by both ligands together (Fig. 4.49).

Cbfa1 is not a direct target gene of BMP2 and TGF-β. Its upregulation is Smad-dependent but an additional step of de novo protein synthesis is required. Whereas Smad is upregulating JunB, JunB is inducing Cbfa1. But it has been shown that p38 pathways are crucial for Cbfa1/Runx2 expression as well. Thus, both the Smad and p38 MAPK pathways converge at the Cbfa1/Runx2 gene to control cell differentiation (Lee et al., 2002). Vinals and colleagues, however, claim the Cbfa1 pathway to be p38 and PI3K independent, in contrast to the later stages in osteoblast differentiation that are negatively regulated (Vinals et al., 2002).

To explain the strong synergism of BMP2 and SCF on Cbfa1 induction, further experiments on the involvement of Smad1/5 are necessary. But it seems that SCF itself alone can induce

Cbfa1 in C2C12 cells on protein levels (Fig. 4.49), what excludes activation of Smad1/5. As described, BMP2 as well as SCF induce the p38, that is involved in Cbfa1 induction. Thus, p38 phosphorylation, induced by BMP2 and potentiated by SCF or vice versa, seems to be the key regulator of Cbfa1.

p38 and ERK pathways

It has been described, that BMP2 as well as SCF activate the ERK and the p38-pathway (Gallea et al., 2001), (Kimura et al., 2000), (Palcy and Goltzman, 1999), (Matsui et al., 2004). One of the factors downregulating these pathways is SOCS 6 (Suppressor of cytokine signalling 6), that is specifically binding to Y567 and thus inhibiting the onset of the MAPK dependent pathways via Src family kinases (Bayle et al., 2004). Moreover both cytokines are shown to induce JNK activation as well as the PI3K pathway that leads to Akt phosphorylation (Ghosh-Choudhury et al., 2002), (Vinals et al., 2002), (Lemonnier et al., 2004), (Linnekin, 1999). So, there are many switches and pathways that are potential targets for synergistic or antagonistic actions.

We were testing C2C12 cells for the upregulation of BMP2 and/or SCF induced phosphorylation of Akt, ERK1/2 and p38. Whereas the phosphorylation of Akt was at a continuously high level (data not shown), and neither BMP2 nor SCF induced any changes, the p38- and ERK-phosphorylation was influenced by both ligands (Fig. 4.50). In C2C12 cells SCF alone did not induce p38-phosphorylation, but simultaneous stimulation with both ligands resulted in p38-phosphorylation after 30min of stimulation. BMP2 dependent activation of p38 was observed after 60min of ligand treatment (Fig. 4.50b). Thereupon it seems that addition of SCF is shifting p38 response in C2C12 cells.

Regarding ERK1/2 phosphorylation SCF influences BMP2 signalling drastically. Here addition of SCF leads to a shift in the phosphorylation time course (Fig. 4.50a). Despite the fact that both ligands do activate and phosphorylate ERK, the shift observed here seems to be a backward-shift. This delay might be due to the interaction in a different cellular compartment or due to a direct phosphorylation event. But so far, it can not be explained yet.

5.3.4 Crosstalk between BMP and SCF pathway

The proven interaction of c-kit with BRII-LF might help to elucidate the symptoms of PPH observed in PPH BRII-tail mutants. As Nishihara and coworkers could show, the inhibition of the Smad-pathway of extracellular and kinase PPH mutations was due to a defect in transport to the membrane. The tested BRII-tail mutations exhibited no defects in signalling (Nishihara et al., 2002). We might have found one reason for BRII-tail mutants exhibiting a pathogenic phenotype. As we found several BRII-tail interactors one of them might play

crucial roles in arteries. c-Kit, one of the BRII-tail interactors, is demonstrated to influence BMP2 mediated signalling as well as to promote the osteoblastic phenotype.

As c-kit is expressed in C2C12 cells, it is constantly influencing BMP2 and BRII mediated signal transduction. So far, signalling in the absence of c-kit or with BRII-LF lacking the trans-phosphorylation site has not been investigated yet. Thus, further experiments would include siRNA experiments using c-kit or cells stably infected with siRNA for BRII. These cells can subsequently be transfected with BRII-LF mutants, which can be analyzed without BRII cellular background. Thus, the impact of that interaction could be uncovered.

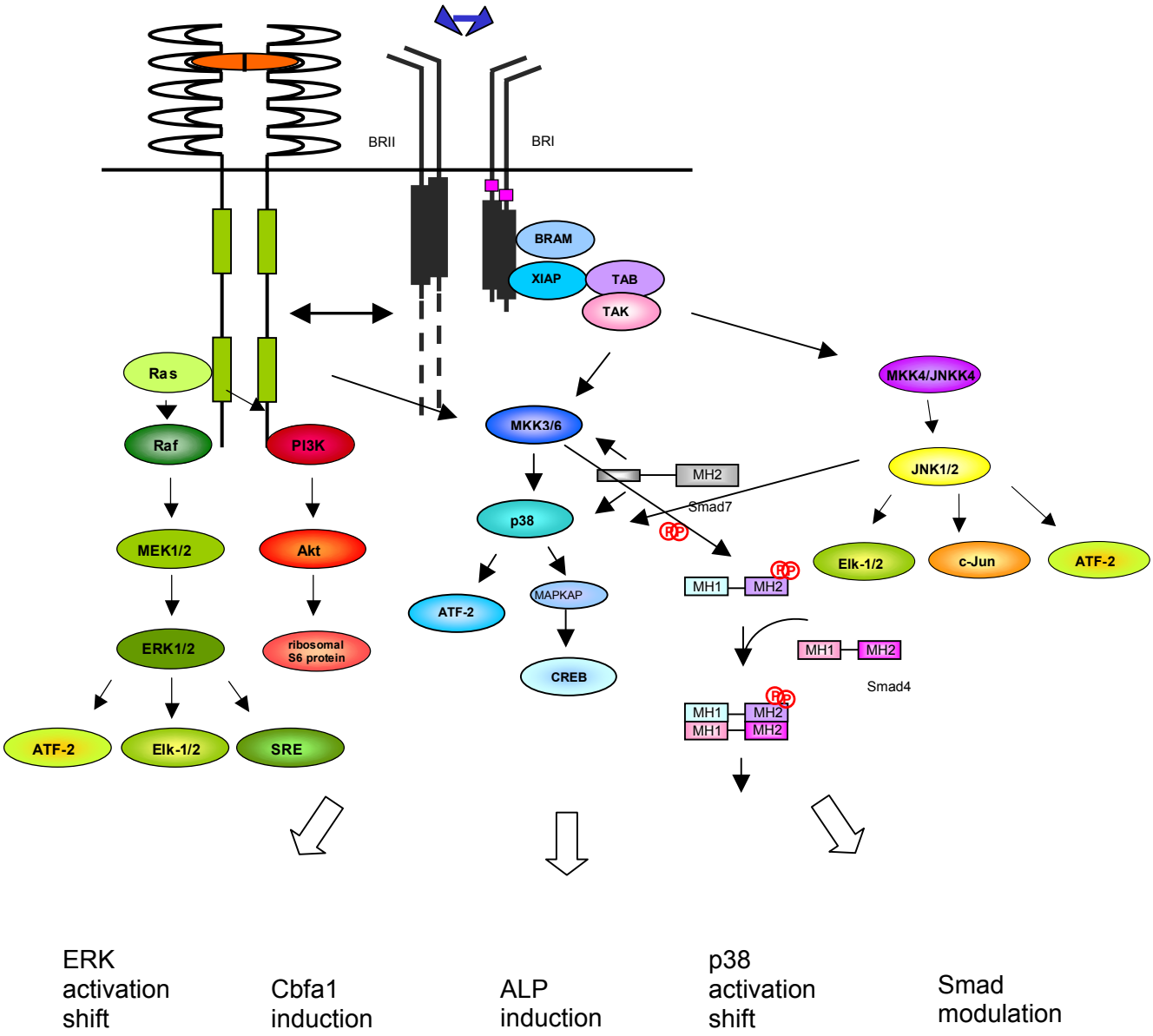


Fig. 5.4 Pathways induced by BMP2 and SCF via BRII and c-kit
Here are all the pathways depicted that are induced by BMP2 and c-kit. The effect of concomitant BMP2 and SCF stimulation upon C2C12 cells are listed below, as they are results of these signalling pathways.

5.3.5 Possible links between c-kit/ SCF and BRII/BMP2

Here we present a link between the BRII/BMP- and the c-kit/SCF pathway. Still, the physiological role of that interplay has to be discovered.

It has been reported that in some systems the TGF-β superfamily and the c-kit signalling are connected. One example are colorectal carcinoma cells, where growth stimulation via the c-kit receptor is inhibited by exogenous TGF-β (Bellone et al., 1997).

Another system are adult mesenchymal progenitor cells. During the culture of adult human mesenchymal progenitor cells in stirred suspension culture, addition of IL3+SCF for 21 days strikingly increases the formation of bone progenitors, compared to adherent cultures. When grown in osteogenic conditions, these cells express bone-specific genes as Cbfa1/Runx2, bone sialoprotein, and osteocalcin, showing that adult BM-derived non-haematopoietic progenitor cells can differentiate to the osteoblastic phenotype under SCF stimulation (Baksh et al., 2003).

The regulation of adult human and murine blood stem cells is still undiscovered. One of the characteristics of these cells is the surface expression of c-kit (Ashman et al., 1991). Mostly LIF has been shown to be involved central regulation processes in these cells. Moreover it has been shown that BMPs either keep those cells in the immature state or induce differentiation, depending on the BMP used (Bhatia et al., 1999). Moreover, BMPs, in collaboration with Stat3, keep the immature state of embryonic stem cells (ES) by blocking the lineage-specific transcription factors via Id proteins (Ying et al., 2003).

In addition, Nakayama showed that BMP4 triggers the differentiation of ES cells, supported by VEGF (Nakayama et al., 2000), and the formation of embryonic bodies, a process that is enhanced by VEGF, SCF, erythropoietin and thyroid hormone (Adelman et al., 2002).

Thus, there are several processes, mostly during stem cell development or stem cell differentiation where BMP2 and SCF act concomitantly or where cells do obviously express and need both receptor types.

Another region where BMP receptors and SCF receptors seem to synergize is the human haematopoietic stem cell-niche (HHSCc). For birth and maturation HHSCs need the contact with spindle-shaped osteoblasts that are lining the inner bone surface, probably via β-catenin and N-cadherin, that helps the cells to adhere. The activity of BMP has been shown to be essential for the development of blood-forming tissues. Manipulation of the BMP-signalling in the spindle-shaped osteoblasts, increased the number of those and the number of long-term HHSCs as well (Lemischka and Moore, 2003), (Calvi et al., 2003), (Zhang et al., 2003a).

In addition, BMPs and SCF might interact in the rat ovar. In a coculture system, BMP15 stimulated SCF expression in granulosa cells, whereas the produced SCF inhibits BMP15 expression in oocytes, thus forming a negative feedback loop. This interplay between SCF,

BMP15 and the respective receptors might play important roles in early folliculogenesis (Otsuka and Shimasaki, 2002).

5.4 BRII Phosphorylation (Hassel et al., submitted)

An efficient and quick method to regulate protein activity and the association of docking partners is phosphorylation of proteins mediated via a big variety of different kinases and the counterplay dephosphorylation through phosphatases. Whereas many kinases and their substrate specificity as well as the consensus sequence have been identified, the knowledge of phosphatases is rather poor. Serine/threonine phosphorylations are removed by (serine/threonine) protein phosphatases (PPs), tyrosine phosphorylation is controlled by the action of protein tyrosine phosphatases (PTPs) (Shi et al., 2004).

One third of the proteins present in a typical mammalian cell can be phosphorylated. Despite the fact that the phosphorylation pattern of many proteins is well described nothing is known so far about the phosphorylation pattern of the BMP receptors. As we are interested in signal transduction mediated via the BRII, our focus was on the phosphorylation of this protein and the impact of certain phosphorylation on signal transduction.

When examining BMP receptor phosphorylation the phosphorylation of structurally and functionally similar receptors can be taken into account. Here, the phosphorylation of TβRI and TβRII and its impact on signalling gives useful hints (Fig. 5.4). As demonstrated the TβRII is a constitutively active kinase when overexpressed. Upon ligand binding it transphosphorylates TβRI which leads to subsequent receptor-activation (Wrana et al., 1994), (Souchelnytskyi et al., 1996), (Luo and Lodish, 1997).

In transiently transfected COS7 as well as non-transfected C2C12 cells BMP2 is enhancing the phosphorylation of BRII-LF (Fig. 4.52 and 4.54), in contrast to the situation for the TGF-β receptors (Luo and Lodish, 1997). The phosphorylation of transfected BRII-SF was very weak. 2-dimensional phosphopeptide-mapping, that could only be performed with overexpressed BRII-LF, revealed 12 spots (Fig. 4.58). For 10 sites the phosphorylated amino acid and their position could be determined (Tab. 4.6). Clearly identified phosphorylation sites in BRII-LF are S336, S757, S815, S818 and Y825.

To distinguish between auto- versus trans-phosphorylation and to detect phosphorylation sites in the tail an in vitro kinase assay with BRII-SF and BRII-LF was performed (Fig. 4.55 and 4. 56). Here, only four phosphorylation sites could be found in the kinase domain, eight more in the tail or in the BRII-LF, due to conformational changes of the long receptor chain (Fig. 4.56). Moreover, it could be seen that many of the phosphorylation sites in BRII-LF in vivo result from auto-phosphorylation. But still, some phosphorylation sites differ in vivo versus in vitro (Fig. 4.57). This demonstrates that physiologic conditions do influence BMP receptor phosphorylation. These observations are in contrast to the findings for the TβRI, where no difference between in vivo phosphorylation and in vitro kinase assays could be proven (Luo and Lodish, 1997).

To examine BRII-LF auto-phosphorylation we performed in vitro kinase assays with GST-fusion proteins of various BMP receptor constructs. BRII can phosphorylate the BRII-tail in trans by an inter-molecular mechanism (Fig. 4.61). Working with overexpressed receptors the BRII-LF can phosphorylate BRII-SF and BRII-tail in trans but the BRII-SF hardly fails to phosphorylate the BRII-LF (Fig. 4.62). As this is in contrast to the data with the purified proteins, it can be concluded that the BRII-SF is able to phosphorylate the BRII-tail, but only if it has access to it. This is more difficult for BRII-LF where the tail might be In a different conformation due to the transmembrane domain of the whole receptor. The tail might be flipped up, interacting with its own kinase and thus not be easily accessible for the kinase of the other BRII in the homo-oligomer. Figure 5.5. is summarizing the data of BRII auto– and trans– phosphorylation.

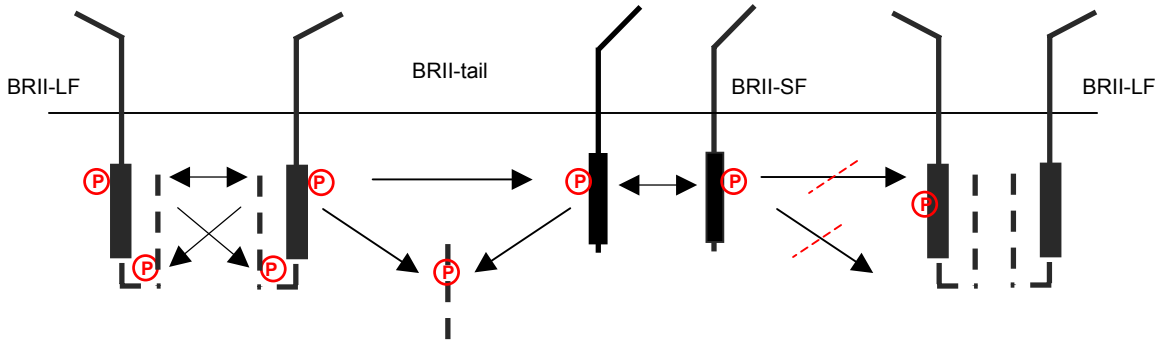


Figure 5.5 BRII auto- and trans-phosphorylation
BRII-LF is able to phosphorylate itself in a cis- or trans-phosphorylation event. Moreover it can phosphorylate bacterially expressed BRII-tail. BRII-SF is phosphorylating itself (in cis or trans), BRII-tail in trans and is weakly phosphorylating BRII-LF, maybe because the tail is difficult to access.

To examine the influence of certain amino acids on BRII-phosphorylation and on signalling, BRII-mutants were created where serine was exchanged to alanine. Only the mutant S336A completely abrogates phosphorylation in an in vitro kinase assay (Fig. 4.64). To test the impact of this mutant on signal transduction, SBE reportergene assays as well as ALP assays were performed. S336A strongly inhibits the expression of the reportergene (Fig. 4.65), but does not show any inhibition of the ALP-pathway (Fig. 4.66). These effects induced by the mutant S336A are not due to low protein expression (Fig. 4.64) or deficient surface expression (Fig. 4.67). Immunofluorescence studies in C2C12 cells showed S336A cell surface expression being identical to the BRII-LF expression (Fig. 4.67).

5.4.1 Prediction of BRII phosphorylation

So far nothing is published on BMP receptor phosphorylation. To get an idea about potential phosphorylation sites, the program NetPhos can be used to predict possible phosphorylation targets.

MLTGDRKQGLHSMNMMEAAASEPSLDLDNLKLLELIGRGRYGAVYKGSLDERPVAVKVFSFA
NRQNFINEKNIYRVPLMEHDNIARFIVGDERVTADGRMEYLLVMEYYPNGSLCKYLSLHTSD
WVSSCRLAHSVTRGLAYLHTELPRGDHYKPAISHRDLNSRNVLVKNDGTCVISDFGLSMRLT
GNRLVRPGEEDNAAISEVGTIRYMAPEVLEGAVKLRDCESALKQVDMYALGLIYWEIFMRCT
DLFPGESVPEYQMAFQTEVGNHPTFEDMQVLVSREKQRPKFPEAWKENSLAVRSLKETIEDC
WDQDAEARLTAQCAEERMAELMMIWERNKSVSPTVNPSTAMQNER
→NLSHNRRVPKIGPYPDYSSSSYIEDSIHHTDSIVKNISSEHSMSSTPLTIGEKNRNSINYER
QQAQARIPSPETSVTSLSTNTTTTNTTGLTPSTGMTTISEMPYPDETNLHTTNVAQSIGPTP
VCLQLTEEDLETNKLDPKEVDKNLKESSDENLMEHSLKQFSGPDPLSSTSSSLLYPLIKLAV
EATGQQDFTQTANGQACLIPDVLPTQIYPLPKQQNLPKRPTSLPLNTKNSTKEPRLKFGSKH
KSNLKQVETGVAKMNTINAAEPHVVTVTMNGVAGRNHSVNSHAATTQYANGTVLSGQTTNIV
THRAQEMLQNQFIGEDTRLNINSSPDEHEPLLRREQQAGHDEGVLDRLVDRRERPLEGGRTN
SNNNNSNPCSEQDVLAQGVPSTAADPGPSKPRRAQRPNSLDLSATNVLDGSSIQIGESTQDG
KSGSGEKIKRVKTPYSLKRWRPSTWVISTESLDCEVNNNGSNRAVHSKSSTAVYLAEGGTAT
TTVSKDIGMNCL

Figure 5.6 Predicted phosphorylation of BRII-LF
According to the NetPhos-Program the predicted phosphorylation sites in BRII-LF are listed. The sequence shown starts with the intracellular part of BRII-LF. The begin of the tail sequence is marked with an arrow. Potentially phosphorylated serines are marked green, threonines red and tyrosines blue. Pink colour indicates mapped phosphorylation sites so far. Grey marked are potential phosphorylation sites. The one in the kinase domain seems to be Spot11, the one in the tail Spot1. Only calculation based on experimental data hints towards these two grey sites.

For the prediction of potential phosphorylation sites in BRII-LF the consensus sequences of more than 100 protein kinases were matched with the BRII-LF sequence (PhoshoBase http://www.cbs.dtu.dk/services/NetPhos/). For serine and threonine kinases the respective consensus sequences were matched. For tyrosine kinases a general consensus sequence was taken into account (Kreegipuu et al., 1999). The following kinases were predicted to phosphorylate BRII (see Tab.5.1).

Serine/Threonine Kinases:

CaMII	**R-X-X-S/T-X**
CKI	Sp/Tp-X_{2-3}-S/T-X
CKII	X-S/T-X-X-D/E
GSK3	X-S/T-X-X-D/E
P70s6k	K/R-X-R-X-X- S/T-X
PKA	R-X_{1-2}- S/T-R/K
PKC	X- S/T-X-R/K
PKG	$(R/K)_{2-3}$-X- S/T-X

Table 5.1 BRII-phosphorylating kinases according to NetPhos
Potentially BRII phosphorylating kinases and their consensus sequences are listed.

Comparing potential and identified phosphorylation sites, there are only two overlaps. S815 which is supposed to be recognized and phosphorylated by CaMKII and Y825, which is phosphorylated by a tyrosine kinase. CaMKII is a multifunctional CaM kinase that is mainly

regulated by the Ca^{2+}/calmodulin complex. It is an ubiquitously expressed enzyme with special impact in the brain, regulating neurotransmitter secretion and transcription factor regulation and glycogen metabolism in various tissues (Bayer and Schulman, 2001).
So far there are no hints that CamKII is phosphorylating BRII. To investigate the involvement experiments using specific CaMKII inhibitors as autocamtide-2 related inhibitory peptide (AIP), pentylenetetrazol (PTZ), KN-62 or KN-93, can be performed (Smyth et al., 2002).

5.4.2 Serine phosphorylation on BRII

Serine phosphorylation sites of BRII-LF were identified in both, the kinase domain and the C-terminal tail. Within the kinase, phosphorylation was detected at serine336, and within the tail domain at serine757, serine815 and serine818. Whereas S336 regulates kinase activity (Fig. 4.64), the function of S815 and S818 remains unknown. S815 is phosphorylated alone or in combination with S818 and Y825 (Fig. 4.58, Tab. 4.6 and 4.9). Besides, it seems to be a important target for auto-phosphorylation, as it is a prominent spot on the 2D-map of the in vitro kinase assay (Fig. 4.57). S757 has been shown to be phosphorylated in a c-kit, BMP2 and SCF dependent manner and thus influencing BMP2 and c-kit mediated signal transduction. Moreover two potential phosphorylation sites are identified at S515 and S863 but their impact has not been investigated so far.

5.4.3 Tyrosine phosphorylation of BRII

As depicted above, Y825 in the tail domain is a target for phosphorylation. Single phosphorylation of Y825 could not be discovered. Either S818 is phosphorylated besides Y825 or a triple phosphorylation of S815, S8181 and Y825 can be observed. In addition, a second tyrosine phosphorylation could be demonstrated in the kinase domain using phospho-tyrosine-specific antibodies. Unfortunately the exact site could not be determined yet.
BRII-LF is tyrosine phosphorylated without ligand stimulation, cotransfection of BRIa and subsequent BMP2 stimulation for 2h enhances that signal (Sylke Hassel, Diploma thesis). As this was demonstrated by western blotting the phosphorylated amino acid might be within the kinase or the Y825. In addition, the kinase performing this phosphorylation might be either BRIa, that is phosphorylating BRII in a “back-phosphorylating mechanism”, exhibiting dual-kinase activity (Sylke Hassel, Diploma thesis) or c-kit, that has been shown to interact with BRII and BRIa. Back-phosphorylation can not be excluded, as it has been shown by Huse and coworkers that the TβRI exhibits certain structural features at the catalytic center that are characteristic of tyrosine kinases rather than Ser/Thr kinases (Huse et al., 1999). Moreover the phosphorylation observed could originate from auto-phosphorylation or the involvement of other kinases as well. As Lawler described auto-phosphorylation on tyrosines, the

participation of other kinases might be unlikely (Lawler et al., 1997). On the other hand the phosphorylation described occurred via auto-phosphorylation and was ligand independent, in contrast to the observed ligand dependent enhancement of phosphorylation for Y825.

5.4.4 Comparison of BRII phosphorylation with TGF-β receptor phosphorylation

Studies on receptors for TGF-β have revealed several mechanisms of receptor activation and modulation that might also be shared by other receptors in this family. As phosphorylation is a key event in receptor activation the phosphorylation pattern of TβRI, TβRII and BRII should be compared.

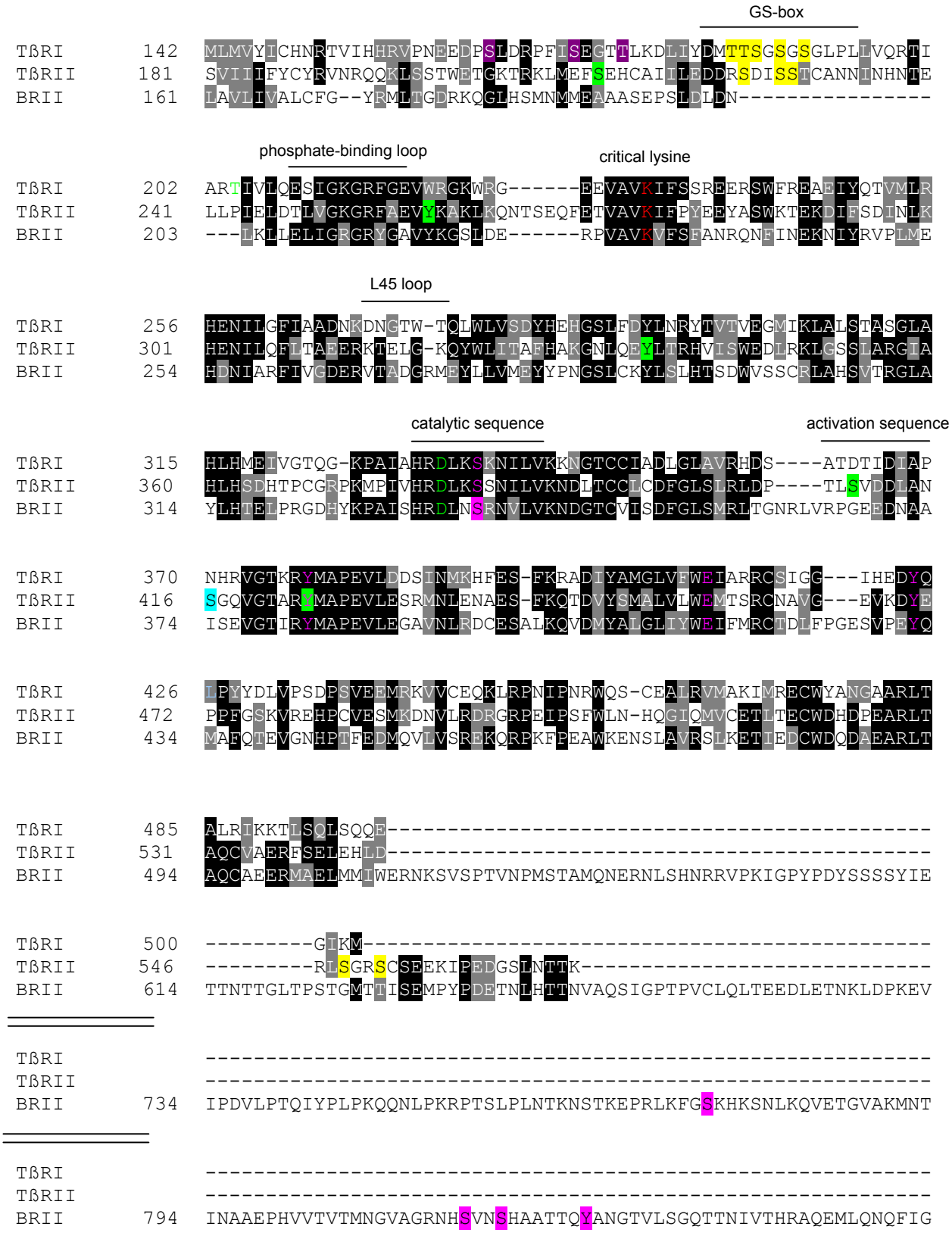


Figure 5.7 Alignment of BRII with TβRI and TβRII and indication of phosphorylation sites
Boxshade alignment of TβRI, TβRII and BRII. The location of the GS-box and the L45 loop in TβRI and the catalytic sequence, the critical lysine as well as the phosphate-binding loop in all three kinases are indicated. Activating phosphorylations in the membrane-proximal region and in the TβRII tail are indicated in yellow, in violet receptor-modulating phosphorylations are indicated, in green positively activating phosphorylations are marked. Turquoise marks the inhibitory phosphorylation. In pink

proven BRII phosphorylation sites are highlighted. In pink the amino acids complexed with S336 are shown.
TGF-β receptor phosphorylation sites are marked according to literature (Souchelnytskyi et al., 1996), (Chen and Weinberg, 1995), (Luo and Lodish, 1997), (Wrana et al., 1994), (Lawler et al., 1997).

As sequence comparisons have shown, the TGF-β receptors contain classical serine/theronine kinase domain motifs, but this family clusters close to the tyrosine kinases in terms of overall sequence similarity (Hanks and Hunter, 1995), (Huse et al., 1999).
Many kinases become activated by direct phosphorylation of the activation segment, which leads to a confomational change that enables kinase activity (Johnson et al., 1996b). So far there is no evidence that TβRI is phosphorylated in this region (Wrana et al., 1994), (Souchelnytskyi et al., 1996). For TβRII activating (S409) as well as inhibiting phosphorylation (S416) has been observed here. But none of these two phosphorylations has been shown to be essential for signalling (Luo and Lodish, 1997).

In the absence of ligand, overexpressed TGF-β receptors exhibit strong phosphorylation of more than ten different sites each. For TβRII the phosphorylation can not be increased by cotransfection of the type I receptor or addition of ligand, as overexpressed TGF-β receptors have the tendency to form heterooligomers (Chen and Weinberg, 1995). In case of TβRI, ligand is inducing TβRII mediated phosphorylation at 5 sites, mostly in the GS-box, named according to the conserved TTSGSGSGLP sequence at its center (Wieser et al., 1995), (Saitoh et al., 1996) (Souchelnytskyi et al., 1996). T185, T186, S187, S189, S191 in the GS-box are phosphorylation targets, among them T186, S189 and S191 are indispensable for receptor activation (Souchelnytskyi et al., 1996), (Saitoh et al., 1996). Moreover S165, S172 and T176 are phosphorylated in a ligand-dependent manner. All three phosphorylation events modulate signalling outcome, mostly regulating cell growth (Souchelnytskyi et al., 1996), (Saitoh et al., 1996). Nothing is known about the approximately 11 sites in TβRI that are phosphorylated in the absence of ligand. Dephosphorylation of TβRI is partially mediated by PP1c (Shi et al., 2004).
In in vitro kinase experiments, that were claimed to mimic wildtype situation, TβRII exhibits phosphorylation on serines, threonines and tyrosines. Whereas the phosphorylated threonines were not determined (Lin et al., 1992), S213, S409 and S416 as phosphorylated serines (Luo and Lodish, 1997) and Y259, Y336, Y424 as phosphorylated tyrosines were identified (Lawler et al., 1997). Except for S416 all phosphorylation events are receptor-activating. Moreover it is postulated that S213, S409 and Y259 are essential for receptor activation (Luo and Lodish, 1997), (Lawler et al., 1997). In vivo and complexed with TβRI, TβRII is phosphorylated at 17 sites, among which S223, (S226), S227, S549, and S551 were identified (Souchelnytskyi et al., 1996) (Fig. 5.6).

Still many phosphorylation sites in the TGF-β receptors are not identified. So far, for BRII only few phosphorylation sites are determined. Surprisingly, there is no overlap between BRII and TGF-β receptors concerning phosphorylation. On the other hand, some of the identified sites in BRII have been predicted using NetPhos. But taking into account that the prediction is rather loose and that only 2 out of 5 really phosphorylated amino acids could be predicted the program is rather useless for relatively unknown kinases. Therefore it is more likely that the match between the TGF-β family receptors is higher. The corresponding phosphorylated amino acids might just not be published. Moreover, there are major differences between BMP and TGF-β receptors, concerning signalling and oligomerization (Nohe et al., 2002). These differences might result in a different phosphorylation and thus activation pattern. As phosphorylated sites serve as docking sites for associated molecules and the proteins found with BRII and TβRII exhibit differences (see chapter 1.5 and Fig. 4.20), the different signalling might find its correspondence in phosphorylation.

When comparing the alignment and modelling the structure of BRII according to the solved structure of TβRI and comparing the sites, it can be realized that important structures are well conserved as the phosphate-binding-loop, the catalytic sequence and the region around the critical lysine, that binds the α and β phosphates for ATP (see Fig. 5.8). The kinase domain of the receptors consists of two lobes, the upper lobe is binding the nucleotide ATP and supporting the transfer of the phosphate to the substrate bound in the substrate binding pocket. The lower lobe is responsible for peptide binding and catalysis of the phosphate-transfer. The deep cleft between the lobes is the site of catalysis. In Fig 5.8 the structure of TβRI with the two lobes is depicted. The phosphate binding loop in the upper lobe and the catalytic sequence in the lower lobe are highlighted in yellow.
Most protein kinases are activated by phosphorylation on a centrally located loop known as the "activation loop". Interestingly, the type-I TGF-β receptor does not become activated by phosphorylation of the activation loop. Instead, multiple phosphorylations of serine and threonine residues in a segment known as the GS-box, result in receptor activation. TβRI exhibits two structural conformations, a locked form, bound to the inhibitor FKBP12, and an open form, that is able to bind Smads and ATP (Huse et al., 2001). In general, the structure of TβRI exhibits a canonical protein kinase fold, similar to that which was first observed in the structure of PKA (c-AMP dependent protein kinase) (Knighton et al., 1991). The most related kinase, though, seems to be the insulin receptor kinase (IRK), a tyrosine kinase, that shares approximately 30% identity with TβRI and can be aligned over 250 amino acids (Huse et al., 1999).

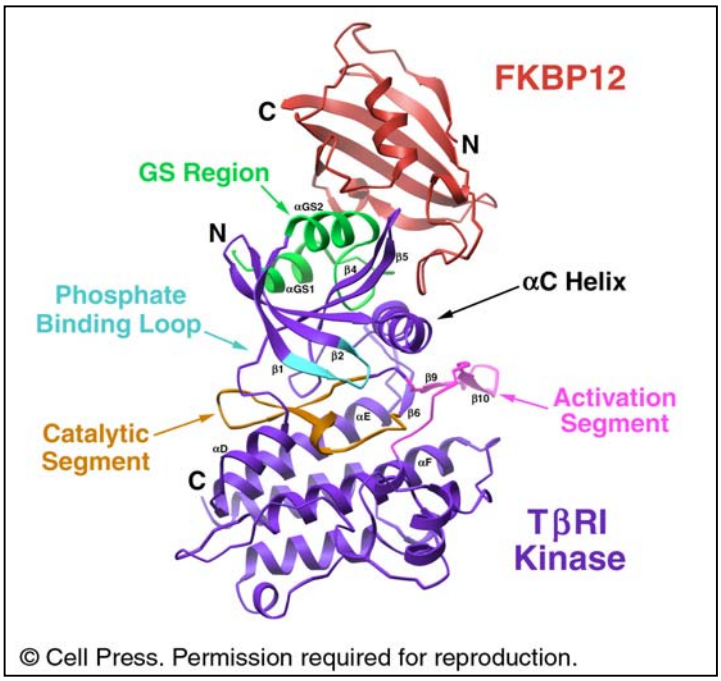


Figure 5.8 Ribbon diagram of TβRI structure in complex with FKBP12 (Huse et al., 1999)
The upper lobe contains the phosphate-binding loop depicted in turquoise and the GS region in green. The catalytic segment is highlighted in gold, the activation segment in the lower lobe is depicted in pink.

TGF-β receptor signalling complex is consisting of at least homodimers of TβRI and TβRII (Henis et al., 1994), (Chen and Derynck, 1994). It has been pointed out that G216 and G322 are important for the functional interaction between type I and type II receptors (Weis-Garcia and Massague, 1996). Surprisingly mutations of these amino acids can be compensated by cotransfection of TβRI K232R, a kinase-inactive mutant, suggesting that both receptors can interact in trans.

Being a member of the TGF-β superfamily, the BRII sequence was taken for structure determination using the solved TβRI structure. Fig. 5.9 depicts the potential structure of BRII. As there is no known sequence homology to the BRII-tail, only the kinase domain was taken into account. In this region only one phosphorylation site in the BRII could be identified so far. Phosphorylation of S336 in BRII is highly upregulated by BMP2 in vivo (Fig. 4.58, Fig. 4.59, Spot12). Moreover S336 is a target for auto-phosphorylation, as seen in the in vitro kinase assay of BRII-SF and BRII-LF (Fig. 4.58). Phosphorylation of that site seems to regulate BRII activity, as S336A shows no auto-phosphorylation and exhibits dominant negative effects upon SBE-mediated reportergene expression. Therefore S336 and its surrounding crystal lattice should be examined.

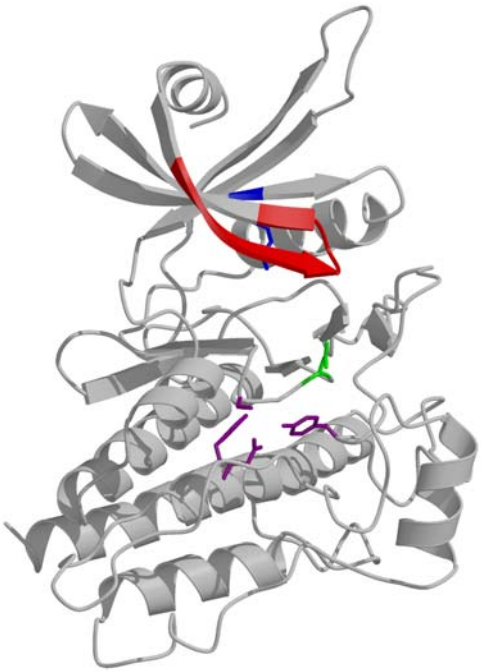


Figure 5.9 Structure modelling of BRII kinase according to the structure of TβRI (Huse et al., 1999)
The upper lobe contains the phosphate-binding loop, highlighted in red, the D333 in the catalytic sequence in the lower lobe is depicted in green. S336 in the catalytic sequence and the amino acids binding it are highlighted in violet (Model Th. Müller).

S336 is directly situated in the catalytic center of the kinase in a groove inside the molecule. A close neighbour, aspartate 333 plays an important role in phosphorylation of the substrate. The mutant BRII S336A exhibits no auto-kinase activity. From the structure it can be concluded that the mutation of serine to alanine is not influencing the protein folding. Thus phosphorylation of S336 might have regulatory effects.

In the closed conformation of TβRI S336A is forming a ring-structure of hydrogen bonds together with Y378, Y424 and E410 (see Fig. 5.10 and 5.11). In the TβRII the same ring-structure is formed by S382 with Y470, E456 and Y424. As it has been demonstrated by Lawler et al., Y424 is phosphorylated in TβRII and is a target for activating auto-phosphorylation (Lawler et al., 1997). The same seems to hold through for BRII, where S336 is linked via hydrogen-bonds to Y382, E415 and Y432.

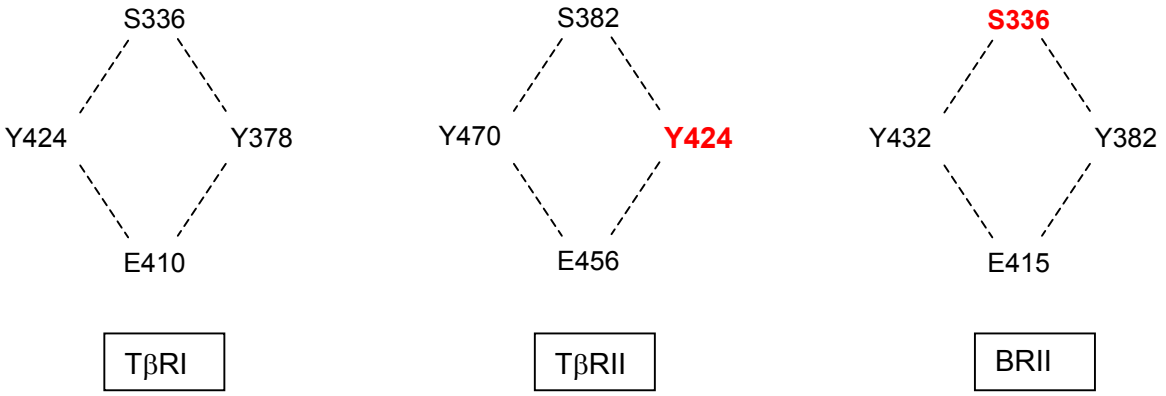


Figure 5.10 Scheme of the ring structure formed by S336/S382 in TGF-β superfamily receptors
On the left hand, the hydrophobic ring structure, as observed in the crystal structure, is depicted. The situation for TβRII (middle) and BRII (right) is analogous to that for TβRI, as the model is based on that structure

Despite the fact of being in a groove Y424 in TβRII and S336 in BRII are targets for phosphorylation. In both cases this phosphorylation seems to promote receptor activation.
The crystal structure helps to elucidate the potential mechanism. All four amino acids of that ring are not exposed to the surface of the protein, being situated in a deep groove that is not well accessible. For BRII, Arg337 is hanging in front of the groove, resembling a curtain and thus stabilizing the negative charge within that groove (Fig. 5.11). Structurally, one phosphorylated side chain at one of these amino acids would fit into the groove, despite the fact that it is very tight, but the acidic character of the phosphate moiety would probably induce structural changes within the groove. For this and for spatial reasons, the kinase structure would open up and thus expose other amino acids. With this mechanism the inactive receptor could be activated and start to transmit signals.

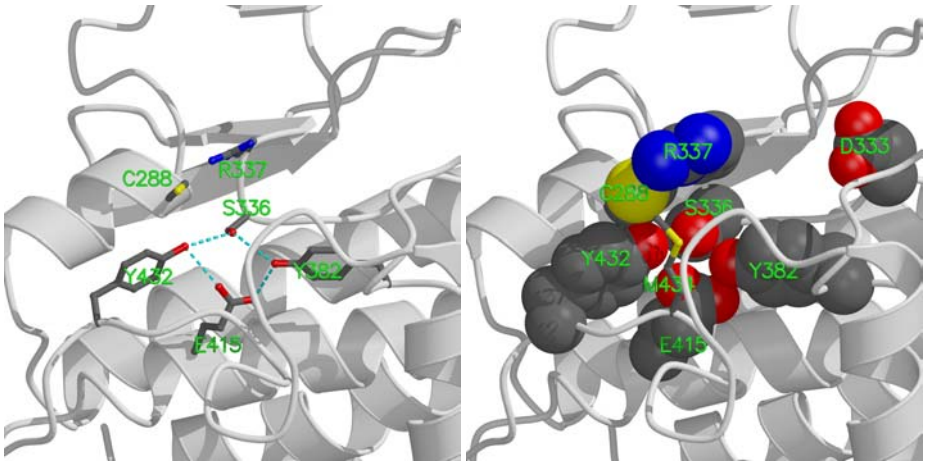


Figure 5.11 Close view on the interactions of amino acids in the catalytic sequence of BRII
Left: Hydrophobic bonds in the groove of S336 connecting S336 with Y382, E415 and Y432. The structure is stabilized by a structure-protecting positively charged amino acid (K337 for TβRI and R337 for BRII). (Model Th. Müller)
Right: Tight packing of the amino acids in the groove of S336, demonstrated by electron-density model Structure according to Huse (Huse et al., 1999).

The phosphorylation of S336 (BRII) or Y424 (TβRII) can only occur via auto-phosphorylation as the groove is not accessible from outside. Instead, the phosphate group of ATP could be transmitted via the arginine residue from the back-side in the inactive, closed form of the kinase. In this closed conformation trans-phosphorylation of either S336 or Y424 by another kinase would be impossible for sterical reasons. After the respective amino acid is phosphorylated, the cleft opens up and thus the kinase becomes activated. Structurally, the environment surrounding the amino acids in the ring, is suitable to stabilize the phosphorylation (Th. Müller, personal communication).

To further examine the activation and the phosphorylation of BRII, BRII-mutants can be used. The S336D or S336E mutation, introducing a negative charge, results in a conformational change similar to that caused by phosphorylation of this residue. Therefore these mutations should activate the BRII, but this hypothesis has to be tested.

6. Summary

BMPs influence a variety of cellular processes. They have been shown to regulate proliferation, differentiation, migration and apoptosis and thus play central roles during developmental processes and tissue homeostasis. Ligand mediated signal transduction is transmitted via BMP type I and BMP type II receptors, both members of the serine/threonine kinase superfamily. The BMP receptor mediated signal transduction is not explored in detail. Therefore our aim was to address different aspects of BMP mediated signal transduction with main focus on BRII and its regulation. Due to the existence of two alternative splice variants, a long and a short form, the function of the two variants and the impact of the C-terminal extension are of general interest. Moreover, mutations in the *BMPR2* gene were identified to be responsible for PPH, a autosomal dominant lung disease.

In this thesis, BRII phosphorylation and signalling mediated by different receptor oligomers were investigated and multiple BRII associated proteins were identified.

We could show that the oligomerization pattern of BMP receptors exhibits a higher degree of flexibility compared to other receptors of that superfamily. In the present work the BMP2 mediated signal transduction should be examined, depending on the receptor oligomerization pattern. Using kinase-deficient mutants, it could be demonstrated, that signalling via preformed BMP receptor complexes is mediated by the well characterized Smad1/5/8 pathway, whereas signalling initiated by BMP2 induced recruitment of the receptors activates the p38 pathway and leads to Alkaline Phosphatase production.

To further study signalling events triggered directly from the BRII a proteomics-based screen for BRII associated proteins was performed. 53 associated proteins were found, the majority being signal transducing molecules, but in addition metabolic proteins, transcriptional regulators and others were identified. These proteins enable to gain a deeper insight in BMP mediated signalling.

One of the interactors, the receptor tyrosine kinase c-kit, was characterized in more detail. It could be demonstrated, that BRII and c-kit form a complex in vitro and in vivo, and the interaction is enhanced upon BMP2 stimulation. 2D phosphopeptid mapping showed that BRII is phosphorylated at S757 upon activation of c-kit by SCF. Moreover, c-kit and its ligand SCF are modulating BMP2 pathways, by enhancing Smad1/5 phosphorylation, Smad-transcriptional activity, Alkaline Phosphatase production and expression of Cbfa1. All these pathways hint towards modulation of the osteoblast development via c-kit. Thus, we were able to develop a novel paradigm for the BMP2 meditated signalling.

One of the initial triggers for BRII is the auto-phosphorylation of BRII. Here we analyze ligand-independent as well as ligand-dependent phosphorylation of BRII. Some phosphorylation sites in BRII were identified. The general phosphorylation occurs mostly on serines. S815, S818 and Y825 are identified targets of phosphorylation whose function is still unclear. However phosphorylation of S336 is demonstrated to be essential for BRII activation.

The elucidation of BMP receptor phosphorylation and oligomerization as well as the impact of a number of BRII associated proteins (such as c-kit), demonstrated in this thesis that BMP signalling has to be regulated precisely on multiple levels. This can be useful for the development of selective signalling inhibitors for basic research and therapeutic approaches of PPH and other diseases.

6. Zusammenfassung

BMPs regulieren eine Vielzahl zellulärer Prozesse, unter anderem Zellwachstum, Zelldifferenzierung, Bewegung und Zelltod. Dies macht sie zu einem bedeutenden Faktor während zahlreicher Entwicklungsvorgänge und im adulten Organismus. BMP-Signale werden über BMP Typ I und Typ II Rezeptoren weitergeleitet, die beide Serin/Threonin Kinase Rezeptoren sind. Während der Typ I Rezeptor Signale zu den Smad-Proteinen weiterleitet, fungiert der Typ II Rezeptor seinerseits als Aktivator des Typ I Rezeptors und ist somit für die Signalweiterleitung essentiell. Alternative Signalwege sind nicht sehr gut untersucht. Deswegen war es unser Ziel verschiedene Aspekte der BMP vermittelten Signaltransduktion, mit Schwerpunkt BRII, zu untersuchen.

Der BRII ist interessant, da er in zwei alternativen Spleißvarianten vorliegt, einer langen und einer kurzen Form, die sich intrazellulär um mehr als 500 Aminosäuren unterscheiden und deren Unterschiede hinsichtlich Signalweiterleitung noch nicht erforscht sind. Darüber hinaus führen Mutationen im *BMPR2* Gen zu einen Gefäßerkrankung der Lunge, PPH.

In dieser Arbeit wurden die BRII Phosphorylierung und Signalweiterleitung, initiiert von unterschiedlichen BMP Rezeptorkomplexen, untersucht. Hierbei wurde eine Vielzahl von BRII assoziierten Proteine identifiziert und hinsichtlich ihres Einflusses auf BMP Signalweiterleitung charakterisiert..

Unser Labor konnte zeigen, daß das Oligomerisierungsmuster der BMP Rezeptoren weitaus flexibler als das der verwandten TGF-β Rezeptoren ist. Aus diesem Grund wurde die Siganltransduktion der BMP Rezeptoren im Hinblick auf die Komplexbildung untersucht. Mit Hilfe Kinase-defekter Mutanten konnte gezeigt werden, dass präformierte Rezeptorkomplexe den Smad-Signalweg benutzen, wohingegen liganden-induzierte Komplexe ihre Signale über den p38-Weg weiterleiten, welcher die Produktion von Alkalischer Phosphatase steuert.

Um darüber hinaus Signalwege ausgehend vom BRII zu untersuchen und mehr über Smad-unabhängige Signalwege zu erfahren, wurde ein Proteomics-basierter Screen nach BMP Typ II Rezeptor interagierenden Proteinen durchgeführt. Unter den 53 Rezeptor-assoziierten Proteinen befanden sich viele Signaltransduktionsmoleküle, aber auch metabolische Proteine, Proteine, die an der Regulation der Transkription beteiligt sind und andere. Mit Hilfe dieser Proteine kann ein Einblick in die Vielfalt der BMP Rezeptor vermittelten Signaltransduktion gewonnen werden.

Der Tyrosinkinase Rezeptor c-kit ist eines der assoziierten Proteine. Es konnte gezeigt werden, dass die Interaktion durch BMP2 Zugabe in vivo verstärkt wurde. Phosphorylierung an Serin 757 des BRII, vermittelt von aktiviertem c-kit und SCF, konnte mittels 2-dimensionalem Phosphopeptidmapping gezeigt werden. Außerdem beeinflusst der SCF/c-kit Signalweg auch bereits beschriebene BMP Signalwege. So konnten synergistische Effekte beider Liganden auf dem Smad1/5 Weg, bei der Produktion der Alkalischen Phosphatase und bei der Transkription von Cbfa1 beobachtet werden, alles Wege, die die Entwicklung von Osteoblasten steuern. So konnte mit dem Einfluß von c-kit auf BMP2 vermittelte Osteoblastenetwicklung ein neuer Faktor bei BMP gesteuerter Entwicklung aufgedeckt werden.

Einer der initialen Trigger bei der BMP Signaltransuktion ist die Aktivierung des BMP Typ II Rezeptors durch Phosphorylierung. Es wurde Liganden-unabhängige wie Liganden-abhängige Phosphorylierung des BRII untersucht und die jeweiligen Phosphorylierungssites identifiziert. Hauptsächlich findet die Phosphorylierung an Serinen statt. So wurden S815 und S818, aber auch Y825 als Target-sites identifiziert. Im Gegensatz zu der Phosphorylierung von S815, S818 und Y825, deren Funktion noch unklar ist, konnte gezeigt werden, daß Phosphorylierung von S336 essentiell für die Rezeptoraktivierung ist.

Die Erforschung der BMP-Rezeptor Phosphorylierung, Oligomerisierung und Rezeptor-assoziierter Proteine (wie c-kit), wie in dieser Arbeit vorgestellt, zeigt, dass BMP vermittelte Signaltransduktion präzise auf mehreren Ebenen reguliert werden muß.

Die hier erstmalig beschriebenen Wege der Regulation der BMP Signaltransduktion tragen sowohl zu einem besseren Verständnis molekularer Zusammenhänge bei, als auch zu der Entwicklung selektiver Inhibitoren und therapeutischer Ansätze zur Behandlung der PPH und anderer Krankheiten.

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A1 Abbreviations

Chemicals/Materials

AA	Acrylamid
ab	Antibody
Ambic	Ammoniumbicarbonat
Amp	Ampicillin
APS	Ammoniumpersulfat
ATP	Adenosine Triphosphate
BA	Methylenbisacrylamid
BCA	Bicinchoninic acid
β-gal	β-galactosidase
BSA	Bovine serum albumin
CMV	Cytomegalovirus
DEAE	Diethylaminoethyl
DMEM	Dulbecco´s modified essential medium
DMSO	Dimethylsulfoxid
dNTP	2´-Desoxy-nucleosid-5´triphosphat
DSS	Disuccinimidyl suberate
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylendiammintetraacetate
EGFP	enhanced green fluorescent protein
EMEM	Eagle´s minimal essential medium
env	Envelope
EtBr	Ethidiumbromide
EtOH	Ethanol
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
gag	Group specific antigen
GFP	Green fluorescent protein
HA	Haemagglutinin
HEPES	N-2-hydroxyethylpiperazine-2´-ethansulfonic acid
HRP	Horseradish peroxidase
IAA	Iodacetamide
IEF	Isoelectric focusing
IPTG	Isopropyl-β-thiogalactopyranoside
IRES	Intervening ribosomal entry site
LB	Luria bertani
LTR	Long terminal repeat
Luminol	3-Aminophtalhydrazide
MMLV	Mouse Moloney murine leukemia virus
MTS	Methyl tetrazolium salt
MTT	Thiazolyl blue
MuLV	Murine leukemia virus
P/S	Penicillin/Streptomycin
PAGE	Polyacrylamide gelelectrophoresis
PAS	Protein A sepharose
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PEI	Polyethylenimmine
Pen	Penicillin
PES	Phenazine ethosulfate
Pfu	*Pyrococcus furiosus*
PMSF	Phenylmethylsulfonylfluorid
pNPP	Para-Nitrophenylphosphate
pol	Polymerase
pox	Peroxidase
PVP	Polyvinylpyrrolidon
RNase A	Ribonuclease A
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecylsulfate
STE	Sodium chloride, Tris, EDTA
Strep	Streptomycin
Taq	*Thermophilus aquaticus*
TBS	Tris buffered saline
TCA	Trichloric acid
TEMED	N, N, N, N,-tetramethylethylenediamine
TFA	Trifluoracetic acid
Tris	Tris-(hydroyxmethyl)-aminomethan
VSV	Vesicular stomatitis virus
X-gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
zeo	Zeocin

Terms

2D	2-dimensional
α–	anti-
aa	amino acid
ActRII	Activin type II receptor
AIP	autocamtide-2 related inhibitory peptide
ALK	Activin like kinase
ALP	Alkaline phosphatase
AMH	Anti Mullerian Hormone
AML	Acute Myelogenous Leukemia
AP1	activator protein-1
ARP	Actin related protein
ATF2	activating transcription factor-2
BAMBI	BMP and Activin membrane bound inhibitor
BCR	B cell receptor
BIR	baculovirus IAP repeats
BISC	BMP induced signalling complex
BMP	Bone morphogenetic protein
bp	basepairs
BRAM	BMP receptor associated molecule
BRI/II	BMP receptor type I/II
bZIP	basic region/leucine zipper transcription factor
CaMKII	Calmodulin-Dependent Protein Kinase II
Cbfa1	core binding factor α 1
CBP	CREB-binding protein
CCE	capacitative calcium entry
CD	cluster of differentiation
cGK	cyclic GMP dependent protein kinase
CHIP	C-terminus of HSC70-interacting protein
CHL1	chordin-like 1
CIZ	cas-interacting zinc finger
CKII	casein kinase II
CMV	Cytomegalovirus
CR domains	cystine-rich domains
CREB	cAMP response element binding protein
CSF-1R	colony stimulating factor 1 receptor
CtBP	C-terminal binding protein
CTGF	Connective-Tissue Growth Factor
CV2	Crossveinless2
HECT	homologous to E6-AP C-terminus
Dab-2	Disabled 2
DAG	Diacylglycerol
DAN	differential screening-selected gene aberrant in neuroblastoma
Daxx	death-associated protein 6
deltaEF1	δ-crystallin enhancer binding factor 1
dH_2O	deionized water
Dlx5	distal-less homeobox 5
DNA	Deoxyribonucleic acid
Dpp	Decapentaplegic
ds	double stranded
EC	endothelial cell
ECL	enhanced chemoluminescence
ECM	extracellular matrix
eco	ecotropic
EEA1	early endosome antigen 1
EGF	epidermal growth factor
EHZF	early haematopoietic zinc finger
ELISA	Enzyme Linked ImmunoSorbent Assay
Eng	Endoglin
ER	Endoplasmatic reticulum
ERK	extracellular signal-regulated kinase
ES cells	embryonic stem cells
Evi3	ecotropic viral integration site 3
FGF	Fibroblast Growth Factor
Fig.	figure
FKBP12	FK506-binding protein 12
FMS	identical with CSF-1R
FOP	Fibrodysplasia Ossificans Progressiva
FT	farnesyltransferase
GADD45	growth arrest DNA-damage 45
GDF	Growth and Differentiation Factor
GDNF	Glial cell-line Derived Neurotrophic Factor
GEF	guanine nucleotide exchange factor
gp130	glycoprotein 130
GS-domain	glycine/serine rich region
GST	Glutathione S-transferase
HAT	Histone actetyl transferase
HBF	heterotopic bone formation
HGF	Hepatocyte Growth Factor

HGP	human genome projekt
HHSC	human haematopoietic stem cells
HHT	Hereditary Hemorrhagic Telangiectasia
Hoxc-8	homeodomain transcription factor 8
HPAEC	human pulmonary-artery endothelial cells
IAP	insulinoma amyloid polypeptide
ICS	image correlation spectroscopy
Id1	inhibitor of differentiation 1
Ig domain	Immunglobulin domain
IL-2	interleukin 2
ILK	integrin linked kinase
IP	immunoprecipitation
IRES	internal ribosome entry site
IRK	insulin receptor kinase
IVKA	in vitro kinase assay
JAK	Janus kinase
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
JPS	Juvenile Polyposis
LIF	Leukemia Inhibitory Factor
LIMK1	LIM kinase 1
MAD	mothers against Dpp
MALDI-TOF	matrix assisted laser disorption - time of flight
MAP	mitogen-activated protein
MAPK	mitogen activated protein kinase
MASP2	mannan-binding lectin serine-protease 2
MEK1	MAP/Erk kinase-1
MH	MAD-homology
MIS	Mullerian Inhibiting Substance
MKK3	MAP kinase kinase 3
ML promoter	major late promoter
mLC7	murine light chain 7
MLD	metachromatic leukodystrophy
MLK	mixed-lineage protein kinase
MPP	mitochondrial processing peptidase
MS	Mass spectrometer
MS	Mass Spectrometry
MSG1	melanocyte specific gene
Msx	Muscle segment homeobox
MyoD	myogenic differentiation antigen 1
mZnf8	mouse Kruppel-type zinc finger protein 8
NES	nuclear export sequence
NF_KB	nuclear factor $_K$ B
NGF	Nerve Growth Factor
NIC	Notch intracellular domain
OAZ	Olf1/EBF associated zinc finger
OC	osteocalcin
OD	optical density
Osx	Osterix
p-	phospho-
PAI-1	plasminogen activator inhibitor 1
PASMC	primary artery smooth muscle cell
pCAF	p300/CBP associated factor
PCR	polymerase chain reaction
PDGF	Platelet-Derived Growth Factor
PDZ domain	domain first identified in PSD-95, Discs-Large, ZO-1
PFC	preformed complex
PH-domain	Pleckstrin Homology domain
PI3K	Phosphatidyl-inositol-3-kinase
PKC/PKD	protein kinase C/D
PLC	phospholipase C
PMDS	Persistant Mullerian Duct Syndrome
PP1c/PP2A	protein phosphatase 1c/2A
$PPaR_\gamma$	peroxisome proliferator-activating receptor gamma
PPH	Primary Pulmonary Hypertension
PRD	proline-rich domain
PRDC	protein related to DAN and cerberus
PTB site	phosphotyrosine binding site
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
PTP	protein tyrosine phosphatase
PTPRO	protein tyrosine phosphatase receptor type O
PTZ	pentylenetetrazol
RING	really interesting new gene
RLTK	Renilla luciferase under the control of a thymidine kinase promotor

RNA	Ribonucleic acid
ROS	rat osteosarcoma
RSK	receptor serine/threonine kinase
RTK	receptor tyrosine kinase
Runx	Runt-related transcription factor
SANE	Smad1 Antagonistic Effector
SAPK	stress-activated protein kinase
SARA	Smad anchor for receptor activation
SBE	Smad binding element
SCF	stem cell factor
SH2 domain	src homology 2 domain
SH-domain	src homology-domain
SHP1	src homology 2 domain-containing tyrosine phosphatase 1
SIP	Smad interacting partner
SMIF	Smad-interacting factor
Smurf	Smad ubiquitination regulatory factor
SNIP	Smad nuclear interacting protein
SNX6	sorting nexin 6
SOSC6	suppressor of cytokine signalling 6
SOST	Sclerostin
SRE	serum response element
ss	single stranded
Stat3	signal transducer and activator of transcription 3
STRAP	serine/threonine kinase receptor associated protein
SUMO	small ubiquitin-related modifier
TAB	TAK binding protein
TAK1	TGF-β activated kinase 1
TβRI/II	TGF-β receptor type I/II
TC	truncation
Tctex-1	T-complex testis-specific protein 1 homolog
tfr	Transferrin receptor
TGF-β	Transforming growth factor-β
TK	thymidine kinase
TLP	Trap like protein
Tob	transducer of ERB2
TOF	time of flight
TRAP1	TGF-β receptor associated protein
TRIP-1	TGF-β receptor interacting protein
trp	transient receptor potential channel
Ubc9	ubiquitin conjugating enzyme 9
UV	ultraviolett
VEGF	vascular endothelial growth factor
WB	western blot
wit	wishful thinking
Wnt	wingless-type MMTV integration site family
wt	wild-type
XIAP	X-chromosome linked inhibitor of apoptosis
XMAN1	integral inner nuclear membrane protein
YY1	yin and yang 1
ZEB	zinc finger homeodomain enhancer-binding protein

Units

°C	Degree celsius
µl	Microliter
A	Ampere
bp	Basepairs
Bq	Bequerel
Ci	Curie
Da	Dalton
g	Gram
h	hours
k	Kilo-
kDa	Kilodalton
l	Liter
M	Molar
min	Minutes
ml	Mililiter
mM	Millimolar
n	Nano-
OD600	Optical density at a wavelength of 600nm
p	Pico-
rpm	Rounds per minute
RT	Room temperature
sec	Seconds
U	Unit
V	Volt
Vol	Volume

A2 Sequences of oligonucleotides

Oligonucleotides for BRII

GST-1	GGA GGA **GGA TCC** GAC TAC AAG GAC GAC GAT GAC AAG GGT GGT GGT ATG GCT GAA CTT ATG ATG ATT TGG (starting after the kinase, GST-Fusion, BamHI site)
BRII-20 (5)	CAT TTA ACC GAG ATG AG (internal forward primer)
BRII-27	AGC AGC ACC ATG GCT GAA CTT ATG ATG ATT (NcoI-site 5`of the tail)
BRII-33	GGA GGA **GGA TCC** GAC TAC AAG GAC GAC GAT GAC AAG GGT GGT GGT AAC CTG TCA CAT (starting from SF, GST-Fusion, BamHI site)
BRII-34	AGC TGG **GAA TTC** AGG GAT GAC TTC CTC G (5`of BRII EcoRI-site)
BRII-35	AG CTC CGC GGC CGC TCA CAG ACA GTT CAT TCC TAT (3´of BRII NotI-site)
BRII-36	ACC AAT TTT TGG CAC ACG (internal reverse primer)
BRII-37	GAT GAT **GGA TCC** GGC CCA GGG ATG ACT TCC (5´of BRII BamHI site)
BRII-38	GTC GTC GAA TTC CTA ATC TAG ATC AAG (3´of TC1 EcoRI-site)
BRII-39	GGA GGA **GGA TCC** GAC TAC AAG GAC GAC GAT GAC AAG GGT GGT GGT TTG ACA GGA GAC CGT AAA CAA GGT (GST BamHI cytoplasmatic part)
BRII-40	GCA ACG GAA TTC TCA TCA TCA CTA CCT ACG TTC ATT CTG CAT AGC (3´of BRII-SF EcoRI-site)
BRII-41	AGA GAC CTA CTA GTT TGC C (internal forward primer)

S307A forw	CGT CTT GCT CAT **G**CT GTT ACT AGA GGA CTG GC
S307A rev	GC CAG TCC TCT AGT AAC AG**C** ATG AGC AAG ACG
S757A forw	CTT CCC AAG AGA CCT ACT **GC**T TTG CCT TTG AAC ACC
S757A rev	GGT GTT CAA AGG CAA A**GC** AGT AGG TCT CTT GGG AAG
S980A forw	G AAA ACT CCC TAT **G**CT CTT AAG CGG TGG CGC
S980A rev	GCG CCA CCG CTT AAG AG**C** ATA GGC AGT TTT C
S1011A forw	GT AAC AGG GCA GTT CAT **G**CC AAA TCC AGC AC
S1011A rev	GCT GGA TTT GG**C** ATG AAC TGC CCT GTT AC
T768A forw	C TTG AAA CAA GTC GAA **G**CT GGA GTT GCC AAG ATG
T768A rev	CAT CTT GGC AAC TCC AG**C** TTC GAC TTG TTT CAA G
Y314F forw	GA GGA CTG GCT T**T**T CTT CAC ACA GAA TTA CCA CG
Y325F forw	CCA CGA GGA GAT CAT T**T**T AAA CCT GCA ATT TCC CAT C
Y325F rev	G ATG GGA AAT TGC AGG TTT A**A**A ATG ATC TCC TCG TGG
K230R forw	CGT CCA GTT GCT GTA A**G**A GTG TTT TCC TTT GCA AAC

K230R rev	GTT TGC AAA GGA AAA CAC T**C**T TAC AGC AAC TGG ACG
S815A forw	GCA GGT AGA AAC CAC **GC**T GTT AAC TCC CAT GCT G
S815A rev	CAG CAT GGG AGT TAA CA**G C**GT GGT TTC TAC CTG C
S818A forw	AAA CCA CAG TGT TAA C**G**C CCA TGC TGC CAC
S818A rev	GTG GCA GCA TGG G**C**G TTA ACA CTG TGG TTT
Y825F forw	CT GCC ACA ACC CAA T**T**T GCC AAT GGG ACA G
Y825F rev	C TGT CCC ATT GGC A**A**A TTG GGT TGT GGC AG

Oligonucleotides for BRI

BRIa-Smad-ko forw	TTC ATA GCG GCA GAC **GC**T AAA G**C**T **G**CA GGT TCC TGG ACT CAG (mutation: IGT → AAA)
BRIa-12(5)	CAG TCA CAA AGT TCT GGT (internal forward primer)
BRIa-13(3)	TGG AGA AGT ATG GAT GGG C (internal reverse primer)
BRIa-14(5)	GCA CAC AGA AAT TTA TGG C (internal forward primer)
BRIa-15(5)	CAC AGG AGG GAT CGT GGA A (internal forward primer)

Other Oligonucleotides

pGEX-linker	GGC GAC CAT CCT CCA AAA TCG (sequencing primer)
Kit K623N	GCC ATG ACT GTC GCT GTA AA**T** ATG CTC AAG CCG AGT GCC

Oligonucleotides for real time PCR

mCbfa1-F23	AAC CAC AGA ACC ACA AGT GCG
mCbfa1-R141	AAA TGA CTC GGT TGG TCT CGG
mALP-F1080	AAT CGG AAC AAC CTG ACT GAC C
mALP-R1181	TCC TTC CAC CAG CAA GAA GAA
mId1-F	AGG TGA ACG TCC TGC TCT ACG A
mId1-R	CAG GAT CTC CAC CTT GCT CAC T.

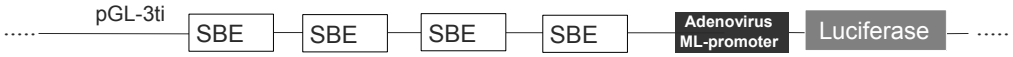
A2 Primers used

Here the primers used are listed. The functions of the primers are indicated in brackets. For oligonucleotides introducing a point mutation the function is not indicated.

A3 Reportergene constructs

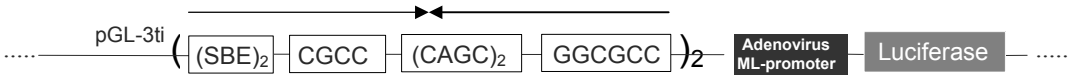
The following constructs were used for reportergene analyses. Whereas pSBE-luc is binding BMP and TGF-β Smads, the Id1, or the part from it, the BRE, binds only BMP Smads, Smad1/5/8. Here the constructs used are depicted.

pSBE-Luc



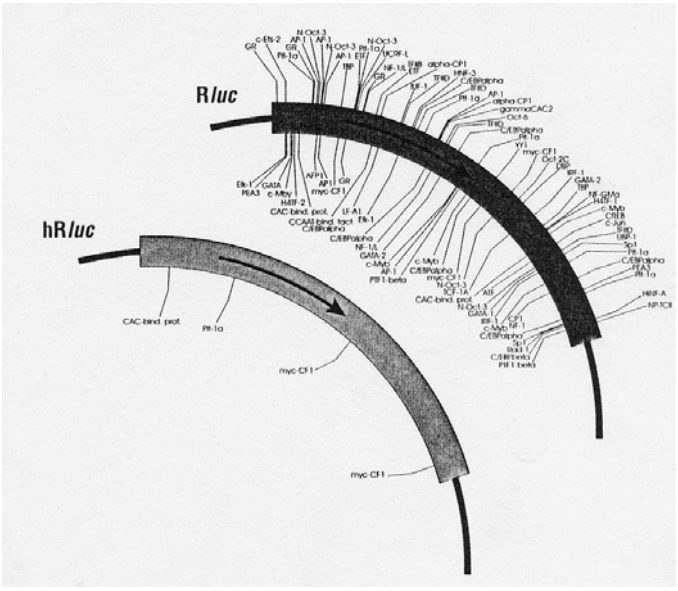
see Jonk et al (Jonk et al., 1998)

pBRE-Luc



see Lopez-Rovira for the Id1 promoter and Korchynskyi for Id1 and BRE (Lopez-Rovira et al., 2002), (Korchynskyi and ten Dijke, 2002)

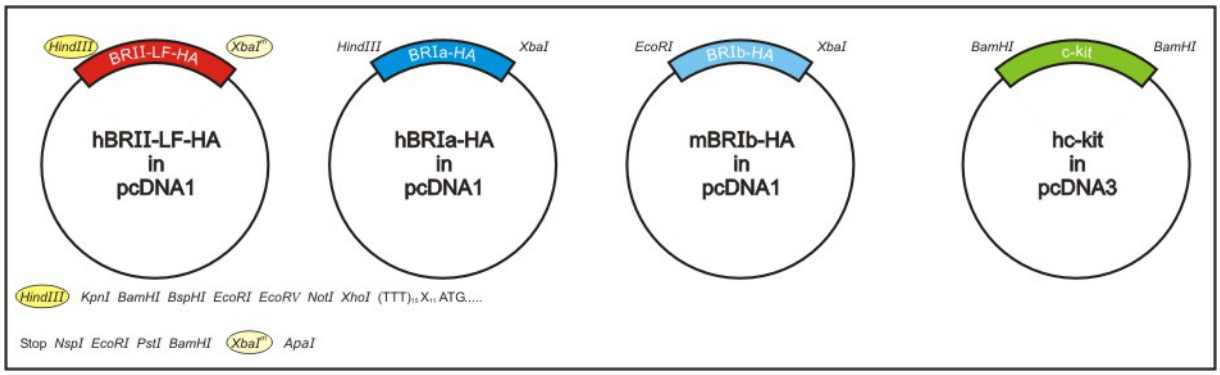
pRLTK



According to Promega manual

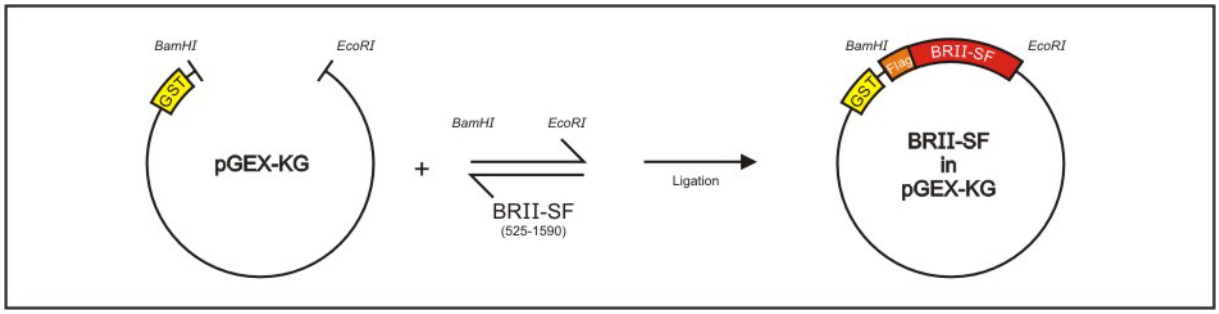
A4 Clone cards

For the further cloning experiments the four constructs depicted below were the initial ones.



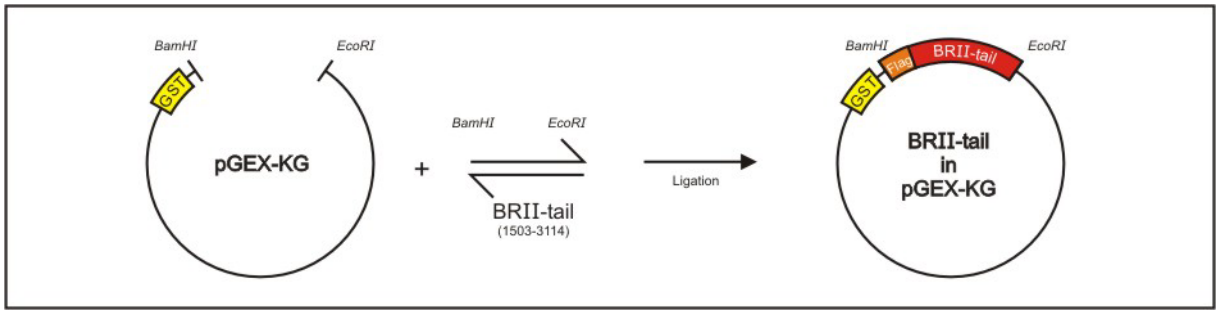
A4.1 Cloning of BRII parts in pGEX vectors to generate GST fusion proteins

A4.1.1 BRII-SF in pGEX-KG



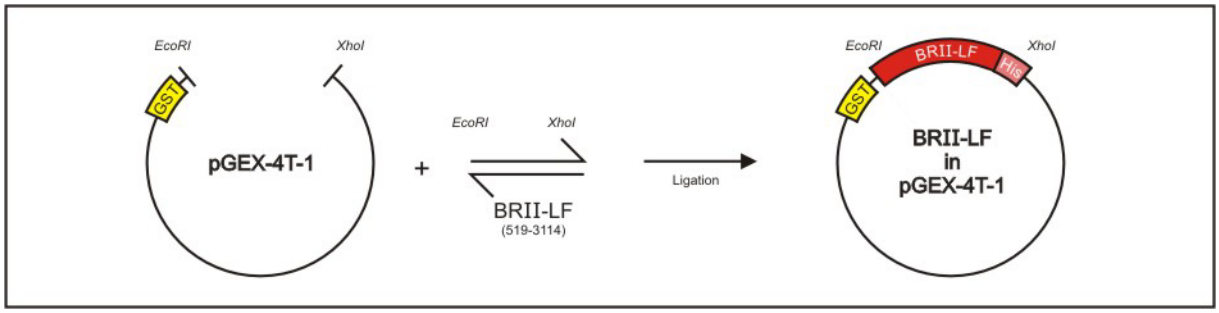
pGEX-KG was digested with BamHI and EcoRI, BRII-SF was amplified with PCR, introducing a Flag-tag and restriction sites. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.1.2 BRII-tail in pGEX-KG



pGEX-KG was digested with BamHI and EcoRI, BRII-tail was amplified with PCR, introducing a Flag-tag and the BamHI restriction site. The EcoRI site is within the amplified vector construct (pcDNA1) 3´of BRII. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

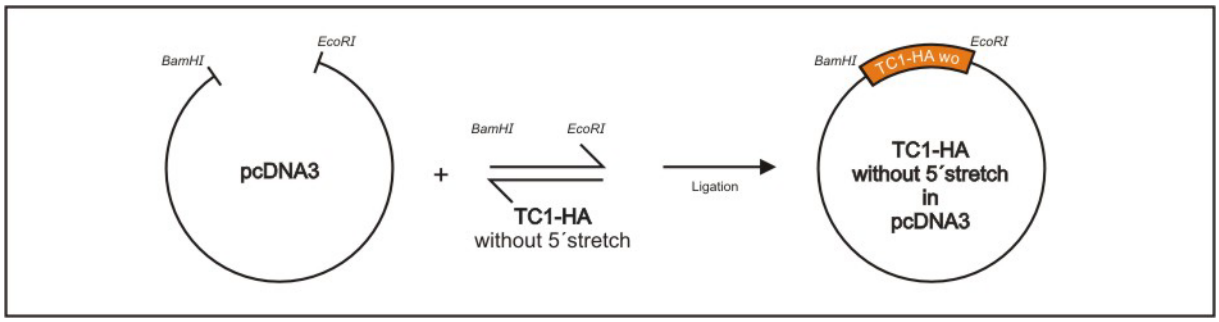
A4.1.3 BRII-LF in pGEX-4T-1



pGEX-4T-1 was digested with EcoRI and XhoI, BRII-LF was amplified with PCR, introducing a HIS-tag and restriction sites. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

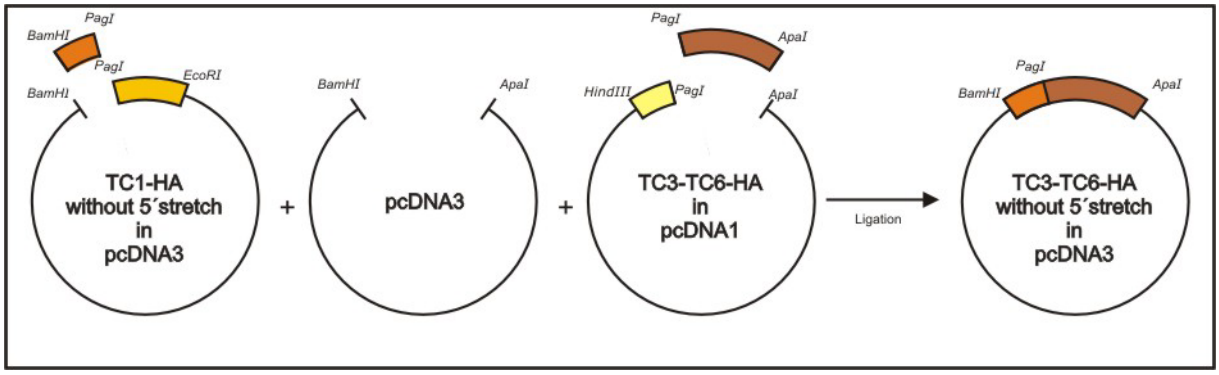
A4.2 Cloning of the BRII-TCs in pcDNA3

A4.2.1 BRII-TC1 in pcDNA3



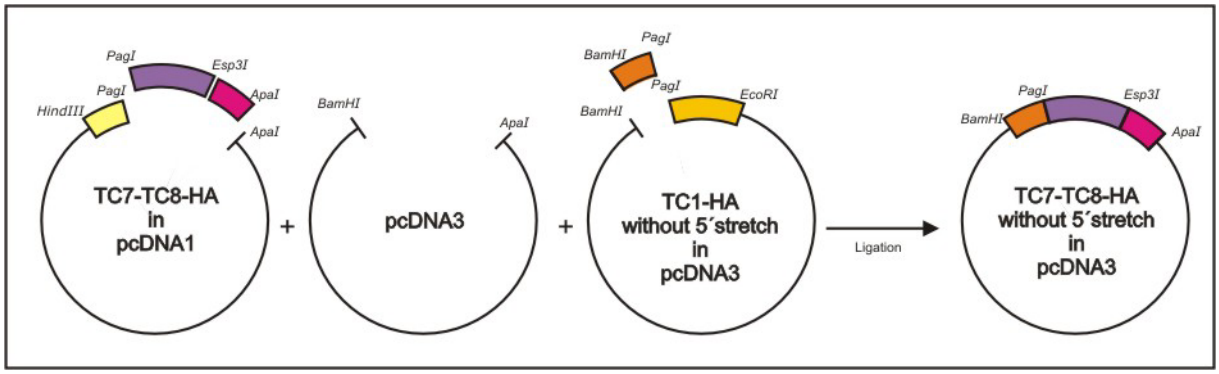
pcDNA3 was digested with BamHI and EcoRI, TC1-HA was amplified with PCR, removing the 5´stretch and introducing restriction sites. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.2.2 BRII-TC3-6 in pcDNA3



TC1-HA in pcDNA3 was digested with BamHI and PagI, pcDNA3 was digested with BamHI and ApaI and TC3-TC6-HA in pcDNA1 were digested with PagI and ApaI. Into the pcDNA3 backbone BamHI and ApaI the TC1-HA fragment without 5´stretch with BamHI/PagI and the 3´end of the respective TC 3-6 was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

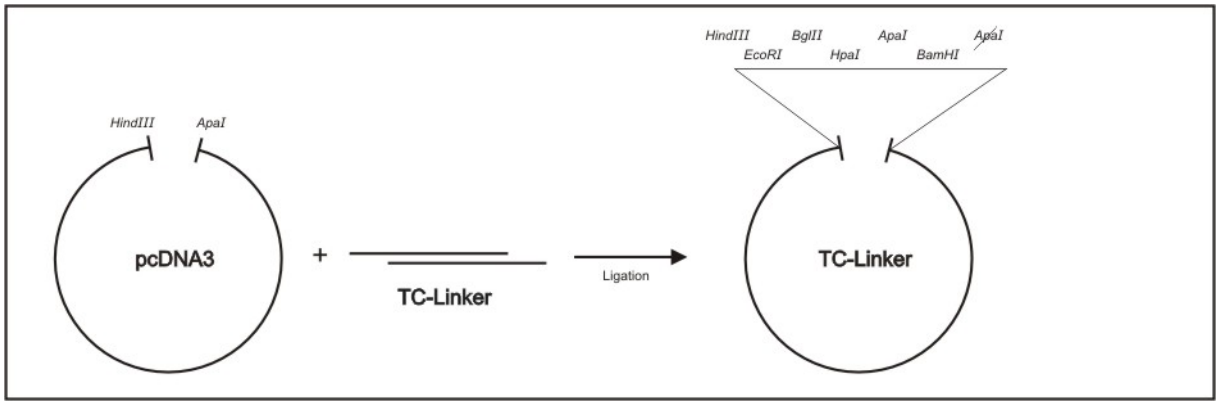
A4.2.3 BRII-TC7-8 in pcDNA3



TC1-HA in pcDNA3 was digested with BamHI and PagI, pcDNA3 was digested with BamHI and ApaI and TC7-TC8-HA in pcDNA1 were digested with PagI, Esp3I and ApaI. Into the pcDNA3 backbone BamHI and ApaI the TC1-HA fragment without 5´stretch with BamHI/PagI and the 3´end of the respective TC7-8 (PagI/Esp3I fragment and Esp3I/ApaI fragment) were inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

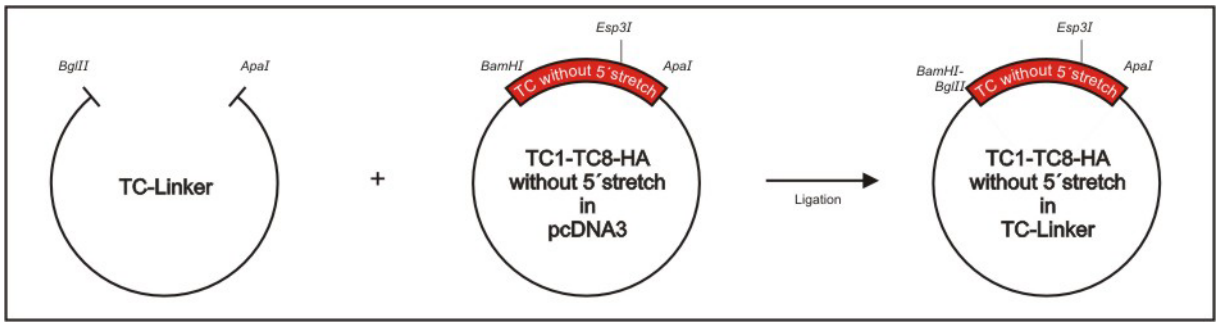
A4.3 Cloning of the BRII-TCs in pczCFG5 IEGN via TC-Linker

A4.3.1 Generation of TC-Linker



pcDNA3 was digested with HindIII and ApaI. The oligonucleotide TC1 was dimerized and ligated into pcDNA3, inserting new restriction sites and destroying ApaI at the 3´end of the multiple cloning sequence. Digestion with HpaI confirmed insertion. The construct was confirmed by sequencing.

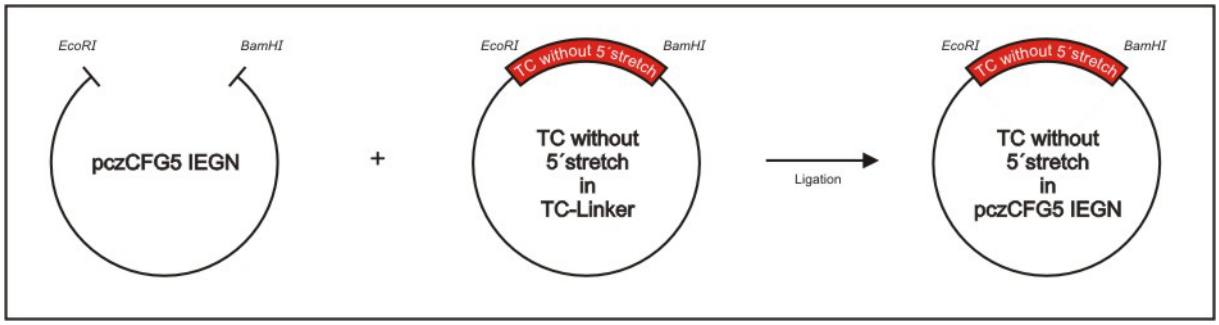
A4.3.2 TC1-TC8-HA/-myc in TC-Linker



TC-Linker was digested with BglII and ApaI, TC1-TC6-HA in pcDNA3 were digested with BamHI and ApaI. TC7-8-HA were digested with BamHI/Esp3I as well as Esp3I/ApaI. Into the TC-Linker backbone BglII and ApaI the TC1-6-HA fragment with BamHI/ApaI or both TC7/8 fragments were

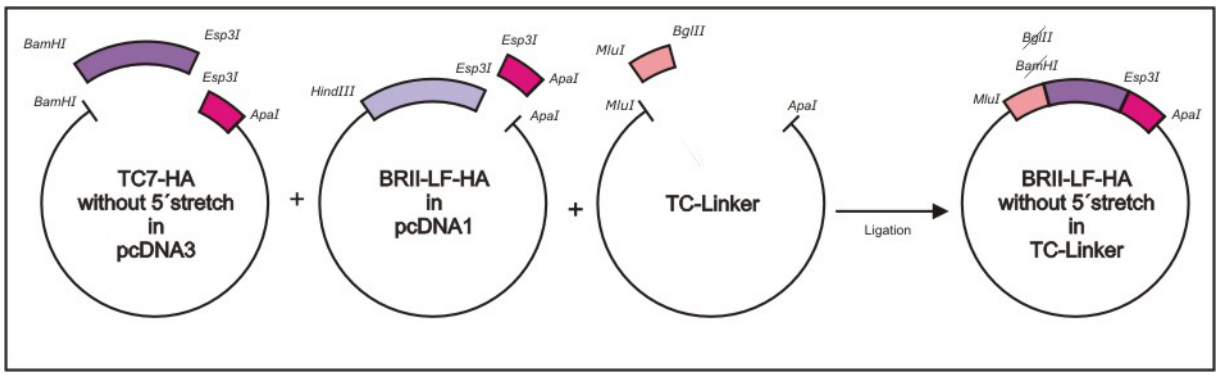
inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.3.3 TC1-TC8-HA in pczCFG5 IEGN



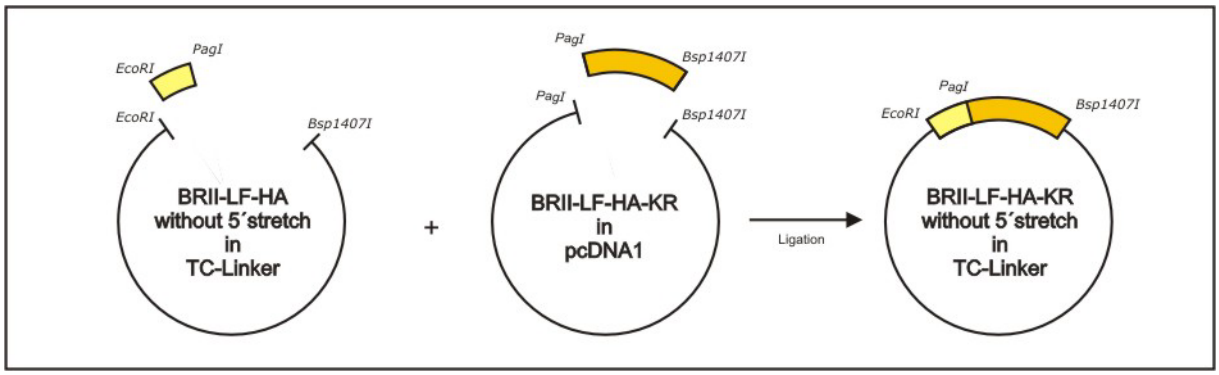
pczCFG5 IEGN was digested with EcoRI and BamHI, TC1-TC8-HA in TC-Linker were digested with EcoRI and BamHI as well. Into the pczCFG5 IEGN backbone EcoRI and BamHII the TC1-8-HA fragment was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.3.4 BRII-LF-HA in TC-Linker



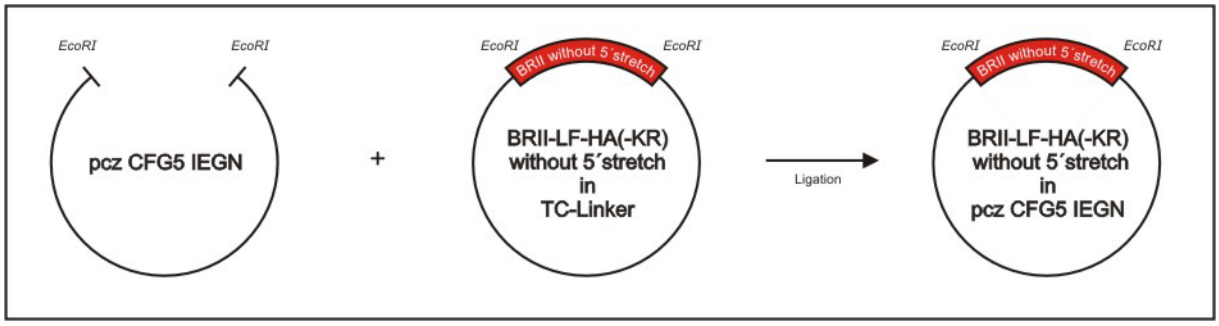
TC-Linker was digested with MluI and ApaI, TC7-HA in pcDNA3 was digested with BamHI and Esp3I. BRII-LF-HA in pcDNA1 with 5´stretch was digested with Esp3I and ApaI. Into the TC-Linker backbone digested with MluI and ApaI, the TC7-HA fragment with BamHI/Esp3I and the Esp3I/ApaI fragment were inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.3.5 BRII-LF-KR-HA in TC-Linker /pcDNA3



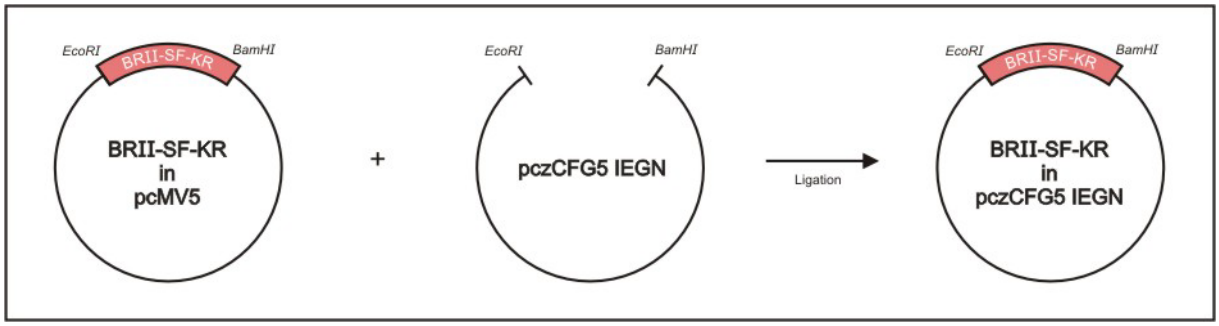
BRII-LF-HA in TC-Linker was digested with EcoRI and Bsp1470I. BRII-LF-HA-KR in pcDNA1 was digested with PagI and Bsp1470I. The BRII-LF-HA-KR fragment PagI/Bsp1470I as backbone was ligated with the BRII-fragment in TC-Linker EcoRI/Bsp1470 as well as the EcoRI/PagI fragment. The product was tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.3.6 BRII-LF (-KR)-HA in pczCFG5 IEGN



pczCFG5 IEGN was digested with EcoRI. BRII-LF-HA or BRII-LF-HA-KR in TC-Linker were digested with EcoRI as well. Into the pczCFG5 IEGN backbone EcoRI the BRII fragment was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

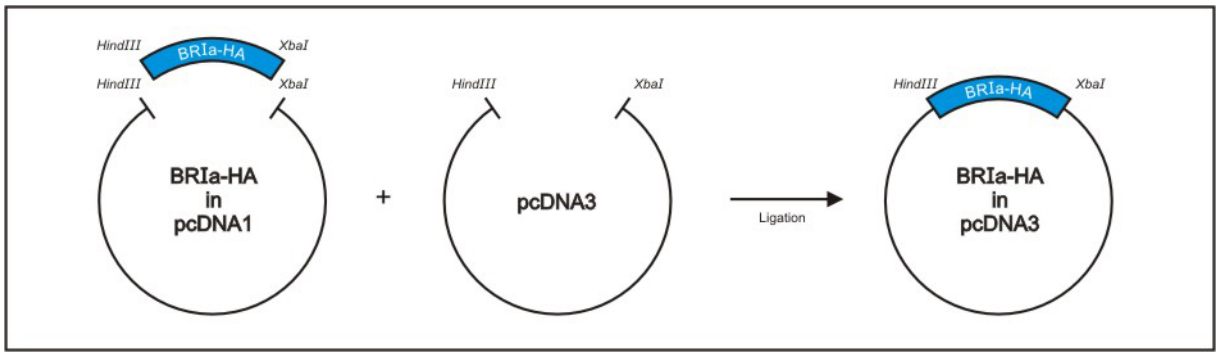
A4.3.7 BRII-SF-KR-HIS in pczCFG5 IEGN



pczCFG5 IEGN was digested with EcoRI and BamHI, BRII-SF-KR in pcMV5 was digested with EcoRI and BamHI as well. Into the pczCFG5 IEGN backbone digested EcoRI and BamHII the BRII-SF-KR fragment was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

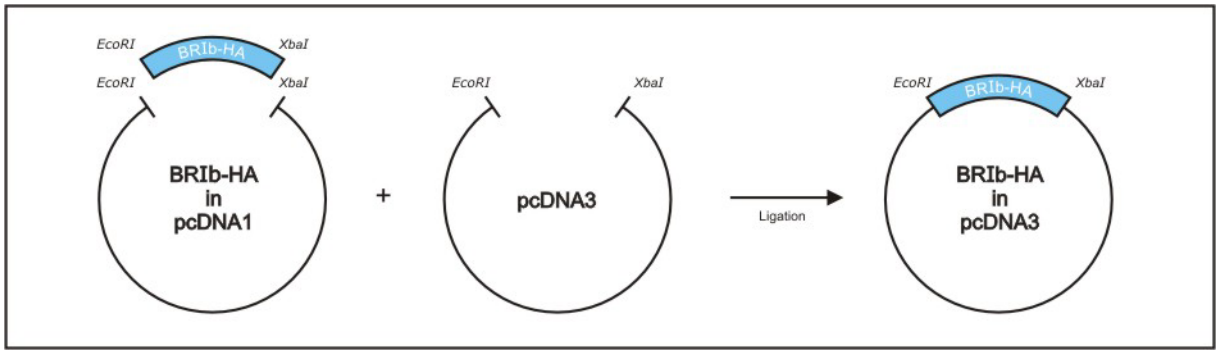
A4.4 Cloning of the BRI in pcDNA3

A4.4.1 BRIa-HA/-myc in pcDNA3



pcDNA3 was digested with HindIII and Xbal, BRIa-HA in pcDNA1 was digested with HindIII and Xbal as well. Into the pcDNA3 backbone digested with HindIII and Xbal the BRIa-HA fragment was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

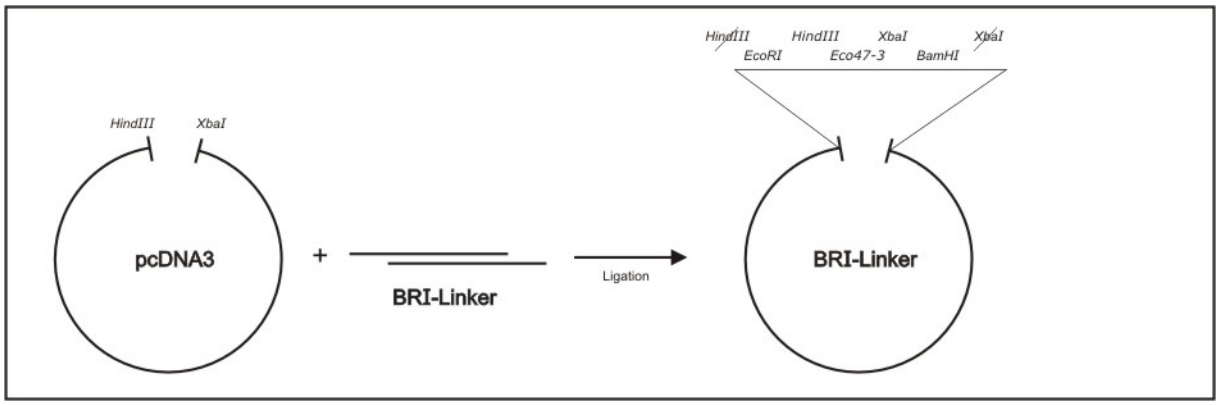
A4.4.2 BRIb in pcDNA3



pcDNA3 was digested with EcoRI and Xbal, BRIb-HA in pcDNA1 was digested with EcoRI and Xbal as well. Into the pcDNA3 backbone digested with EcoRI and Xbal the BRIb-HA fragment was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

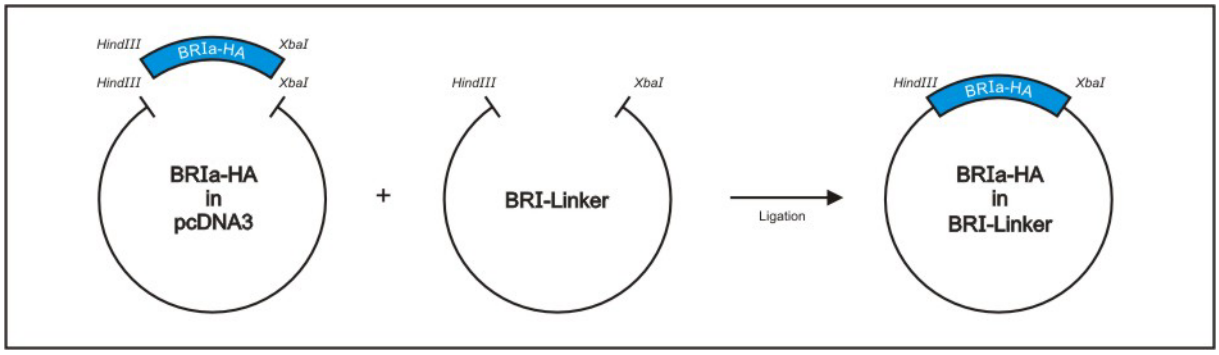
A4.5 Cloning of the BRI in pczCFG5 IEGN via BRI-Linker

A4.5.1 Generation of BRI-Linker



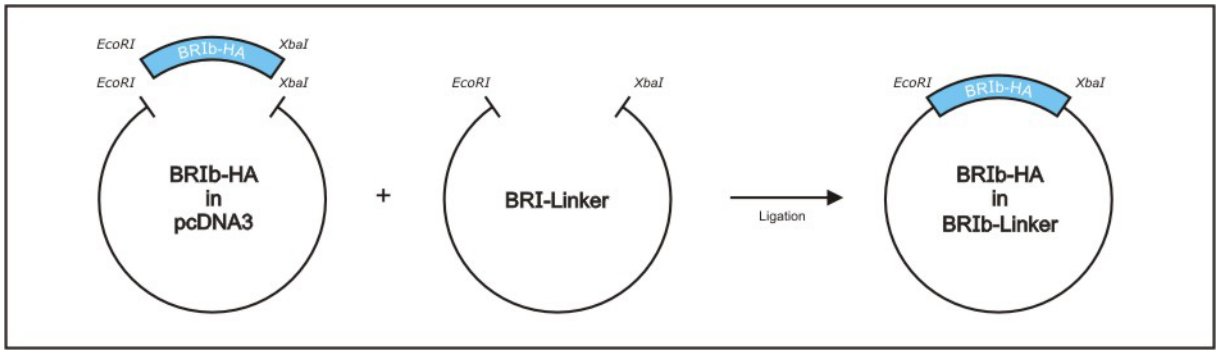
pcDNA3 was digested with HindIII and Xbal. The oligonucleotide BRI-Linker was dimerized and ligated into pcDNA3, inserting new restriction sites and destroying the HIndIII site at the5´end as well as the Xbal aite at the 3´end of the multiple cloning sequence. Digestion with Eco47-3 confirmed insertion. The construct was confirmed by sequencing.

A4.5.2 BRIa-HA/-myc in BRI-Linker



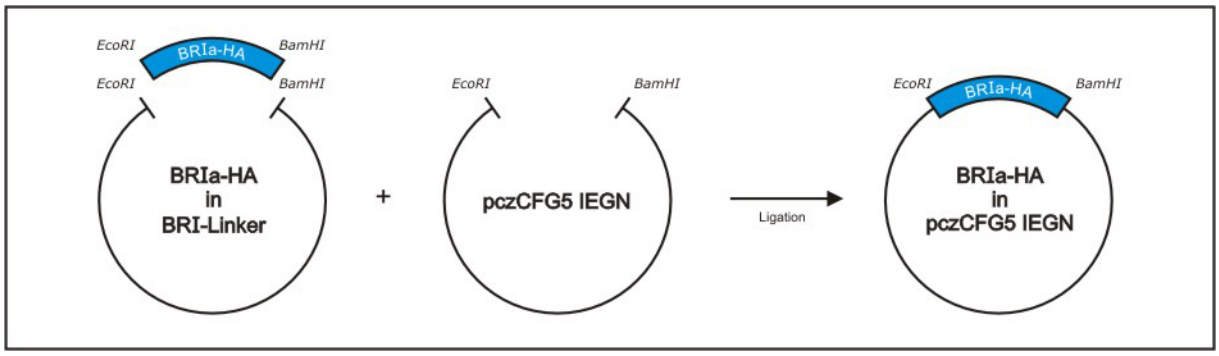
BRI-Linker was digested with HindIII and Xbal, BRIa-HA in pcDNA3 was digested with HindIII and Xbal. Into the BRI-Linker backbone HindIII and Xbal the BRIa-HA fragment was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.5.3 BRIb-HA in BRI-Linker



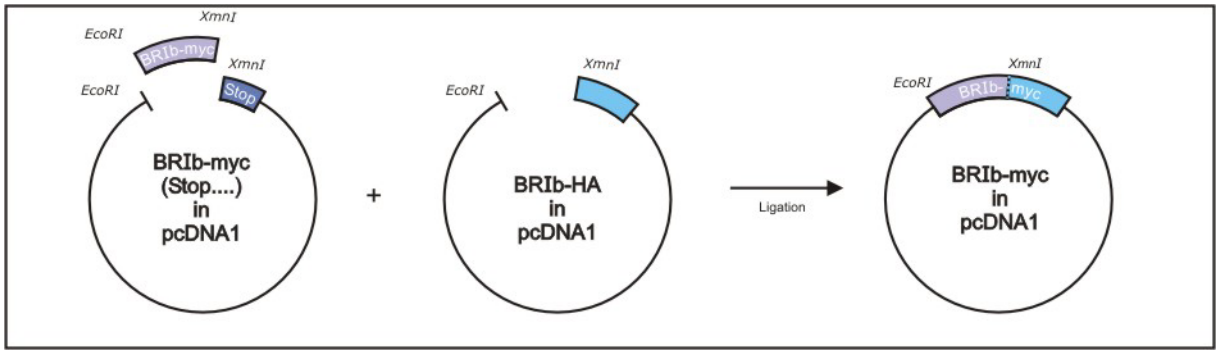
BRI-Linker was digested with EcoRI and XbaI, BRIb-HA in pcDNA3 was digested with EcoRI and XbaI. Into the BRI-Linker backbone EcoRI and XbaI the BRIb-HA fragment was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.5.4 BRIa/b-HA in pczCFG5 IEGN



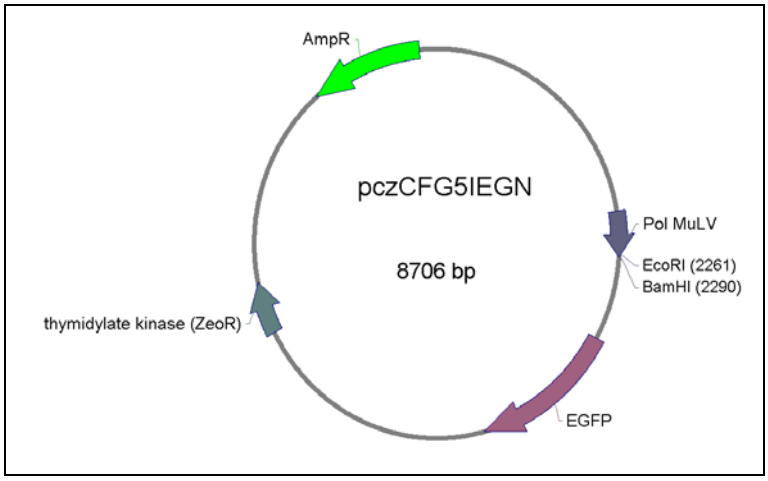
The same strategy was followed for BRIa-HA and BRIb-HA
pczCFG5 IEGN was digested with EcoRI and BamHI, BRI-HA in BRI-Linker were digested with EcoRI and BamHI as well. Into the pczCFG5 IEGN backbone EcoRI and BamHII the BRI-HA fragment was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.6 Repairing of the BRIb-myc in pcDNA1



BRIb-myc pcDNA1, having an internal Stop-Codon, was digested with EcoRI and XmnI, BRIb-HA in pcDNA1 was digested with EcoRI and XmnI Into the BRIb-pcDNA1 backbone EcoRI and XmnI the 5´fragment of BRIb-myc was inserted. The digested products were ligated and tested with digestion for correct insertion. The construct was confirmed by sequencing.

A4.7 Vector card of pczCFG5 IEGN



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Für das Entstehen und Gelingen dieser Arbeit war die wissenschaftliche sowie persönliche Unterstützung zahlreicher Personen von Bedeutung. Daher gilt mein Dank allen, die mir diese Hilfe entgegen gebracht haben:

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Lebenslauf

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2000 - 2002	Stipendium des Boehringer Ingelheim Fonds für biomedizinische Grundlagenforschung
05.2002	Second European Conference on Bone Morphogenetic Proteins, Zagreb, Preis für “outstanding paper presentation”

Publikationen

Artikel

Nohe A.*, **Hassel S.***, Ehrlich M., Neubauer F., Sebald W., Henis YI., Knaus P.
“The mode of BMP receptor oligomerization determines different BMP-2 signaling pathways”,
Journal of Biological Chemistry, 277(7): 5330-8

Pohl F., **Hassel S**., Nohe A., Flentje M., Knaus P., Sebald W., Koelbl O.
“Radiation-induced suppression of the Bmp2 signal transduction pathway in the pluripotent mesenchymal cell line C2C12: an in vitro model for prevention of heterotopic ossification by radiotherapy”,
Radiation Research, 159(3): 345-50

Hassel S., Schmitt S., Hartung A., Roth M., Nohe A., Petersen N., Ehrlich M., Henis YI., Sebald W., Knaus P.
”Initiation of Smad-dependent and Smad-independent signaling via distinct BMP-receptor complexes”,
Journal of Bone and Joint Surgery Am., 2003, 85-A Suppl 3: 44-51

Hassel S., Eichner A., Yakymovych M., Hellman U., Knaus P., Souchelnytskyi S.
“Proteins associated with type II bone morphogenetic protein receptor (BMPR-II) and identified by two-dimensional gel electrophoresis and mass spectrometry”,
Proteomics (2004) 4(5): 1346-1358

Hassel S., Yakymovych M., Hellman U., Rönnstrand L., Knaus P., Souchelnytskyi S.
“Interaction of serine/threonine kinase bone morphogenetic protein type II receptor with tyrosine kinase stem cell factor receptor”,
Submitted

Hassel S., Yakymovych M., Wernstedt C., Mueller T., Sebald W., ., Souchelnytskyi S., Knaus P.
“Mechanism of activation and phosphorylation of the BMP type II receptor”,
Submitted

* both authors contributed equally

Kongressteilnahme – Vorträge

Second European Conference on Bone Morphogenetic Proteins**,** Zagreb, 2002
Hassel S., Nohe A., Ehrlich M., Neubauer F., Sebald W., Henis YI. and Knaus P.
The mode of BMP receptor oligomerization determines different BMP-2 signalling pathways
Awarded for “outstanding paper presentation”

TGFß meeting, Ludwig Institute for Cancer research, Uppsala, Sweden, 2002
Hassel S., Eichner A., Yakymovych M., Hellman U., Knaus P. and Souchelnytskyi S.
Identification of BMP receptor II associated proteins

TGFß meeting, Ludwig Institute for Cancer research, Uppsala, Sweden, 2003
Hassel S., Wernstedt C., Souchelnytskyi S. and Knaus P.
BMP type II receptor phosphorylation – mechanisms and sites

TGFß meeting, Ludwig Institute for Cancer research, Uppsala, Sweden, 2004
Hassel S., Eichner A., Yakymovych M., Hellman U., Souchelnytskyi S., Knaus P.
BMP receptor interactom

Kongressteilnahme - Poster und Abstracts

Osteologie 2000, Würzburg
Nohe A., **Hassel S.**, Neubauer F., Sebald W. und Knaus P.
Ligandenunabhängige Heterooligomere der BMP-Rezeptoren sind Vorraussetzung für Aktivierung des Smad-Pathways

Gordon Research Conference on „Peptide Growth Factors", New Hampshire, USA, 2000
Knaus, P., Nohe, A., **Hassel S.**, Kirsch, T., Henis YI., Dreyer, M. and Sebald W.
BMP receptors: crystal structure, mode of oligomerization and initiation of different signalling pathways

American Society of Therapeutic Radiology and Onkology (ASTRO) Conference, San Francisco, USA, 2001
Pohl, F., Flentje, M., **Hassel S**., Knaus P., Sebald W. and Kölbl, O.
The mechanism of radiotherapy responsible for the prevention of heterotopic ossification: a cellular and subcellular analysis of an in vitro model using the pluripotent mesenchymal C2C12 cell line from mouse

Deutsche Gesellschaft für Radiologie und Onkologie DEGRO, Hamburg, 2001
Pohl, F., Flentje, M., **Hassel S**., Knaus P., Sebald W. and Kölbl, O.
Heterotope Ossifikation im Zellmodell: Bestrahlungswirkung auf Ebene der Signaltransduktion bei der pluripotenten mesenchymalen C2C12 Mauszellinie

Second European Conference on Bone Morphogenetic Proteins**,** Zagreb, 2002
Hassel S., Nohe A., Ehrlich M., Neubauer F., Sebald W., Henis YI. and Knaus P.
The mode of BMP receptor oligomerization determines different BMP-2 signalling pathways

Gordon Research Conference on „Peptide Growth Factors", New Hampshire, USA, 2002
Hassel S., Hartung A., Schmitt S., Nohe A., Souchelnytskyi S., Henis YI., Sebald W. and Knaus P.
BMP receptor oligomerization determines the initiation of different signalling cascades

4th international Conference on Bone Morphogenetic Proteins, Sacramento, USA, 2002
Hassel S., Hartung A., Schmitt S., Nohe A., Henis YI., Sebald W. and Knaus P.
Initiation of Smad-dependent and Smad-independent signalling via distinct BMP-receptor complexes

FASEB Summer research conference, The TGF beta superfamily: signaling and development, Tuscon, USA, 2003
Hassel S., Hartung A., Schwappacher R., Schmitt S., Nohe A., Souchelnytskyi S., Henis YI., Sebald W. and Knaus P.
BMP receptor oligomerization determines the initiation of different signalling cascades

Deutsche Gesellschaft für Zellbiologie Meeting, Berlin, 2004
Hartung, A., **Hassel, S**., Henis, Y.I., Knaus, P.
Initiation of Smad-dependent and Smad-independent signalling via distinct BMP-receptor complexes and plasmamembrane domains

Deutsche Gesellschaft für Zellbiologie Meeting, Berlin, 2004
Schwappacher R., **Hassel S.**, Roth M., Scholz S., Souchelnytskyi S., Knaus P.
Characterisation of BMP Type II Receptor associated proteins

Strategies in Tissue Engineering, Würzburg, 2004
Hartung, A., **Hassel, S.**, Henis, Y.I., Knaus, P.
Initiation of Smad-dependent and Smad-independent signalling via distinct BMP-receptor complexes and plasmamembrane domains

Strategies in Tissue Engeneering, Wuerzburg, 2004
Schwappacher R., **Hassel S.**, Roth M., Scholz S., Souchelnytskyi S., Knaus P.
Characterisation of BMP Type II Receptor associated protein

FASEB Summer research conference, Protein Lipidation, Signaling and membrane domains, Tuscon, USA, 2004
Hartung, A., **Hassel, S.**, Noskov, A., Harms, G.S., Knaus, P.
Localization of BMP receptors in specific plasma membrane microdomains

Erklärung

Erklärungen gemäß §4 Absatz 3 der Promotionsordnung der Fakultät für Biologie der Bayerischen Universität Würzburg vom 15. März 1999.

1. Ich erkläre ehrenwörtlich, die vorliegende Dissertation selbständig angefertigt zu haben und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt zu haben.

2. Ich erkläre desweiteren, daß die vorliegende Arbeit weder in gleicher noch ähnlicher form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

3. Ich erkläre weiterhin, daß ich früher keine akademischen Grade erworben habe oder zu erwerben versucht habe.

Würzburg, den 28 10.2004 Sylke Haßel