Establishment of Hey-triple-KO-ES cells and characterisation of Bre, a Hey binding partner

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1. Summary

1.1 Summary

Hey1, Hey2 and HeyL are downstream effectors of the Notch signalling pathway. Hey genes play decisive roles during embryonic development for example in cardiovascular development. However, the precise transcriptional programmes and genes, which are affected by each single Hey gene, are still poorly understood. One drawback for the analysis of Hey1, Hey2 or HeyL single gene function is that these genes are co-expressed in many tissues and share a high degree of functional redundancy. Thus, it was necessary to establish a system, which is either devoid of Hey expression, or just comprises one single Hey gene family member.

For this, $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$- as well as Hey-triple- knock out (KO)-ES cells (embryonic stem cells) were generated in this work, because ES cells and their differentiation as EBs (embryoid bodies) represent a valuable tool for the *in vitro* analysis of embryonic developmental processes. After the establishment of $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$- and Hey-triple-KO-ESC, it could be seen by ALP staining and pluripotency marker expression that loss of Hey expression did not affect ES cell pluripotency features. Thus, these ES cells represent *bona fide* ES cells and could be further used for the differentiation as EBs. Here, differences in gene expression between $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$- and Hey-triple- KO-ESC (after the loss of Hey1) E3-ligase could be observed in realtime-RT-PCR analysis for the endodermal marker AFP as well as for neural and myogenic markers in d10 EBs. However, the establishment of inducible Hey1, Hey2 or HeyL ES cell lines will be essential to confirm these findings and to search for novel Hey target genes.

To get further insight into the mode of Hey action, the analysis of Hey interaction partners is necessary. One such binding partner, the Bre protein, has previously been found in a yeast-two-hybrid screen. Bre has been described to be a member of two distinct complexes (i.e. the nuclear BRCA1-A complex with a function in DNA damage response and the cytoplasmic BRISC complex), to directly interact with the TNF-receptor and Fas and to interfere with apoptotic signalling.

The Hey-Bre interaction could be further corroborated in this work; yet, it was not possible to narrow down the interaction site of Bre with Hey1. It rather seems that non-overlapping parts of the Bre protein may bind to Hey. This interaction may be direct– pointing to more than one interaction site inside the Bre protein – or via a common binding partner such as the endogenous Bre protein itself. Besides the interaction studies, functional assays were

performed for a more detailed characterisation of Hey1 and Bre interaction. Here, it could be shown that Hey1 over-expression did not have any influence on Bre sub-cellular localisation. Interestingly, it could be demonstrated that Bre positively interfered with Hey1 repressive function in luciferase assays at three of four promoters analysed. Moreover, interaction with Bre seems to lead to a stabilisation of Hey1. As Bre has been described to modulate the E3-ligase activity intrinsic to the BRCC complex it was analysed whether Bre over-expression results in an ubiquitination of Hey1. Yet, this could not be observed in the present work. Furthermore, an interaction of Bre with ubiquitinated proteins could not be demonstrated in an ubiquitin binding assay.

To obtain a better insight into Bre function, Bre LacZ gene trap-ES cells and animals were generated. However, realtime-RT-analyses revealed that these cells and mice did not show a loss of Bre expression on mRNA level indicating that insertion mutagenesis did not occur as expected. However, embryos derived from these mice could nevertheless be used for the detection of tissues with Bre expression by β-galactosidase staining. Bre deficiency on mRNA levels was only achieved after the deletion of the floxed exon 3 resulting in the generation of Bre del-mice. Bre del-mice were fertile and without any obvious phenotype and they were used for the generation of Bre del- and wt-MEFs (murine embryonic fibroblasts). Characterisation of these cells showed that proliferation was not affected after loss of Bre (neither under normal nor under stress conditions). However, loss of Bre notably resulted in a reduction in the BRCA1 DNA damage response, in a slightly increased sensitivity towards apoptosis induction by FasL treatment and in an increase in the K63-poly-ubiquitin content in Bre del-cytoplasmic fractions, probably linked to a change in the BRISC de-ubiquitinase activity. Even though these results have the same tendencies as observed in former studies, the effects in the present work are less striking. Further studies as well as intercrossing of Bre del- to Hey KO-animals will be necessary to further understand the functional relevance of Hey and Bre interaction.

1.2 Zusammenfassung

Hey1, Hey2 und HeyL sind Zielgene des Notch Signalwegs und spielen eine entscheidende Rolle während der Embryonalentwicklung, z. B. bei der Bildung des kardiovaskulären Systems. Die genauen Effekte eines jeden einzelnen Hey Gens auf Transkriptionsprogramme und einzelne Gene sind allerdings noch relativ unbekannt. Einer der Gründe hierfür liegt vermutlich in der Koexpression von Hey-Proteinen in vielen Geweben bzw. in der daraus resultierenden funktionellen Redundanz. Daher sollte in dieser Arbeit ein System entwickelt

werden, in dem entweder keines oder jeweils nur eines der Hey-Gene intakt ist. Hierzu wurden $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$ und Hey-triple-knock out (KO) ES-Zellen (embryonale Stammzellen) etabliert. ES-Zellen stellen ein hervorragendes Modellsystem für die Embryonalentwicklung dar, weil ihre *in vitro* Differenzierung als sog. „embryoid bodies" (EBs) embryonale Entwicklungsprozesse widerspiegelt. Der Verlust der Hey-Genexpression hatte keinen Einfluss auf den Stammzellcharakter der etablierten Zellen, da sowohl die generierten Hey-triple-KO- als auch die $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$-ES-Zellen eine positive ALP-Färbung sowie eine hohe Expression von Pluripotenzmarkern zeigten. Daher konnten die Zellen im Folgenden als EBs differenziert und auf Genexpressionsunterschiede während der Differenzierung untersucht werden. Zwischen $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$- (mit intakter Hey1-Expression) und Hey-triple- KO- ES Zellen konnten an EB Tag 10 mittels realtime-RT-PCR Unterschiede in der Genexpression für den endodermalen Marker AFP, sowie für neurale und myogene Marker festgestellt werden. Um diese Ergebnisse zu bestätigen, aber auch, um neue Hey Zielgene ausfindig machen zu können, ist jedoch die Etablierung induzierbarer ES-Zellen (für Hey1, Hey2 bzw. HeyL) notwendig.

Um einen tieferen Einblick in die Funktionsweise der Hey-Gene gewinnen zu können ist die Untersuchung von Hey Interaktionspartnern wichtig. Das Bre-Protein ist ein solcher Bindepartner und wurde zuvor in einem Yeast-two-hybrid Assay gefunden. Bre ist in zwei verschiedenen Komplexen beschrieben worden: dem nukleären BRCA1-A-Komplex, der eine Rolle bei der Detektion von DNA-Schäden spielt und dem cytoplasmatischen BRISC-Komplex. Es ist außerdem bekannt, dass Bre direkt mit dem TNF-Rezeptor und mit Fas interagiert und die apoptotische Antwort in der Zelle beeinflusst.

Die Interaktion zwischen Bre und Hey1 konnte in dieser Arbeit zunächst bestätigt werden; in weiteren Ko-immunpräzipitations-Experimenten war es aber nicht möglich, den Bereich des Bre-Proteins zu bestimmen, der die Interaktion mit Hey1 vermittelt, da verschiedene nicht überlappende Bereiche des Bre-Proteins eine Interaktion mit Hey1 zeigten. Ob es sich hierbei um direkte Interaktionen handelte und Bre somit mehrere Bindestellen für Hey1 aufweist oder ob die Interaktion indirekt über einen gemeinsamen Bindepartner wie z.B. das endogene Bre-Protein selbst vermittelt wird, ist noch nicht geklärt.

Für eine weitere Charakterisierung der Interaktion zwischen den beiden Proteinen wurden funktionelle Versuche durchgeführt. Hierbei konnte gezeigt werden, dass die Überexpression von Hey1 keinen Einfluss auf die subzelluläre Lokalisation des Bre Proteins hat. Mit Hilfe von Luziferase Assays konnte aber interessanterweise nachgewiesen werden, dass Bre bei drei von vier untersuchten Promotern positiv auf die Repression durch Hey1 einwirkte.

Außerdem scheint die Überexpression von Bre möglicherweise eine Stabilisierung des Hey1-Proteins zu bewirken. Da Bre eine Verstärkung der E3-Ligasefunktion des BRCC-Komplexes zugeschrieben wird, wurde außerdem untersucht, ob die Überexpression von Bre zu einer Ubiquitinylierung von Hey1 führt. Dies konnte allerdings nicht festgestellt werden. Desweiteren konnte in einem Ubiquitin-Bindeassay keine Interaktion von Bre mit anderen ubiquitinylierten Proteinen gezeigt werden.

Die Etablierung von Bre LacZ gene trap-ES Zellen und -Mäusen sollte weiteren Aufschluss über mögliche Funktionen des Bre-Proteins geben; allerdings konnte in diesen Mäusen und Zellen mittels realtime-RT-PCR kein Verlust der Bre-Expression auf mRNA-Ebene nachgewiesen werden; die Insertionsmutagenese ist somit nicht wie erwartet erfolgt. Dennoch konnten in Bre LacZ-Embryonen Gewebe mit Bre-Expression mittels β-Galaktosidase-Färbung dargestellt werden. Der Verlust der Bre-Expression auf mRNA-Ebene wurde schließlich über Deletion des gefloxten Exon 3 erreicht: Die erhaltenen Bre del- Mäuse waren fertil und zeigten keinen auffälligen Phänotyp. Zur weiteren Charakterisierung wurden Bre del-MEFs (murine embryonale Fibroblasten) isoliert und mit Wildtyp-Zellen verglichen. In der Proliferationsrate wiesen die deletierten Zellen weder unter Standard- noch unter Stressbedingungen eine Veränderung im Vergleich zum Wildtyp auf; der Verlust von Bre führte jedoch bemerkenswerterweise zu einer verminderten BRCA1-Antwort nach Induktion von DNA-Schäden. Bre del-MEFs zeigen außerdem eine leicht erhöhte Sensitivität gegenüber einer Apoptoseinduktion mit FasL. Darüber hinaus hatten Zytoplasma-Fraktionen von Bre del-Zellen einen höheren Anteil an Proteinen mit K63 poly-Ubiquitin-Modifikation; dies kann wahrscheinlich auf eine veränderte BRISC-Deubiquitinase-Aktivität zurückgeführt werden. Diese Ergebnisse konnten zwar tendenziell Resultate früherer Studien bestätigen, allerdings waren die in dieser Arbeit beobachteten Effekte im Vergleich weniger schwerwiegend.

Weitere Versuche sowie die Etablierung von Mäusen mit Bre- und Hey-KO werden notwendig sein, um weitere Einblicke in die Bedeutung der Interaktion von Hey1 und Bre erlangen zu können.

2. Introduction

2.1 The canonical Notch signalling pathway

Notch signalling has first been analysed in Drosophila wing and neural development. Additional studies in C. elegans and in mammals revealed that Notch signalling components are highly conserved: A membrane bound ligand of the DSL family (Drosophila: Delta and Serrate, C. elegans: Lag2, mammals: Dll1, 4 and Jagged1, 2) interacts with a transmembrane Notch receptor. Only one Notch receptor exists in Drosophila, but there are two receptors in C. elegans (Lin12, glp1) and four in mammals (Notch1-4). The Notch receptor is cleaved in two steps upon interaction with the ligand via its EGF repeats: first, an ADAM metallo-protease and second a γ-secretase is active on the Notch substrate. This leads to the generation of the NICD (Notch intracellular domain). The NICD translocates to the nucleus, where it leads to the release of a repressive complex (consisting of N-CoR, SHARP, CtBP) from Drosophila Su(H), C. elegans Lag1 or mammalian RBP-Jκ/CBF1, respectively. In turn, Notch recruits co-activators such as Mastermind (MAML in mammals) or p300 (CBP) to RBPJκ. This ternary complex then leads to the induction of the Hes (Hairy and enhancer of split in Drosophila) and Hey genes amongst others. As the Notch signalling pathway does not rely on any second messengers, it is seemingly a quite simple and straight-forward process.

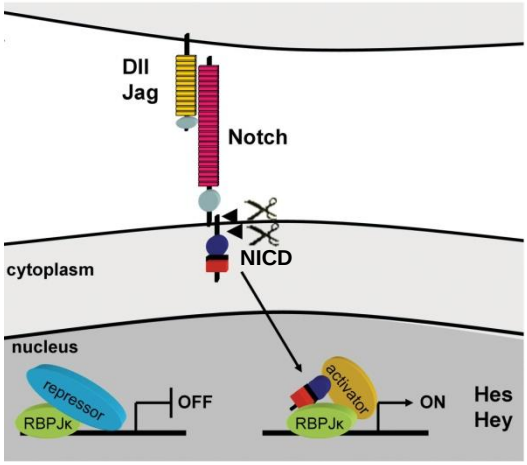


Fig 1. The canonical Notch signalling pathway (modified after Fischer and Gessler, 2007)

However, there is a strict regulation of ligand and receptor via endocytosis and by ubiquitination, so that a correct and only once activation and termination of the pathway is ensured. Furthermore, glycosylation and fucosylation of the receptor and participation of components such as Numb also add on Notch signalling pathway modification (Greenwald 1998; Bray 2006; Fischer and Gessler 2007; Kopan and Ilagan 2009). For a schematic overview of Notch signalling see Fig.1.

Besides the conservation of the Notch signalling components, a conservation of the processes regulated by Notch signalling exists, too. It could be demonstrated in early mouse embryos that loss of Notch1 and even more severe of RBPJκ result in a similar outcome as observed

in the fly: the deregulation of Notch downstream effectors leads to an increase of Dll1 ligand and of proneural Mash1 gene expression (de la Pompa *et al.* 1997).

2.2 Principle of lateral inhibition and lateral induction

The process in which one cell with activated Notch signalling leads to the concomitant down-regulation of ligand on its surface, thereby preventing Notch activation on its neighbouring cell is referred to as lateral inhibition. Further examples besides the neural system for this mechanism are the suppression of an endocrine cell fate in the pancreas by active Notch (Apelqvist *et al.* 1999) or the promotion of a ciliary cell fate over Clara cell development in the lung (Morimoto *et al.* 2010). Furthermore, Notch also plays a major role in the formation of the T cell system by inhibiting the B cell fate (Pui *et al.* 1999). The mechanism of lateral inhibition also occurs during the development and branching of the vascular system: tip cells (which develop filopodia for VEGF gradient sensing) highly express Dll4, whereas in the neighbouring cells Notch1 is active, which in turn results in the adoption of a stalk cell fate in these cells (Hellstrom *et al.* 2007).

Notch signalling not only leads to the formation of "salt-and-pepper" patterns, but also to tissues with a highly uniform Notch signalling activity. Here, the activation of a ligand promotes ligand activation on the neighbouring cell. This so-called "lateral induction" can be found during the regulation of EMT in the developing heart (Timmerman *et al.* 2004) and during the formation of secondary lens fibres (Saravanamuthu *et al.* 2009) or of prosensory patches during the development of the inner ear (Hartman *et al.* 2010).

2.3 Hey genes are Notch downstream repressors belonging to the bHLH transcription factor family

Many of the processes and fate decisions regulated by Notch signalling ligands and receptors are exerted by the Hes and Hey gene family members, which are homologues to the Drosophila Hairy and Enhancer of split Notch downstream effector genes. The Hes genes have been the first Notch downstream effectors to be discovered and they have been found to be primarily involved in neural development. For instance, Hes1 knock out (KO) leads to severe neurulation defects incompatible with life (Ishibashi *et al.* 1995). In 1999, several groups described the discovery of another hairy related gene family, the Hey genes. In mammals, three Hey gene family members (Hey1, Hey2 and HeyL) exist, which are also referred to as Hesr, HRT, CHF and HERP (Leimeister *et al.* 1999; Kokubo *et al.* 1999; Nakagawa *et al.* 1999; Chin *et al.* 2000; Iso *et al.* 2001a; Iso *et al.* 2001b). Hey genes show a

broad expression pattern during mouse embryonic development (Leimeister *et al.* 1999; Kokubo *et al.* 1999; Nakagawa *et al.* 1999; Leimeister *et al.* 2000b) and are highly conserved among different species (Fischer *et al.* 2002). Hey genes have functional RBPJκ binding sites in their promoters and show responsiveness to Notch receptor induction or ligand stimulation; (Lin *et al.* 2000; Maier and Gessler 2000; Nakagawa *et al.* 2000; Iso *et al.* 2001a; Iso *et al.* 2002; Winkler *et al.* 2003). Consistent with this, loss of ligand expression also correlates with a loss of Hey gene expression sites (Kokubo *et al.* 1999; Leimeister *et al.* 2000a). Thus, Hey genes are *bona fide* Notch signalling pathway down-stream effectors. Their mode of action is primarily characterised by the repression of target genes. It has been demonstrated in *in vitro* assays that Hey genes show a preference for E boxes as binding sites in promoters (Nakagawa *et al.* 2000; Iso *et al.* 2001b; Fischer *et al.* 2002). Even though E box sequences present in target gene promoters such as those of the Gata transcription family or the VEGFR2 promoter are dispensable for the repression by Hey proteins (Elagib *et al.* 2004; Fischer *et al.* 2005; Holderfield *et al.* 2006), an E box is necessary for efficient repression of the Jagged1 promoter, for instance (Heisig *et al.* 2012). Like the Hes genes, Hey genes belong to the family of bHLH (basic helix-loop-helix) transcription factors and both protein families display a high degree of homology (compare Fig. 2, abbreviations are explained in the text).

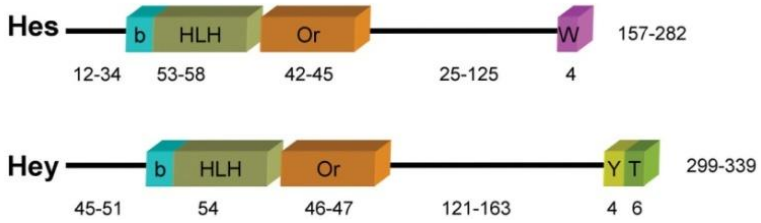


Fig. 2 Structural comparison of Hes and Hey gene family members (Fischer and Gessler 2007)
The basic (b), helix-loop-helix (HLH) and Orange (Or) domain are common in Hes and Hey genes. Hes genes have a WRPW (W) motif in their C-terminus, whereas Hey genes have a YXXW (Y) and TEIGAF (T) motif. The numbers represent the length of the amino-acid sequence of the single domains.

The close relationship between these two gene families is especially true for the N-terminal parts of the proteins: both protein families harbour an N-terminal basic domain, which is responsible for DNA binding. In this domain, one striking difference between Hes and Hey genes can be found: whereas in the Hes protein there is a conserved proline, in Hey proteins a glycine is found instead (Leimeister *et al.* 1999; Kokubo *et al.* 1999; Nakagawa *et al.* 1999; Chin *et al.* 2000). By mutating the glycine to a proline Holderfield and co-workers could abolish Hey1 repressive function on the VEGFR2 promoter (Holderfield *et al.* 2006). The

basic domain is followed by a helix-loop-helix domain. This structure is important on one hand for homodimerisation and on the other hand for heterodimerisation with other proteins (Murre *et al.* 1994). Protein-protein interactions are also modulated via the Orange domain, which may contribute to the specificity of the binding partner (Dawson *et al.* 1995), exert transcriptional repression (Castella *et al.* 2000) and serve as a dimerisation domain (Leimeister *et al.* 2000a).

The C-termini of Hes and Hey genes are less well conserved. Whereas Hes genes have a terminal WRPW (W) motif, which is essential for the recruitment of the co-repressor TLE (Grbavec and Stifani 1996), Hey1 and Hey2 have an YRPW, or in the case of HeyL, a YHSW motif. This YXXW (Y) motif has been shown to be dispensable for the Hey repressive function (Iso *et al.* 2001b; Sun *et al.* 2001; Fischer *et al.* 2002; Pichon *et al.* 2004; Fischer *et al.* 2005; Fuke *et al.* 2005; Holderfield *et al.* 2006). The repressive function of Hey is rather intrinsic to the bHLH and/or Orange domain (Chin *et al.* 2000; Pichon *et al.* 2004; Taelman *et al.* 2004; Holderfield *et al.* 2006), but the C-terminal part also contains repressive function (Sun *et al.* 2001; Belandia *et al.* 2005; Holderfield *et al.* 2006). The bHLH domain may interact with co-repressors such as hSirt1 (Takata and Ishikawa 2003) and the mSin3 complex (consisting of HDAC1 and N-CoR; (Iso *et al.* 2001b)). A conserved TE(I/V)GAF motif (T) can be found in the very C-terminal end of the Hey genes (Leimeister *et al.* 1999). The function of this motif has not been determined so far. Besides repression by promoter binding, Hey genes also prevent gene transcription by sequestering transcription-activating factors and thus hindering them from DNA binding. For instance, upon interaction with Hey2, the ARNT/EPAS1 heterodimer is inhibited from the localisation to its DNA binding target site, the HIF1 binding site (Chin *et al.* 2000).

2.4 Hey genes and their role in cardiovascular development

Gene function analysis in Hey knockout mice revealed that the genes are involved in the formation of the cardiovascular system. First evidence came from Hey2 mutants, which display severe cardiac phenotypes. Depending on the genetic background, loss of Hey2 results in diverse malformations (Fischer *et al.* 2004a; Sakata *et al.* 2006) often leading to an increased (postnatal) lethality. Examples are: hypertrophic hearts, ventricular septum defects, changes in wall thickness and disturbed cardiomyocyte structure (Gessler *et al.* 2002; Donovan *et al.* 2002; Sakata *et al.* 2002; Fischer *et al.* 2004a; Kokubo *et al.* 2004; Sakata *et al.* 2006). Loss of Hey2 (which is normally expressed in the ventricle) also correlates with a mis-expression of atrial genes in the ventricle (Fischer *et al.* 2005; Koibuchi and Chin 2007;

Xin *et al.* 2007). In the chicken and zebrafish heart, a similar effect can be observed (Rutenberg *et al.* 2006). Interestingly, Hey2 mis-expression has a positive and protective impact on heart function after heart stress conditions (Xiang *et al.* 2006; Yu, M. *et al.* 2009). In zebrafish, rather than heart defects a vascular phenotype resulting in loss of blood flow to the tail and loss of arterial identity can be observed (Weinstein *et al.* 1995; Zhong 2000; Zhong *et al.* 2001). In the mouse, however, a similar vascular phenotype is absent in Hey2 KO animals. Yet, in Hey1/2 double KO-mice a strong vascular phenotype is present, which leads to early embryonic lethality due to disturbed vascular remodelling and haemorrhages. Furthermore, arterial marker gene expression is lost in these animals (Fischer *et al.* 2004b; Kokubo *et al.* 2005). Arterial identity is promoted amongst others by Hey2 via the induction of an arterial specific transcription programme (Chi *et al.* 2003). Hypoxic conditions (at least in cell culture) promote Notch signalling and the arterial fate leading to the suppression of the vein determining factor COUP-TF II, an opponent to Notch signalling (Diez *et al.* 2007; You *et al.* 2005).

The high degree of redundancy is not only true for Hey1 and Hey2 during vascular development, but can also be observed for Hey1 and HeyL in cardiac development: Hey1- and HeyL-single KO-mice are without any obvious defect, but Hey1/HeyL double KO-mice show a ventricular septum defect. The mechanism behind this anomaly can be attributed to a disturbed EMT hindering the proper formation of cardiac septum and valves (Fischer *et al.* 2007). Hey genes also affect the function of VSMCs (vascular smooth muscle cells), which take part in the proper organisation of the vascular system. Following injury, Hey genes stimulate VSMC proliferation and inhibit apoptosis as well as the differentiation of the VSMCs (Wang, W. *et al.* 2003; Sakata *et al.* 2004; Tang, Y. *et al.* 2008).

2.5 Hey genes in the neural system and in other organ systems

Hey genes are not only expressed throughout the cardiovascular system, but also in neural tissues (Leimeister *et al.* 1999; Kokubo *et al.* 1999) pointing to a possible role of these genes during neural commitment. Indeed, mis-expression of Hey1 and Hey2 in neural progenitor cells leads to effects comparable to Hes1 and Hes5 over-expression (Ohtsuka *et al.* 2001): neural progenitor cells are maintained at the expense of neuronal differentiation. Furthermore, Hey2 over-expression results in an expansion of the ventricular zone. Over-expression of Hey1 and Hey2 at later developmental stages results in the preference of astrocyte development over neuronal differentiation, partially due to the suppression of proneural genes (Sakamoto *et al.* 2003). Moreover, Hey2 promotes gliogenesis in the retina, another model

system for neurogenesis (Satow *et al.* 2001). Whereas Hey1 and Hey2 have been demonstrated to negatively influence the process of neurogenesis, HeyL has been ascribed a neuronal differentiation promoting activity (Mukhopadhyay *et al.* 2009; Jalali *et al.* 2011). Yet, further studies are needed to verify and better understand these observations.

Hey gene activity is also essential in other organ systems. For instance, during skeletal muscle formation, Hey1 and HeyL regulate satellite stem cell numbers and differentiation: loss of both Hey genes leads to a loss of progenitor cells and therefore to an increased and premature differentiation of the satellite cells (Fukada *et al.* 2011). In bone, similar observations (i.e. the loss of mesenchymal progenitor cells and increased bone formation) can be seen. At later time points osteopenia phenotypes can be observed due to the loss of osteoblasts (Salie *et al.* 2010; Tu *et al.* 2012). Further examples for Hey gene function in a proper organ organisation are the cochlea (Hayashi *et al.* 2008; Doetzlhofer *et al.* 2009) or lens formation (Jia *et al.* 2007).

2.6 The Notch signalling pathway in embryonic stem cells

Since the establishment of murine ES cell cultures (Evans and Kaufman 1981; Martin 1981), these cells have been broadly used for the analysis of gene function in KO-models. One interesting aspect of this model system is that the pluripotent ES cells cannot only be used for the generation of genetically manipulated animals, but also for the *in vitro* differentiation into cells originating from all three germ layers, thereby nicely reflecting *in vivo* embryonic development (Doetschman *et al.* 1985). For Notch signalling the usage of the ES cell differentiation system recapitulated many findings from KO animals and hence, this *in vitro* system offers also a very powerful tool for the investigation of yet unknown Notch function. For instance, inhibition of the Notch signalling pathway increases the differentiation of neural progenitor cells into neuronal progeny (Hitoshi *et al.* 2002; Crawford and Roelink 2007; Abranches *et al.* 2009) and furthermore, loss of Notch activity leads to a concomitant loss of arterial marker gene expression during endothelial differentiation of ES cells (Lanner *et al.* 2007). Expression levels of Hes1 and the outcome during differentiation also reflect *in vivo* findings: first, Hes1 shows an oscillating expression in ES cells, a mechanism also found in neural stem cells in the brain, and second, only cells with low Hes1 expression lead to a neuronal fate (Shimojo *et al.* 2008; Kobayashi *et al.* 2009; Kobayashi and Kageyama 2010). KO of Hey1 and Hey2 genes during ES cell differentiation (Fischer *et al.* 2005) also verified the findings from KO-animals: loss of Hey1 and Hey2 leads to a deregulation of cardiac marker genes.

2.7 Notch signalling and crosstalk with other pathways

The highly diverse functions of Notch also implicate that this signalling pathway does not work completely on its own, but that it also integrates signals of other pathways. Cross-talk with the TGFβ/BMP signalling pathways plays a very prominent role, for example during tip cell formation in the process of vessel outgrowth (Moya *et al.* 2012). Further interference has been described for the JAK/Stat or Ras signalling pathways, for instance (Kamakura *et al.* 2004; Androutsellis-Theotokis *et al.* 2006; Fitzgerald *et al.* 2000). Moreover, besides the canonical Notch signalling pathway via Hey and Hes downstream effectors, the NICD also directly binds to other promoters such as NFκB or LEF target promoters (Oswald *et al.* 1998; Ross and Kadesch 2001) or induces transcription of Cyclin D1 (Ronchini and Capobianco 2001).

2.8 Discovery and structure of the Bre gene

Bre has first been discovered in a screen for stress-responsive genes (Li, L. *et al.* 1995). As the authors of this paper found Bre to be highly expressed in testes, ovary and brain, they referred to it as "Brain and reproductive organ expressed". However, today it is well established that Bre expression is not only limited to these organs, but that it can also be found in most other organs of the body as analysed in human, mouse and the golden hamster tissues (Miao *et al.* 2001; Chan, B. C. *et al.* 2008; Poon *et al.* 2004). The Bre protein is highly conserved in eukaryota and can even be found in Arabidopsis. The human and mouse Bre genes encode for a mRNA of 1.7-1.9kb resulting in a protein of 383 aa (amino acids) of 13 exons. There is high homology between the human and murine Bre protein (with 99% identity).

The only annotated domains, which have been proven to take part in Bre function, are the two UEV-domains (Wang, B. *et al.* 2009; Patterson-Fortin *et al.* 2010; Hu, X. *et al.* 2011), which are closely related to Ubiquitin-conjugating enzyme domains, but lack the characteristic catalytic cysteine (Hurley *et al.* 2006).

2.9 Bre as a stress responsive gene and its role in steroidogenesis

Consistent with a role of Bre as a stress responsive gene, diverse stress inducing agents lead to alterations of Bre expression (Li, L. *et al.* 1995; Miao *et al.* 2001; Chan, J. Y. *et al.* 2010). Furthermore, Bre transgenic livers show a constant stress response in that Bre over-expression results in high expression levels of stress response proteins (Tang, M. K. *et al.* 2009). Stress

induction also correlates with steroid hormone production. Interestingly, moderate to high Bre expression can be found in sites of steroidogenesis (i.e. adrenal gland, testis and regions in the brain; (Miao *et al.* 2001). Moreover, analysis of Bre deficient Leydig tumour cells suggested amongst others interference of Bre with steroid hormone production (Miao *et al.* 2005). Therefore, Bre likely has a functional impact on stress response via interference with the process of steroidogenesis.

2.10 Bre and apoptosis

Bre is also called TNFRSF1A modulator, because following a yeast-two-hybrid screen, the juxtamembrane region of the p55 TNFα receptor was discovered as an interaction partner of Bre (Gu *et al.* 1998). In the same study, NFκB expression following TNFα stimulation was found to be down-regulated after Bre over-expression. In the same line, the authors claim that Bre over-expression correlates with increased apoptosis in different cell lines. In another study, however, opposite findings were reported: The interaction of Bre with the TNFR was confirmed and binding to the endogenous Fas receptor was also found. Yet, by modulation of Bre expression levels, an anti-apoptotic effect by Bre after treatment of cells with TNFα or FasL (Fas Ligand) was observed. This is achieved by the attenuation of the mitochondrial apoptotic pathway (Li, Q. *et al.* 2004). Such an anti-apoptotic effect after FasL treatment is also observed *in vivo*, because Bre transgenic mice are protected from FasL induced lethal hepatic apoptosis (Chan, B. C. *et al.* 2008). Association of Bre with the mitochondrial pathway may be mediated by Prohibitin (Tang, M. K. *et al.* 2006). Furthermore, the regulation of apoptosis via the mitochondrial pathway by Nur77 (TR3), which exerts pro-apoptotic as well as anti-apoptotic effects, is correlated with Bre expression (Liu, J. J. *et al.* 2010; Wu, H. *et al.* 2011; Yao *et al.* 2012).

2.11 Bre is a member of distinct complexes

Diverse studies revealed that Bre is not only working as a single protein, but that it can also be a part of different complexes. First, it has been found that Bre (also referred to as Brcc45) modulates the E3-ligase activity of the BRCC (BRCA1 containing complex) with the core members BRCA1, BRCA2 and BARD1. Together with another protein called Brcc36, Bre enhances the BRCC activity (i.e. the transfer of ubiquitin) on the p53 substrate (Dong *et al.* 2003). The Bre complex partner BRCA1 plays a very prominent role in DNA damage repair. Besides the BRCA1-BACH1 (Cantor *et al.* 2001) and BRCA1-CtIP (Yu, X. *et al.* 1998; Chen, L. *et al.* 2008) complexes, which have been shown to participate in DNA damage

repair, in the last few years a Bre containing complex especially involved in BRCA1 recruitment to sites of DNA double strand breaks (DSBs) has been investigated in detail. This complex is also referred to as BRCA1-A (Wang B. et al, 2007a). For the detection of DSBs, the UIM domains of a phosphorylated RAP80 protein function as a sensor for DNA damage by recognising K63-ubiquinated-γH2AX generated by the Ubc13/Rnf8 E3-ligase complex (Kim *et al.* 2007a; Sobhian *et al.* 2007; Wang, B. *et al.* 2007; Yan *et al.* 2007; Sato, Y. *et al.* 2009; Sims and Cohen 2009; Wu, J. *et al.* 2009). RAP80 localisation to DSBs is necessary for a proper recruitment of BRCA1 (Kim *et al.* 2007a; Kim *et al.* 2007b; Sobhian *et al.* 2007; Wang, B. and Elledge 2007; Wang, B. *et al.* 2007). RAP80 does not interact directly with BRCA1, but here a phosphorylated Abraxas protein serves as a bridge for the interaction (Kim *et al.* 2007b; Liu *et al.* 2007; Wang, B. and Elledge 2007; Wang, B. *et al.* 2007). The role of Bre in this complex together with another protein known as Merit40 or Nba1 is to stabilise the interaction between the different complex components. Consistent with this, a loss of Merit40 or Bre expression leads to a decrease in BRCA1 IRIF (irradiation induced foci) localisation (Feng *et al.* 2009; Shao *et al.* 2009; Wang, B. *et al.* 2009). Both proteins also regulate the stability of another complex, the BRISC (Brcc36-containing isopeptidase complex) complex, which includes Brcc36 and ABRO, an Abraxas-related protein (Hu, X. *et al.* 2011), but does not contain RAP80, BRCA1 or Abraxas. Contrary to the BRCA1-A complex, the BRISC complex is not located in the nucleus, but in the cytoplasm. Interestingly, depletion of ABRO leads to a more robust Brcc36/BRCA1 localisation at IRIF. Thus, there seems to be a balanced regulation of the two complexes depicted in Fig. 3 (Cooper *et al.* 2009; Feng *et al.* 2009; Cooper *et al.* 2010; Patterson-Fortin *et al.* 2010). The BRISC complex displays DUB (de-ubiquitinase) activity on until now unknown protein targets; the DUB activity is intrinsic to the Brcc36 protein, which belongs to the subclass of JAMM/MPN+ proteases and shows specificity towards K63 cleavage. In the nuclear BRCC complex, the Brcc36 protein also displays DUB activity and is responsible for de-ubiquitination of poly-ubiquitinated chromatin structures (Feng *et al.* 2009; Shao *et al.* 2009), thereby removing the chromatin signature for BRCA1 DSB repair recruitment. In line with a role of BRCA1-A members in

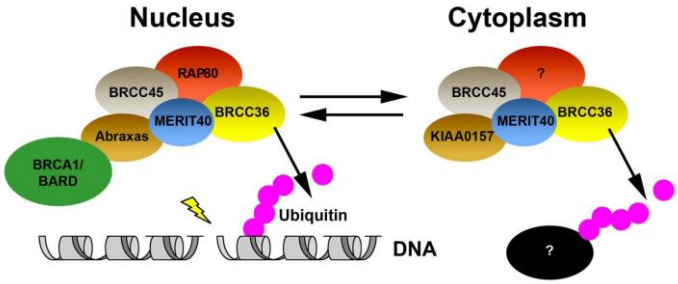


Fig 3. Bre is present in two distinct complexes with DUB activity in nucleus and cytoplasm (Patterson-Fortin et al, 2010)

the regulation of repair mechanisms (Coleman and Greenberg 2011; Hu, Y. *et al.* 2011) is the observation that loss of different BRCA1-A components leads to a disturbed G2M checkpoint (Dong *et al.* 2003; Kim *et al.* 2007a; Liu *et al.* 2007; Wang, B. *et al.* 2007; Feng *et al.* 2009).

2.12 Bre and tumourigenesis

The role of Bre as an anti-apoptotic protein and as a member of the BRCA1 containing DNA damage response complex points to a possible role of Bre in tumour biology. Indeed, altered Bre expression levels compared to normal surrounding tissue can be found in tumours of different origin. Here, down-regulation or a complete loss of Bre (Miao *et al.* 2001) as well as over-expression of Bre can be found (Chan, B. C. *et al.* 2008; Chen, H. B. *et al.* 2008). Furthermore, Bre haplotype expression also correlates with breast or ovarian cancer risk (Rebbeck *et al.* 2011). Bre protein expression in tumour cells leads to different outcome depending on the tumour type. Whereas high Bre expression in pediatric and adult AML with MLL fusion protein is a prognostic marker for a favourable outcome (Balgobind *et al.* 2010; Noordermeer *et al.* 2011), high Bre expression in other cell types promotes tumourigenesis: In human and in murine HCC models, in Lewis cell carcinoma and in esophageal carcinoma Bre over-expression leads to enhanced tumour growth (Chan, B. C. *et al.* 2005; Chan, B. C. *et al.* 2008; Chen, H. B. *et al.* 2008; Tang, M. K. *et al.* 2009; Chui *et al.* 2010). However, Bre does not *per se* lead to tumour formation as it was demonstrated in a HCC tumour model, where there was no difference in the number, but only in the size of tumour nodules (Chui *et al.* 2010). With regard to the formation of metastases Bre does not have an effect either (Chan, B. C. *et al.* 2005). Therefore, Bre is probably not a tumour-initiating oncogene, but a modulator gene for example due to its anti-apoptotic effects.

2.13 Aims of the thesis

The role of the Hey genes is well-established in a multitude of differentiation processes. However, as there is a strong redundancy between the three Hey genes, effects exerted by one single gene are often very difficult to assess if the other two members are still present. Furthermore, Hey1/Hey2 double KO-mice are early embryonic lethal preventing *in vivo* analyses at later developmental time points at least in mice with a global gene knock out. To overcome these problems, Hey-less ES cells should be established from Hey1$^{fl/fl}$/Hey2/HeyL mice and differentiated as embryoid bodies (EBs). This *in vitro* differentiation system is a good model for the main aspects of embryonic development. In this work, ES cells with intact Hey1 expression should be compared to Hey-triple-KO-ES cells in regard to their differentiation potential and differences should be quantified by gene expression analysis.

Even though Hey gene function as bHLH repressors could be shown in many settings, the way how Hey genes exert their repressive function is still not well characterised. To further elucidate Hey function, it is therefore necessary to gain closer insight into the interaction of Hey with binding partners. For this, Bre, a previously found interaction partner of Hey1 should be further characterised in detail in this work. Binding studies should determine the domain(s) of Bre involved in the interaction with Hey1 and functional assays should reveal whether the interaction with Bre interferes with Hey1 function such as Hey1 repressor activity or Hey1 stability. Furthermore, Bre deficient mice and cells should be generated and analysed for potential defects to better understand Bre function and hence possible modes of interaction with Hey1.

3. Material and methods

3.1 Material

3.1.1 Instruments

Amaxa® Nucleofector® II Device (Lonza, Köln)

BD LSR II Flow Cytometer (BD Biosciences, San Jose, USA)

Faxitron CP160 (Faxitron Bioptics, Tucson, USA)

Glomax luminometer (Promega, Madison, USA)

NanoDrop ND 1000 spectrophotometre (peqLab, Erlangen)

Sunrise Absorbance Reader Magellan® (Tecan, Crailsheim)

Varian 50 UV-VIS spectrophotometer (Varian Medical Systems, Zug, Switzerland)

W-250D digital sonifier® (Branson Ultrasonics, Dietzenbach)

Fluorescence Microscopes

Eclipse Ti/ Eclipse C1si Spectral Imaging Confocal Microscope (Nikon, Tokyo, Japan)

Inverted Fluorescence microscope DMI 6000B (Leica, Wetzlar)

Realtime-RT-PCR cycler

iCycler iQ®Real-Time PCR Detection System (BioRad, München)

Mastercycler® ep *realplex2* (Eppendorf, Hamburg)

3.1.2 Mouse strains

BL/6: $Hey1^{fl/fl}$/Hey2/HeyL , Bre LacZ/del

BALB/c

CD1

3.1.3 Cell lines

3.1.3.1 Cells grown in ES cell medium

KO SR 2i medium

$Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$: 52765.c1

$Hey1^{del/del}/Hey2^{-/-}/HeyL^{-/-}$: 5276.5 c1 del #5, 7, 12

ES LIF medium

Bre LacZ $^{+/-}$: # 11B, 23B (i.e. in the following het #1,2)

Bre LacZ $^{-/-}$: # 8B, 10B, 31B, 35B (i.e. in the following KO #1-4)

3.1.3.2 Cells grown in D10 medium

HeLa cells

MEFs

BALB/c MEFs for feeder layer

Bre MEFs$^{+/+}$: 1788.9, 1882.6, 1884.5, 2107.7, 2221.6, 3171.5

Bre MEFs$^{-/-}$: 1788.4, 1788.5, 1788.6, 1882.3, 1884.2, 2107.2, 2221.10, 3171.4, 3171.6

HEK 293 derived cell lines

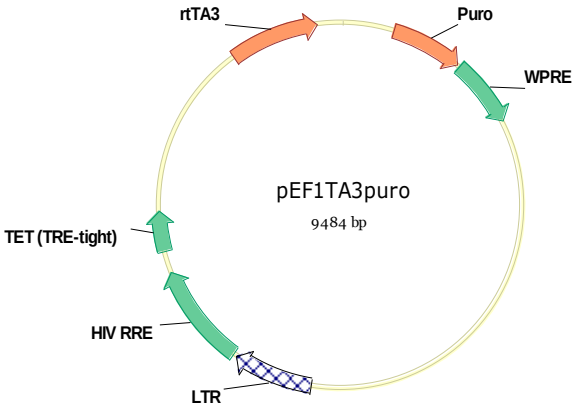
HEK 293 T

HEK293-pWHE134-p199 FS-mHey1 cells (i.e.293tet-FS-mHey1cells; cellular system as described by Heisig *et al.* 2012)

3.1.4 Plasmids

pEF1TA3p

Lentiviral construct with a doxycycline dependent transactivator (TA), which allows the induction of target gene expression upon addition of Doxycyline (unpublished, compare vector map below)



pLL3.7

Lentilox construct expressing GFP (Rubinson *et al.* 2003)

pMBP parallel

MBP tagged vector, allowing induction of protein expression in bacterial BL21/DE3 Lys strains (Sheffield *et al.* 1999)

pCMV-VSVg, pPAX2

packaging plasmids for viral protein production (obtained from D.Frono)

pTurbo Cre

Construct encoding for CRE-recombinase, allowing extremely high expression rates (obtained from M.Morkel)

All chemical reagents not listed below were purchased at Applichem (Darmstadt), ROTH (Karlsruhe) or Sigma-Aldrich (Taufkirchen).

3.1.5 Cell culture materials

Plastic ware was purchased from Greiner (Frickenhausen) except for 4 well plates, which were provided by Nunc (Wiesbaden) and ibidi treat 15µ-Slides VI 0.4, which were purchased from ibidi (Martinsried).

Accutase® (PAA, Cölbe)
amaxa ® ES cell kit (Lonza, Köln)
β-mercaptoethanol (PAN, Aidenbach)
CHIR 99021 (axon Medchem BV, Groningen, The Netherlands)
DAPI Nr. D-1306 (Molecular Probes, Eugene, USA)
DMEM (PAN-Biotech, Aidenbach; Sigma Aldrich, Taufkirchen)
FasL (produced in Prof. H. Wajant´s laboratory)
FCS (PAN-Biotech, Aidenbach)
FuGene ® HD (Roche, Grenzach-Wyhlen/Mannheim)
GlutaMAX-I supplement (GIBCO/Invitrogen, Karlsruhe)
G418 (PAA, Cölbe)
KO DMEM (GIBCO/Invitrogen, Karlsruhe)
LIF (produced by C. Söder)
Metafectene ® Pro (Biontex, Martinsried)
MEM-NEAA (PAN, Aidenbach)
Mitomycin C (medac, Hamburg)
PD 0325901 (axon Medchem BV, Groningen, The Netherlands)
Penicillin/Streptomycin (PAN, Aidenbach)
Sodium pyruvate (GIBCO/Invitrogen, Karlsruhe)
TNFα (produced in Prof. H. Wajant´s laboratory)
Trypsin-EDTA (PAN-Biotech, Aidenbach)

3.1.6 DNA preparation kits

Mini, Mid and Maxi kits were purchased from peqLab (Erlangen), Promega (Madison, USA), Omega Biotek (Norcross, USA) or Sigma-Aldrich (Taufkirchen).

3.1.7 PCR and realtime-RT-PCR

3.1.7.1 Chemical reagents

Acetylated BSA (Amersham Biosciences, Freiburg)

dNTPs (ROTH, Karslruhe)

FITC (BioRad, München)

Sybr Green (Cambrex, Apen)

Taq polymerases (produced in the own laboratory)

3.1.7.2 RNA preparation and cDNA synthesis

peqGOLD TriFast® reagent (peqLab, Erlangen)

RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot)

3.1.7.3 Primer (sequence: 5´→3´)

Primers were synthesised by MWG (Ebersberg) or Sigma-Aldrich (Taufkirchen)

Genotyping primers

Floxed Hey1 vs del

Eagrev	ACAAAGCAAAGCAGGCAGTC
Clikseq2	TGAGATCTTGCAGATGACTGTG
clik-atg	GCGGGATCCACATGAAGAGAGCTCACCCAG

Hey2

Hey2ko-test3'	TCGGTGAATTGGACCTCATCACTGAGC
Hey2ko-test5'	GCTGTCTCAAGGCCTCAACAGCATTG
Z3L	ATCGGTGCGGGCCTCTTCGCTATTA

HeyL

mHeyL-ex2-r	TGTTGCACACTCACCCCTCT
mHeyL-ex2-lb	GGATCCTTCAGCTCTGAGAAA
M13	CGCCAGGGTTTTCCCAGTCACGAC

Bre LacZ

Bre-flox-1	AGGAATAGGCCTGTGCAGAA
Bre-flox-2	GGGAGGTGTCCTTACCCACT

Bre-K (= lacZ present, but floxed allele deleted)

Lucseq2	ACAAATAAAGCAATAGCATCACAA
Bre-flox-1	AGGAATAGGCCTGTGCAGAA
Bre-flox-2	GGGAGGTGTCCTTACCCACT

Bre-N (= lacZ and floxed exon deleted)

Bre-flox-1	AGGAATAGGCCTGTGCAGAA
Bre-flox-2	GGGAGGTGTCCTTACCCACT
Bre-flox-3	TTTGTGCTTTCCTGCAGATG

FSmHey1 in ES cells

FlagStrep-Tag KpnIfor	GCGGTACCGCCGCCACCATGGATTATAAAG
clk2	CTGGCCAAAACCTGGGAC

realtime-RT-primers

Primers for realtime-RT-PCR analysis are ideally chosen in such a way that the resulting PCR products have a size of approximately 200 bp. Furthermore, the primers are designed over exon-intron boundaries if possible, so that problems with genomic DNA contamination can be ruled out.

mOct4-real5'	CCGTGAAGTTGGAGAAGGTG
mOct4-real3'	GAAGCGACAGATGGTGGTCT
Nanog_fwd	AAGTACCTCAGCCTCCAGCA
Nanog_rev	GTGCTGAGCCCTTCTGAATC
huclik-3	TGAGCTGAGAAGGCTGGTAC
Clikseq5	ACCCCAAACTCCGATAGTCC
mHey2-real-e3for	TGAGAAGACTAGTGCCAACAGC
mHey2-real-e5rev	TGGGCATCAAAGTAGCCTTTA
mHeyL-real-ex2/3	GAAGCGCAGAGGGATCATAG
mHeyL-real-ex4rev	GGCATGGAGCATCTTCAAGT
mAFP_ex11	CTCAGCGAGGAGAAATGGTC
mAFP_ex12	GGTGATGCATAGCCTCCTGT
Brachyury T_ex2	GCGTCTACATCCACCCAGAC
Brachyury T_ex3	CCTCATTCTGGTAGGCAGTCA
mNestin _ex3	ACTCTCGCTTGCAGACACCT
mNestin_ex4	ATTAGGCAAGGGGGAAGAGA
mVE-Cadherin_5'real	GGATGTGGTGCCAGTAAACC
mVE-Cadherin_3'real	ACCCCGTTGTCTGAGATGAG
mTie1-real5'	TGCAGACTTTGGCCTTTCTC
mTie1-real3'	TGCCTCCAAGGCTCACTATC
mneurog2_5'real	GACATTCCCGGACACACAC
mneurog2_3'real_b	CCCAGCAGCATCAGTACCTC
mMash1_5'real	TCTCCTGGGAATGGACTTTG
mMash1_3'real	CCCCTGTTTGCTGAGAACAT

mOlig2_5'real	CAGCGAGCACCTCAAATCTA
mOlig2_3'real	CACAGTCCCTCCTGTGAAGC
mDat1_5'real	TGGAGGTTTCCCTACCTGTG
mDat1_3'real	CCAGCAGCTCCTTCTCTGTT
mtubb3_5'real	GTCAGCATGAGGGAGATCGT
mtubb3_3'real	GCAGGTCTGAGTCCCCTACA
Mus_myoG-f01	AGGAGCGCGATCTCCGCTACAGA
Mus_myoG-r01	GACATATCCTCCACCGTGATGCTGT
Mus_myoT-f01	GTGTGACCACGTGTAACACACGATTAGA
Mus_myoT-r01	GGATTGAGCTGCCAGGCGCTGAA
mGATA4-real5'	TCAAACCAGAAAACGGAAGC
mGATA4-real3'	CTGCTGTGCCCATAGTGAGA
mGATA6-real5'	GCCAACTGTCACACCACAAC
mGATA6-real3'	TGTTACCGGAGCAAGCTTTT
mSnail_5'real	CTTGTGTCTGCACGACCTGT
mSnail_3'real	CTTCACATCCGAGTGGGTTT
mBre-realex12-for	CGTGGAATATGATGCAGAAGG
mBre-realex13-rev	AGGCTGATCTCGAGGGAAAA
mNkx2-5-real5'	TGACCCAGCCAAAGACCCTCGG
mNkx2-5-real3'	GTCTCGGCTTTGTCCAGCTCCAC
Bre ex2 for	CCTTGAACCGAATTTCTCCA
Bre ex4 rev	TGAAATCAGGAGGCAGCTCT
BreLacZTest for	TCCCGAAAACCAAAGAAGAA
BreLacZTest rev	GGCGATTAAGTTGGGTAACG

3.1.8 Protein biochemistry

3.1.8.1 Chemical reagents

Bradford reagent (Biorad, München)

Cycloheximid (ROTH, Karlsruhe)

MG132 (Sigma-Aldrich, Taufkirchen)

NEM (Sigma-Aldrich, Taufkirchen)

PMSF (Applichem, Darmstadt)

Protein A Agarose (Sigma-Aldrich, Taufkirchen)

Protease inhibitor cocktail tablets Complete EDTA free (Roche, Grenzach-Wyhlen)

3.1.8.2 SDS PAGE and Westernblot

PageRuler^{TM} Prestained Protein Ladder (Fermentas, St.Leon Rot)

Primary antibodies

Mouse α Flag-M2 (Sigma-Aldrich, Taufkirchen)

Mouse α HA-probe F7 **(**Santa Cruz, Santa Cruz, USA)

Human α K63 ubiquitin (Genentech, South San Francisco, USA)

Mouse α β Tubulin AA2 (Sigma-Aldrich, Taufkirchen)

Mouse α Ubiquitin P4 D1, (Santa Cruz, Santa Cruz, USA)

Secondary antibodies

α Mouse-HRP (Chemicon/Millipore, Billerica, USA)

α Human-HRP (Sigma-Aldrich, Taufkirchen)

3.1.8.3 Immunofluorescence

Mowiol 4-88 (Calbiochem, Darmstadt)

Hoechst 33258 (Amersham Biosciences, Freiburg)

Hoechst 33342 (Amersham Biosciences, Freiburg)

Primary antibodies

Goat α BRCA1 I-20 **(**Santa Cruz, Santa Cruz, USA)

Rabbit α Bre GTX111188 antibody (GeneTex Inc, Irvine, USA)

Phospho-Histone H2AX (ser139) 20E3 (Cell Signaling, Danvers, USA)

Secondary antibodies

Alexa 488 goat-anti-rabbit (Molecular Probes/Invitrogen, Karlsruhe)

Alexa 488 chicken-anti-rabbit (Molecular Probes/Invitrogen, Karlsruhe)

Alexa 568 donkey-anti-goat (Molecular Probes/Invitrogen, Karlsruhe)

3.2 Methods

3.2.1 Cell culture

All cells are grown at 37°C and incubated with 5% CO2. MEFs (when not used as a feeder layer for ES cells), HEK 293 derived cells and HeLa cells are cultivated in D10 medium, Hey ES cells in KO SR 2i medium and Bre LacZ ES cells in ES LIF medium.

D10 medium

DMEM
10% FCS
Penicillin (50U/ml), Streptomycin (50µg/ml)

ES LIF medium

DMEM
10% FCS
Penicillin (50U/ml), Streptomycin (50µg/ml)
1% MEM-NEAA
0.1 mM β-mercaptoethanol
1000 U/ml LIF

KO SR 2i medium
Knockout DMEM
15% Knockout serum replacement (SR)
1% Glutamaxx
1 mM sodium pyruvate
1% MEM-NEAA
0.05mM β-mercaptoethanol
Penicillin (50U/ml), Streptomycin (50µg/ml)
1000 U/ml LIF
3µM CHIR 99021
1µM PD 0325901

Passaging of cells (i.e. the transfer to new wells) is achieved by different treatments: MEFs, HeLa cells and Bre LacZ ES cells are passaged by trypsinisation. For this, cells are washed with PBS, 0.25% trypsin-EDTA solution is added and the cells are incubated at 37°C, until they have detached from their plates. In the following the trypsin is inactivated by addition of FCS containing medium or removed by pelleting of the cells (centrifugation at 1000 rpm, 3 min). Hey KO-ES cells are detached by treatment with Accutase and HEK 293 derived cell lines can easily be rinsed from their plates.
When cells are frozen, they are resuspended in freezing medium (i.e. the respective cell culture medium with an additional 10% of FCS and 10% DMSO) and stored at -80°C and in liquid nitrogen.

3.2.2 Generation of mouse embryonic fibroblasts (MEFs)

For the preparation of MEFs, pregnant females are sacrificed on embryonic day E13.5. Embryos are isolated from the uterus; head and extremities of the embryo are cut off, the inner (blood forming) organs are removed and the remaining embryo is washed in PBS. If necessary, the yolk sack or parts of the extremities/head can be used for genotyping. In the following, the embryo is transferred to a 10 cm cell culture dish and 2 ml of pre-warmed trypsin is added. After incubation at 37°C for 10 min in the cell culture incubator, the embryos are cut into small pieces and digested for further 10 min with an additional 2 ml of trypsin. Then, the mixture is pipetted up and down with another 2 ml of trypsin, for the generation of a single cell suspension. After centrifugation (1200 rpm, 5min) the cells are plated on 10 cm dishes in D10 medium. On the following day, medium is changed to remove residual embryonic tissue and the cells are cultured until they have grown to confluence. Cells can directly be used for experiments (up to passage 5) or may be frozen in the following. If cells are used for feeder layer cell culture, they have to be mitotically inactivated. For this,

20 µg/ml Mitomycin C is added to the cells for 4h. After extensive washing, the cells are supplied with fresh medium and can be seeded the next day or frozen away.

3.2.3 Generation of mouse embryonic stem cells (ES cells)

On E3.5, the pregnant female is sacrificed; the uterus is isolated, transferred to a bacterial culture dish and washed in PBS. Both horns are cut off and a syringe with a 21G needle is inserted to flush out the blastocysts with KO SR ES cell medium (i.e. KO SR 2i medium without CHIR 99021 and PD 0325901 inhibitors). The isolated blastocysts are collected in a bacterial culture dish filled with pre-warmed medium. For washing (3x), blastocysts are transferred by mouth-pipetting to small drops of PBS in a bacterial culture dish. Next, the obtained blastocysts are transferred into KO SR 2i medium in 4 well dishes, which have been pre-coated with 0.2% gelatine. After 48h, it is monitored if the blastocysts have settled down and adhered to the culture well. Only attached blastocysts are used for further culture and the KO SR2i medium is changed daily from now on. When the inner cell mass (ICM) of the blastocysts has grown out and proliferated sufficiently (3-5 days after plating), the ICM is collected by treatment with Accutase. The resulting single cells are plated on freshly prepared inactivated BALB/c MEF feeder layers in 96 well plates. After a maximum of further 3 days, the establishment of ES cell-like colonies can be observed. When the compact colonies have reached an adequate size, the cells are split and transferred to a fresh feeder layer into a 48 well plate and in the following to a 4 well plate with inactivated BALB/c MEF. From this time-point on, cells can be frozen away for storage or transferred for culture without feeder layer: For this, the ES cell/feeder cell mixture is pre-plated following Accutase treatment and after 30-45min (when most fibroblasts have settled down) only the supernatant is transferred to a new gelatine-coated well. After 1-2 passages, no more feeder cells are left in the ES cell culture. Now, the cells are also ready for genotyping. If desired, established ES cell lines can be cultivated in KO SR medium without the addition of the two inhibitors CHIR 99021 and PD 0325901

3.2.4 Differentiation of ES cells as embryoid bodies (EBs)

<u>ES differentiation medium</u>

DMEM medium
10% FCS
Penicillin (50U/ml), Streptomycin (50µg/ml)
1% MEM-NEAA
0.1mM β-mercaptoethanol

For the differentiation of ES cells as EBs, the ES cells are counted, washed and $1x10^6$ cells are resuspended in 10 ml of ES differentiation medium. In the following, cells are plated onto non-adhesive bacterial culture dishes and cultured on an orbital shaker rotating with 40 rpm. Medium is changed after three days for the first time and afterwards every second day. For this, EBs are transferred into 15 ml Falcon tubes. When the EBs have settled down to the bottom of the tube (after 5-10 min), the supernatant is aspirated, fresh medium is added and the EBs are transferred back onto their bacterial culture dishes.

3.2.5 Genetical manipulation of cells

3.2.5.1 Transfection

Cells are plated at least one day before transfection so that they have a confluency of about 80% on the day of transfection. Prior to the incubation of cells with their respective transfection reactions, the volume medium in the corresponding wells is adjusted to half of the normal volume to enhance the transfection efficiency. The transfection solutions are added drop-wise to the cells. After 6-8 hours, cells are fed with fresh medium or passaged. In the following, cells are harvested or analysed at the desired time points (after 24-72h).

PEI transfection protocol

PEI is diluted in 150mM NaCl to a 75nM solution. Next, DNA and PEI are prepared in two different reaction tubes in pure medium (i.e. without FCS/Penicillin/Streptomycin or any supplements). After 2 min of incubation, the PEI mixture is added to the DNA sample and vortexed. Following centrifugation and further 15-20 min at room temperature, the transfections reactions are added to the cells.

The volume of the reactions and the DNA amount applied depends on the cell culture well plate chosen for the transfection (for standard conditions compare table below).

Plate	**Volume of the reaction**	**DNA amount**
24 well	50µl	0.6µg
12 well	50µl	1µg
6 well	100µl	2µg
10 cm	500µl	6µg

DNA amounts may vary depending on the experimental setting, but in all cases the proportion of PEI [µl] to DNA [µg] is 2:1 (i.e. for instance: 6µg/10 cm dish and 12µl PEI). Note that the

DNA amounts are adjusted to each other in one experiment so that all transfection reactions have an equal DNA content [µg], i.e. reactions are filled up with PBS-KS or pcS2p plasmids.

Transfection with Metafecten Pro

Transfection with Metafecten Pro is described in the following for the 12 well plate format. Here, 5µl of Metafecten Pro Solution are added to 50µl of PBS and 1µg of DNA to another 50µl of PBS. In the following, the DNA is added to the Metafecten Pro solution and mixed by careful up-and down-pipetting. After an incubation of 20 min, the mixture is added to the cells.

Transfection with FuGene HD

1µg of DNA and 3µl FuGene HD reagent (i.e. ratio DNA: FuGene HD=1:3) are each diluted in 50µl of pure medium for the transfection of cells in a 12 well plate. Next, the FuGene HD solution is added to the DNA and the reaction mixture is vortexed. 30 min later the mixture is added to the cells.

3.2.5.2 Electroporation of ES cells with the amaxa Nucleofector II Device

Cells are prepared following the protocol of the amaxa ES cell kit. Brief, cells are accutase treated and collected, cells are counted and $3.5x10^6$ cells are washed in PBS, followed by centrifugation with low speed (80g, 10 min) and the obtained pellet is resuspended using 90µl of the Mouse ES Cell Nucleofector® Solution. After addition of the DNA (maximal volume <10µl), and careful up and down pipetting, the mixture is transferred to an electroporation cuvette and the cells are electroporated using the Amaxa® Nucleofector® II Device set to the A24 programme. Next, the cells are immediately resuspended in 8ml of pre-warmed ES cell medium and plated in three different dilutions (5ml, 2ml and 1ml, respectively). 24h after electroporation, the medium is changed and the cells are cultured until further use.

3.2.5.3 Viral particle production and transduction of target cells

Production of virus particles is done by transfection of HEK 293T cells following the PEI transfection protocol. Transfected plasmids include the plasmid encoding for the viral construct (in a concentration of 6µg) together with pPAX2 (4.5µg) and CMV-VSVg plasmids (3µg). The following day, cells are treated with 10mM sodium butyrate. After incubation for 6-8h, the medium is changed. 48h later, the viral particles can be harvested by centrifugation of the supernatant from the HEK 293T cells (1000rpm, 5 min). Next, the virus-containing supernatant is filtrated with a 0.45µm sterile filter. The supernatant is distributed into 1 ml

aliquots, which are either directly used for transduction or frozen in liquid nitrogen for storage at -80°C.

One day before transduction, target cells are seeded in 12 well plates so that they have a confluence of about 80% on the following day. The medium is replaced by 1 ml of the viral supernatant supplemented with 8µg/ml polybren. If ES cells are transduced, it is necessary to add fresh LIF to their medium so that the cells do not start to differentiate during the incubation with the viral supernatant. 6-8h after transduction, the medium is changed or cells are passaged. Selection for cells with a stable integration of the viral construct may start 48h after transduction: For this, 1µg/ml puromycin is added to the standard culture medium for viral constructs encoding for a puromycin resistance, for instance. As a control non-transduced cells are also subjected to the selection agent. When all of the control cells have died, clones from the transduced sample can be picked and tested for a successful targeting by viral transduction. Integration of virally encoded DNA into the cell genome can be analysed by PCR with genomic DNA. Furthermore, Westernblot analysis can be performed to determine whether the transduced constructs are expressed on protein level in the cells. For protocols of genotyping PCR and Westernblot analyses see below.

3.2.6 Plasmid DNA preparation from bacterial cultures and precipitation of DNA

LB medium (supplemented with the corresponding antibiotic) is inoculated with bacterial glycerol stocks or clones from LB agar plates and overnight cultures are grown on a shaker at 37°C. The next day, cultures are centrifuged (4000 g, 10 min) and DNA is isolated by Mini, Midi or Maxiprep kits following the instructions of the manufacturer. Next, DNA sample concentration is determined with the Nano Drop ND 1000.

If DNA samples are not sufficiently concentrated or not pure enough, DNA can be precipitated by addition of 0.1 volume of 3M sodium acetate and 0.7 volumes of 100% isopropanol. The mixture is placed on ice for 10 min; afterwards it is spun at 13000 rpm for 20 min at 4°C. The pellet is washed with 70% ethanol followed by centrifugation at 13000 rpm for 5 min at 4°C. The supernatant is discarded, the pellet is air-dried and ddH2O is added to the desired volume. The samples are incubated at 68°C for 10 min and the DNA is finally dissolved by up-and down-pipetting. After that, samples can be stored at -20°C or used for electroporation or other applications.

3.2.7 Generation of Bre homozygous ES cells by high G418 selection pressure

The culture of heterozygous ES cells under high selection pressure may result in the selection for homozygous ES cells, which have undergone spontaneous LOH (loss of heterozygosity). This is especially true if the promoter driving the resistance gene on the target allele is relatively weak. For the generation of homozygous ES cells, the heterozygous clone is cultivated in standard medium under high selection pressure as described by Mortensen and co-workers (Mortensen *et al.* 1992). For this, different amounts of G418 (i.e. 0.5mg/ml, 1mg/ml, 1.5mg/ml, 2mg/ml) are added to the ES LIF culture medium. Surviving clones are picked and it is tested by genotyping PCR, whether spontaneous LOH has led to the generation of homozygous KO-ES cell clones.

3.2.8 Alkaline phosphatase (ALP) staining of mouse ES cells

TM buffer:

0.22 M TRIS-Maleate, pH 9

Staining solution (25,2ml)

15.25mM TM buffer
0.08% $MgCl_2$
10 mg Naphtol AS-MX Phosphate
25 mg Fast Red TR salt

Cells are fixed in 4% PFA at room temperature for 20 min. After washing in TM buffer (three times), cells are incubated with staining solution for 15-20 min followed by extensive washing. Positive ALP staining results in a red staining of pluripotent stem cells and can be monitored with a microscope.

3.2.9 Karyotyping of murine ES cells

Hypotonic solution:

0.8% sodium citrate

Carnoy´s fixative:

75% methanol
25% acetic acid

Giemsa solution:

5% Giemsa solution in Giemsa buffer

Giemsa buffer

115mM KH_2PO_4
115mM NaH_2PO_4

Cells at a confluence of about 80% are treated with 0.1µg/ml colcemide for 3-4h at 37°C. Then, cells are detached by trypsinisation and centrifuged (1000 rpm, 3 min). The supernatant is aspirated, but approximately 0.5ml of the supernatant are retained for the resuspension of the cell pellet by flicking of the falcon tube. Next, 0.5ml of pre-warmed sodium citrate solution (37°C) is added and the resulting suspension is incubated at 37°C for 30 min. After centrifugation, the supernatant is discarded and the pellet is resuspended in 5 ml of Carnoy´s fixative. Cells are fixed at -20°C overnight or can even be stored for a longer time at -20°C. Depending on the pellet size, cells are resuspended in 0.5-1 ml of fixative and 2-3 droplets are dropped from height onto cleansed objective slides with a glass pipette. The suspension is evenly distributed by blowing. Successful bursting of the nuclei can be checked under a light microscope. After drying of the samples at room temperature (for 1h), Giemsa solution is applied to the samples with a paper strip and the samples are stained for 5 min. Washing with distilled water and drying of the slides is followed by mounting of the samples in Histokitt. For analysis, the slides are checked with immersion oil under a 63x objective. At least 50 broken nuclei are counted for their chromosomal content.

3.2.10 FACS cell cycle analysis: Staining protocol

Staining buffer

154mM NaCl
0.1M TRIS pH7.4
1mM $CaCl_2$
0.5mM $MgCl_2$
0.2% BSA
0.1% NP40
1µg/ml DAPI

Cells are harvested by trypsinisation, and centrifuged. After washing with PBS, the resulting pellet is resuspended in staining buffer so that a solution of $1*10^6$ cells/ml is obtained. Samples are incubated for at least 30 min at 4°C in the dark. Cells are directly taken for FACS analysis or chicken erythrocytes are added ($<< 1*10^4$ cells) prior to the measurement. The peak obtained for the chicken erythrocytes is an internal standard, which would allow for the calculation of the content of aneuploid subpopulations simply by measuring the distance of the different peaks to this internal standard.

3.2.13 Stripe assay by laser-induced DNA damage on a confocal microscope

The conditions for the laser induced generation of a stripe pattern in Bre MEFs have been chosen following the description by Kong and colleagues (Kong *et al.* 2009). Cells are pre-stained with Hoechst 33528 and then submitted to laser treatment on a Nikon Eclipse Ti/ Eclipse C1si Spectral Imaging Confocal Microscope. Cell nuclei are first visualised by scanning with the 405 nm laser set at only 1% output. Then, regions of interest (ROI) are set in a stripe-like pattern into several nuclei. The laser output is now changed to 40% and then 2.5µs/pixel are used for scanning (this should equal approximately 75nW/pixel). Scans are repeated for 25 cycles.

3.2.11 MTT-assay

Cell growth is monitored with a MTT-proliferation assay for up to 96h. After counting, $2*10^3$ cells are plated on 96 well plates in 100µl DMEM medium/well. The cells can now be measured at the desired time points. For this, 50µl of MTT solution (5mg/ml MTT in PBS; dissolved for 30 min at 37°C) are added to each well. After incubation for 30 min at 37°C, a blue staining of the cells can be detected. Next, the plate is centrifuged (2800 rpm, 10 min), the supernatant is discarded and the converted MTT reagent is resuspended in 150 µl of DMSO/well. The purple stained solution is measured with a TECAN/Magellan ELISA reader at a wavelength of 540 nm with a reference wavelength of 690 nm.

3.2.12 γ-irradiation of cells

For the induction of double strand breaks, cells are irradiated with a Faxitron CP160 machine with 160 kV and 6,3 mA for 1 min, which approximately equals a dose of 5 Gy. Irradiation can be performed for cells as a suspension in cryo tubes or also for adherent cells.

3.2.14 Genotyping and realtime-RT-PCR

3.2.14.1 Genotyping PCR

Base buffer

25mM NaOH
0.2mM EDTA

Neutral buffer

40mM TrisHCl; pH 5.0

10xPCR buffer

100mM Tris pH8.85
500mM KCl
15mM $MgCl_2$
1% TritonX-100
970µl of the 10xPCR buffer are mixed with 30µl acetylated BSA [2mg/ml]

1xPCR reaction mix

2.5 µl 10xPCR buffer
0,25mM dNTPs
0,3µM Primer1
0,3µM Primer2
0,3µM Primer 3
Taq 3U
ad 25µl H_2O

Samples for genotyping PCR (i.e. cell pellets or yolk sack/tissue from mouse embryos) are incubated at 96°C for 30 min in base buffer (for cell pellets 30-100µl are added depending on the size of the cell pellet; embryonic tissues are incubated in 100µl) and then the same amount of neutral buffer is added. Samples can now be directly used for genotyping PCR: For this, 1µl template is added to 24µl of PCR reaction mix. The PCR programme used is listed below.

94°C	5 min
94°C	30 seconds
60°C	30 seconds (35x)
72°C	45 seconds
72°C	7 min
16°C	Hold

3.2.14.2 RNA extraction, cDNA synthesis and realtime RT PCR

RNA extraction

Cells in a 6 well plate are washed with PBS and 0.5-1 ml of TriFast reagent is added directly to the well. When embryoid bodies are harvested, they are incubated in TriFast reagent after they have settled down in a Falcon tube. The lysate can either be stored at -80°C or directly used for RNA extraction. After incubation in TriFast (5 min, RT), 200µl of Chloroform is added, the tubes are vortexed and left for 3 min at room temperature. After centrifugation (13000 rpm, 20 min, 4°C) the aqueous phase is transferred to a new tube and the RNA is precipitated by addition of 300µl of isopropanol. The mixture is incubated on ice (20 min) and afterwards centrifuged at 13000 rpm for 20 min at 4°C. The resulting pellet is then washed

with 70% ethanol and after centrifugation (13000 rpm, 5 min, 4°C), the pellet is air dried and DEPC-H_2O is added (20µl-100µl, depending on the size of the pellet). Next, the samples are incubated at 55°C for 10 min and the RNA is finally solved by up-and down-pipetting. The RNA is now ready for further analyses or can be stored at -80°C. The RNA concentration is determined with the NanoDrop ND 1000 spectrophotometre.

cDNA synthesis

2µg of RNA are used as a template for cDNA synthesis with the Fermentas RevertAid™ First Strand cDNA Synthesis Kit. The RNA is diluted in 11µl of DEPC H2O and 1µl of oligo-dT primer is added. The mixture is incubated at 70°C for 5 min and after that chilled on ice for 5 min. In the following, 7µl of a mastermix containing 4µl of reverse transcription buffer, 2µl of dNTPs and 1µl of Revert Aid RNase inhibitor are applied to the mixture and incubated for 5 min at 37°C. Finally, 1µl reverse transcriptase is added. After 60 min at 37°C the samples are heated to 70°C for 10 min to inactivate the reverse transcriptase. In the following, the cDNA is diluted to a final volume of 200µl in DEPC-H_2O. The cDNA can be used directly as a template for Realtime-RT-PCR or stored at -20°C.

Realtime-RT-PCR

1x Realtime-RT-PCR mix

2.5µl 10xPCR buffer
0.25mM dNTPs
0.3µM Primer forward
0.3µM Primer reverse
5nM FITC (Note that the addition of FITC is only necessary if the Biorad iCycler iQ® is used)
0.75µl SybrGreen (diluted 1:20000)
3.75U Taq
ad 25µl ddH2O

For the realtime-RT-PCR 23µl of the mastermix are mixed with 2µl cDNA. Samples are cycled with the programme depicted below.

94°C	3 min
95°C	10 seconds (40x)
60°C	20 seconds
55°C (+1°C/cycle up to 100°C)	10 seconds (45x)
16°C	Hold

The realtime-RT-PCR analysis is used for the relative quantification of target genes. Gene expression is measured by the so-called cycle threshold (ct) values. The ct value is the value at which background fluorescence levels are exceeded. Fluorescence signals (obtained by the intercalation of SybrGreen in double strand DNA) increase with increasing amount of PCR product. This means that the lower the ct value, the more product is contained in the reaction. For relative quantification, the different samples are normalised to their respective ct values obtained with a house keeping gene (i.e. Hprt or Gapdh). The use of a melting curve in the end of the PCR programme allows the discrimination between the desired product or primer dimers, because the latter melt at lower temperatures resulting in a peak at a lower temperature. Yet, agarose gel electrophoresis is also included as a quality control to visualise if primer dimers are present in the reaction.

3.2.15 Agarose gel electrophoresis with SB buffer

20xSB buffer: 20mM sodium hydroxide (titrated to pH8 with boric acid)

Loading buffer

50% glycerol
2.25% Ficoll (type 400)
0.04% bromphenol blue
0.9mM EDTA pH8

PCR products amplified by genotyping PCR or realtime-RT-PCR reactions are loaded on 1% agarose gels with 0.1µg/ml ethidium bromide and run in 1xSB buffer. Prior to loading, samples are mixed 1:5 with loading buffer. For separation, the gel is run at 300V for 15 min. DNA fragments are visualised under UV light.

3.2.16 β-galactosidase staining

X-Gal staining solution (in PBS)

1mM $MgCl_2$
1mM $MgSO_4$
7mM $K_3[Fe(CN)_6]$
7mM $K_4[Fe(CN)_6]$
1mg/ml X Gal

Embryos (E9.5-E12.5) are prepared from pregnant females (yolk sacks are removed and serve as template for genotyping PCR) and fixed in 4%PFA on ice for 10-15 min. After washing in PBS (at least 3x), the embryos are incubated in X Gal staining solution at 37°C until staining can be detected (after several hours up to over night incubation). After staining has become

visible, the staining solution is removed, embryos are washed in PBS (3x) and the embryos are re-fixed for 5 min in 4% PFA. After washing (3xPBS), embryos are transferred into a PBS/glycerol (1:1) mixture, in which they can be stored up to several months. The embryos are step-wise transferred to a higher glycerol concentration (i.e. 50%, 75%, 86%) and observed under a binocular for photography of the resulting staining.

3.2.17 Immunofluorescence

Mowiol

2.4g Mowiol 4-88
6 ml Glycerol
6 ml ddH_2O
12 ml 0.2M Tris pH8.5
0.6g DABCO

Cells are seeded and plated on cover slides pre-coated with 0.1% gelatine or in ibidi chamber wells in the desired volume (i.e. $1*10^4$ cells/24 well plate or ibidi chamber well). All incubation steps for the immunofluorescence staining are performed on a shaker.

3.2.17.1 Fixation with PFA

Cells are fixed in 4% PFA for 20 min at room temperature. After vigorously washing, cells are permeabilised by the addition of 0.1% PBST for 10 min. Next, unspecific bindings are blocked by incubation in blocking buffer (5% BSA in PBS) for 1h at room temperature. The primary antibody is diluted in blocking buffer and incubated at 4°C overnight. After washing with PBS (3x), the secondary antibody is added for 1h at room temperature (in the dark).
In the following, samples are washed and cell nuclei are counter-stained with Hoechst 33342 solution (staining for 1 min, followed by three washing steps). Samples are mounted in Mowiol. Stained cells are visualised by fluorescence microscopy.
Antibodies used in PFA fixed cells (and their respective dilution):

Primary antibody

Rabbit α Bre GTX111188 antibody (1:500)

Secondary antibodies

Alexa 488 goat-anti-rabbit (1:1000)

3.2.17.2 Fixation with methanol and acetone

Cells are fixed with methanol (-20°C, 5min) and acetone (-20°C, 2min), washed (3x PBS) and blocked afterwards with 1% BSA (in PBS) for 1h at room temperature. Primary antibodies are diluted in blocking solution and incubated overnight (at 4°C). After washing and incubation with the corresponding secondary antibodies (1h at room temperature, in the dark) cell nuclei

are stained with Hoechst 33342 or 33258, respectively. Samples on cover slides are mounted in Mowiol and samples in ibidi chambers are stored in PBS, respectively, until they are observed under a fluorescence microscope.

Primary antibodies

Goat α BRCA1 I-20 **(**1:500)

Rabbit α Phospho-Histone H2AX (ser139) 20E3 (1:400)

Secondary antibodies

Alexa 488 chicken-anti-rabbit (1:1000)

Alexa 568 donkey anti-goat (1:1000)

3.2.18 Protein sample preparation and Bradford protein determination

RIPA buffer

50 mM Tris pH 8.0
150 mM NaCl
1 % Nonidet P-40
0.5 % Deoxycholat
0.1 % SDS
+ 50 µg/ml PMSF (serine protease inhibitor) + 1x protease inhibitor mix (PI)

1xSDS loading buffer

50 mM Tris-HCl pH 6.8
2 % SDS
0.125 % brome-phenol blue
12.5 % glycerol
100 mM DTT

Cells are washed with PBS and harvested on ice by scratching with a rubber. After centrifugation (1000-3000 rpm), pellets are either directly resuspended in 2xSDS loading buffer or lysed in RIPA buffer in 2 x pcv (packed cell volume). Alternatively, cell pellets may be frozen in liquid nitrogen and stored at -80°C until further use. Cells in RIPA buffer are incubated for at least 30 min at 4°C (on ice or on a rotating wheel) and then centrifuged at maximal speed for 10 min. The obtained supernatant is transferred to a new tube. Protein concentration of the sample can now be determined via Bradford reagent. For this, the Bradford solution is diluted 1:5. 1µl of protein samples are added to 1 ml of Bradford dilution, the sample is vortexed and transferred to cuvettes. In the following, sample concentrations are determined together with a standard curve established with BSA dilution samples. Measurement is done on a Varian 50 UV-VIS spectrophotometer. In the following, protein samples are mixed with 4xSDS loading buffer. Samples in SDS loading buffer are boiled for

5 min at 100°C and centrifuged. Now, samples are ready for SDS poly acrylamide gel electrophoresis or can be stored at -20°C.

3.2.19 SDS PAGE (poly-acrylamide gel electrophoresis)

Stacking gel

1 ml Rotiphorese 30 gel
3 ml H_2O
750µl 1 M Tris, pH 6.8
30 µl 20 % SDS
60 µl 10 % APS
6 µl TEMED

Separating gel (12%)

6 ml Rotiphorese 30 gel
3 ml H_2O
5.6 ml 1 M Tris, pH 8.8
75 µl 20 % SDS
150 µl 10 % APS
6 µl TEMED

1x SDS running buffer

25 mM Tris
192 mM Glycin
1 % SDS

For SDS poly acrylamide gel electrophoresis, 12% gels are prepared in a SDS PAGE device. 10-20µl or 50-100µg, respectively, of protein samples are loaded together with a protein standard marker. Gels are run for 1.5-2h in SDS running buffer at 120V.

3.2.20 Western blot analysis

Blotting buffer

25 mM Tris
150 mM glycine
10 % Methanol

ECL solution

100 mM Tris HCl pH 8.0
250 mM luminol
90 mM coumaric acid
1.05% H_2O_2

Gels destined for Western blot analysis are transferred onto 0.45µm nitrocellulose membranes by blotting either in a semi-dry blot or with a wetblot device. Gels are placed on the

membrane in between layers of Whatman paper; all of the components are transferred into blotting buffer. Conditions for semi-dry blotting are 25V, 360mA for 35-60 min (for 1-4 gels, respectively). Wet blot analysis is performed at 25V for 2h. The latter wet blot conditions are necessary for Western blot analysis with human αK63-ubiquitin as primary antibody. After blotting, membranes are transferred into 5% milk (in PBS) for blocking. After at least 1h at room temperature, primary antibodies are added (incubation for 1h at room temperature or overnight at 4°C). After washing (3x), the membrane is incubated with secondary antibody for 1h at room temperature (for antibody dilutions of primary and secondary antibodies compare lists below). Next, the membrane is washed in PBS (3x) and ECL developing solution is added for 1 min. In the following, the membrane is transferred into a film cassette, a film is put on the membrane (for 30s to 2h, depending on the signal strength) and the film is processed.

Primary antibodies

Mouse α Flag-M2 (1:2000)

Mouse α HA-probe F7 **(**1:1000)

Human α K63 ubiquitin (1:350)

Mouse α β Tubulin AA2 (1:10000)

Mouse α Ubiquitin P4 D1 (1:1000)

Secondary antibodies

α Mouse-HRP (1:5000)

α Human-HRP (1:5000)

3.2.21 Coomassie staining of SDS poly-acrylamide gels

Staining solution

50% methanol
10% acetic acid
0.05% Commassie R-250

Destaining solution

10% Isopropanol
10% acetic acid

After SDS-PAGE, gels are incubated in Coomassie staining solution for 20 min for the visualisation of protein bands. Next, the gel is rinsed with H_2O and incubated in destaining solution for 20 min (2x). In the following, the gel is completely destained by over night incubation in the destaining solution.

3.2.22 Co-Immunoprecipitation

In CoIP (co-immunoprecipitation) experiments, the interaction potential of two proteins is determined after the transfection of the corresponding plasmids encoding for these proteins. For this, 10 cm plates of HEK 293T cells are transfected with 6 µg of each plasmid (alone or as a combination of both plasmids) by PEI transfection. After 48h, cells are washed twice in PBS, harvested (on ice) with a cell scraper and centrifuged at 1000 rpm for 5 min. The resulting pellet is resuspended in 1 ml of RIPA buffer. Cells are incubated for at least 30 min at 4°C on a rotating wheel (all following incubations are also performed on the wheel, at 4°C). Next, cell debris is pelleted by high speed centrifugation. The supernatant is transferred to a new tube and 5% are taken as an input sample (and mixed with 4xSDS loading buffer). The residual solution is incubated with 1µg of antibody (overnight). The following day, 40 µl of protein A agarose beads are added to each tube. After at least 2 h, beads are centrifuged at 3000 rpm for 1 min and washed with PBS (3-5x). The beads are resuspended in 40µl of 2xSDS sample buffer without DTT and boiled at 100°C (5 min). After high speed centrifugation, supernatants are transferred to new tubes and 1mM DTT solution is added. Samples are now ready for Western blot analysis or can be stored at -20°C.

3.2.23 Purification of nuclear and cytoplasmic fractions

Hypotonic buffer

10 mM Hepes pH 7.9
10 mM KCl
1.5 mM $MgCl_2$
+ 50 µg/ml PMSF + 1x PI
(+ 0.4% NP-40)

High salt buffer:

20 mM Hepes pH 7,9
420 mM KCl
1.5 mM $MgCl_2$
2 mM EDTA
20% glycerine
+ 50 µg/ml PMSF + 1x PI

After harvesting of the cells and centrifugation (1000 rpm, 5min, 4°C), the cell pellet is resuspended in 4 x pcv of hypotonic buffer (without NP40). Cells are incubated on ice for 10 min and centrifuged at 1000 rpm (5 min, 4°C). Now, the obtained pellets are resuspended in 4 x pcv of hypotonic buffer with 0.4% NP40. After swelling on ice (10 min), samples are centrifuged (1500 rpm, 5min, 4°C). The resulting supernatant represents the cytoplasmic

fraction; the pellet contains the nuclear fraction and is resuspended in 2 x pcv High-Salt buffer. After incubation on a rotating wheel for 30 min at 4°C, the nuclear debris is pelleted in high speed centrifugation (30 min, 4°C) and the supernatant (= nuclear extract) is transferred to a new tube. Both, the cytoplasmic and the nuclear fraction are mixed with 4xSDS loading buffer, boiled for 5 min and centrifuged (maximal speed, 10 min). Samples can now be analysed by SDS PAGE and Westernblot analysis.

3.2.24 Ubiquitination assay

In an ubiquitination assay, potential substrates and E3-ligases are combined together with a source for ubiquitin and it is analysed whether the substrate is modified by ubiquitination. For this, 10 cm plates of HEK 293T cells are transfected with 3µg of plasmid encoding for the potential substrate together with or without the potential E3-ligase. Additionally, 6µg of pcDNA3-HA-Ubiquitin are added into all transfection reactions as an exogenous source for ubiquitin. 48h after transfection, cells are treated with 20mM of MG132 or left untreated. After further 6h, cells are harvested in RIPA buffer supplemented with 50 µg/ml PMSF and 1x PI as well as with 20mM of the NEM inhibitor (an ubiquitin isopeptidase inhibitor). In the following, input samples are taken and a CoIP is performed with an antibody specific for the potential substrate. Next, samples are analysed by SDS-PAGE and Westernblot analysis.

3.2.25 Induction of bacterial MBP and MBP Bre protein and incubation with cellular lysates on amylose resin

For the induction and purification of bacterially expressed protein, pMBP and pMBP Bre plasmids are transformed into BL21/DE3 Lys competent bacteria. For this, 50 ng of plasmid DNA is added to 50µl of bacteria suspension. After 20 min on ice, samples are heat shocked (42°C, 45s) and then chilled on ice for 2-3 min. Next, 300µl of LB medium are added and the transformation reaction is incubated at 37°C for 1h on a shaker. Then, the suspension is plated on LB agar plates (with Ampicillin/chlorampenicole). One clone each is used for inoculation of 50 ml of overnight culture (incubation on a shaker, at RT). The next morning, 5 ml of the culture are transferred into fresh medium (50ml) and shaken for one further hour. In the following, 0.1mM of IPTG is added for the induction of protein expression. After 3h at room temperature, cultures are harvested by centrifugation. Pellets are resuspended in 2.5ml of PBS and frozen at -80°C. Following two freeze and thaw (on ice-water) cycles, supensions are sonified by an ultrasound sonifier with 10% amplitude for 5-10 cycles (until a clear suspension is obtained). Samples are frozen in aliquots and stored at -80°C until further use.

HEK 293T cells are transfected with pcS2p Flag Hey1 plasmid (6µg/10cm plate) and after 48h cells are either treated with 20mM of MG132 or left untreated. 6h later, cells are lysed in 1ml of RIPA buffer (+PMSF, PI and NEM inhibitors). After 30 min on a rotating wheel at 4°C, samples are centrifuged at maximum speed; an input sample is taken and the residual supernatants are pre-cleared. For this, the samples are incubated with 30µl of amylose beads and 30µl of MBP protein solution for 2h at 4°C on a rotating wheel. After centrifugation (3000 rpm, 5 min) the supernatant is incubated with 30µl of amylose beads (which have been pre-coupled with MBP Bre- or MBP-protein, respectively) for further 2 hours. The coupled beads have been generated by the incubation of 30µl beads with 100µl of MBP- or MBP-Bre protein for 2h. After extensive washing and centrifugation (3000 rpm, 5 min), the beads are ready for the incubation with the pre-cleared cellular lysates. After the incubation with the cellular lysates, the beads are washed extensively (at least 5x) and resuspended in 2x SDS loading buffer. After boiling and centrifugation, samples are ready for SDS-PAGE and Westernblot analysis with αFlag and αubiquitin antibodies, respectively. Furthermore, MBP- and MBP-Bre proteins are detected by Coomassie staining after SDS-PAGE.

3.2.26 Determination of Hey1 turnover after Bre over-expression

Cycloheximide (Chx) is an inhibitor of translational elongation by interfering with the translocation step in protein synthesis. Therefore, after addition of Chx no further protein is synthetized and thus the half life time of any protein can be determined. Additional treatment with MG132 (a proteasome inhibitor) blocks protein degradation via the ubiquitin-proteasome pathway, thereby leaving protein levels unaffected after Chx addition. For the determination of Hey1 turnover 293tet-FS-mHey1 cells are employed. Stimulation with 100ng/ml Doxycycline leads to an efficient and robust induction of FS-mHey1 expression. 72h after FS-mHey1 induction, cells are treated with 0.1mM Chx with or without 20mM MG132 and samples are harvested at the desired time points. Pellets are frozen in liquid nitrogen until further use. After thawing on ice, pellets are resuspendend in RIPA buffer followed by Bradford protein determination. Next, equal protein amounts are loaded on SDS-PAGE and analysed by Westernblot. Plasmids, which shall be tested for their effect on FS-mHey1 expression are transfected 48h after the start of the Doxycycline induction via the PEI protocol. After 6-8h cells are distributed on the desired number of 6 well plates after trypsinisation. In the following, the assay is performed as described for the non-transfected cells.

3.2.27 Luciferase Assay

Luciferase buffer

25mM glycyl-glycine pH 7.8
15 mM $MgSO_4$
15 mM KPO_4 pH 7.8
4 mM EGTA
1 mM DTT (added just prior to analysis)

Lysis buffer

1% Triton X100 in Luciferase buffer

Assay-Puffer

1mM ATP in Luciferase buffer
0.1µg/µl D-luciferin (in 25mM glycyl-glycine)

HEK 293T cells are seeded in 24 well plates in triplicates and are transfected with the desired plasmids following the PEI transfection protocol. Non-transfected cells are included in the analysis for background subtraction after luminescence measurement. 48h after transfection, cells are lysed by incubation in 150µl of lysis buffer (10 min, RT on a shaker). Cells are rinsed from the plate and cellular debris is pelleted by high speed centrifugation for 10min; the supernatants are transferred to new tubes and can either be frozen and stored at -20°C or they can directly be used for measurement. For this, 50µl of sample are pipetted into black FIA 96 well plates. For the measurements, which have a duration of 2s/well, 150µl of assay buffer/well are injected by the Glomax luminometer with a delay of 5s between the different measurements.

3.2.28 Cytotoxicity assay with FasL or TNFα

A cytotoxicity assay is used for the determination of the sensitivity of target cells towards a certain substance. For this, the LD_{50} (i.e. the concentration, at which 50% of the cells have been killed) is determined by generation of survival curves after treatment with the cytotoxic agent. Prior to stimulation with FasL or TNFα, cells are pre-sensitised with Chx (2.5µg/ml, 1h) to inhibit the translation and hence the protective effects of proteins with anti-apoptotic properties. In the following, cells are treated with different amounts of FasL or TNFα starting from 1µg/ml in 1:3 dilution steps. Experiments are performed in 96 well plates in triplicates. After 16h, cell survival is monitored by a MTT assay.

4. Results

4.1 Generation of Hey-triple-KO-ES cells

The differentiation of embryonic stem cells (ES cells) as so-called embryoid bodies (EBs) is a well-established system to study early embryonic development *in vitro*. In this work, Hey-less ES cells were generated as a tool for the analysis of Hey gene function during development. E3.5 blastocysts were flushed from pregnant $Hey1^{fl/fl}$/Hey2/HeyL mice and plated on gelatine coated four well plates. The outgrown inner cell masses (ICMs) were dissociated with Accutase (after 3-4 days) and henceforth cultured on mitotically inactivated BALB/c MEF-feeder layer (in a 96 well format). When cell numbers were increased and the cultures established (after 2-3 passages), cells were pre-plated and then transferred to gelatine-coated dishes. After the successful removal of residual feeder cells, cells were subjected to PCR genotyping. ES cells with a $Hey1^{fl/fl}$/$Hey2^{-/-}$/$HeyL^{-/-}$ genotype were electroporated with 5µg of pTurbo-Cre plasmid. After electroporation, cells were plated at low density to allow picking of single colonies. The derived clones were genotyped by Flox Hey1- vs. Hey1 del-genotyping PCR. Cre recombinase led to an efficient deletion of the floxed Hey1 alleles (Fig. 4 depicts the workflow of the establishment of ES cell cultures).

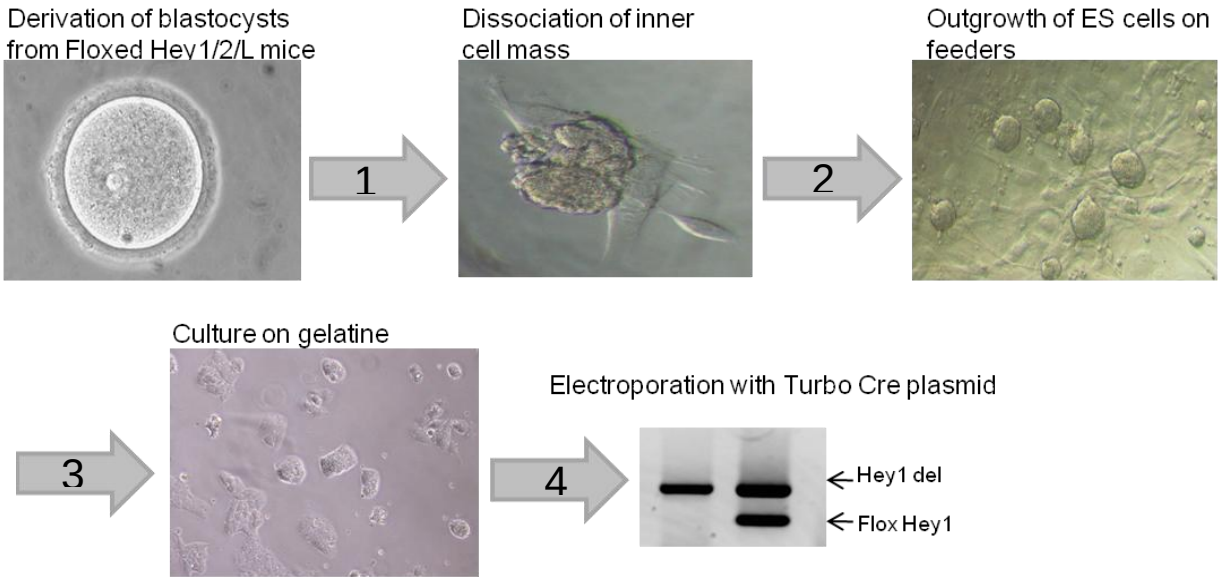


Fig. 4 Schematic overview of ES cell derivation
(1) Plating of flushed blastocyst on gelatine, (2) transfer of resulting inner cell mass on feeder cells, (3) establishment of culture on gelatine coated dishes, (4) genotyping of electroporated ES cell clone after successful deletion of the floxed allele

4.2 Karyotyping of Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$ - and Hey-triple-KO-ES cells

During a first test differentiation, a substantial number of EBs disintegrated between day 2 and day 3. As this was an unexpected behaviour, these cells were tested for chromosomal aberrations by karyotyping. Some of the analysed ES cells indeed displayed karyotype instability (with a chromosomal content of mostly n~60) or showed a tendency to develop tetraploidy. Therefore, cells were further sub-cloned (cells were plated as single cells and then single colonies were picked) and the chromosome content per cell was counted in the derived clones (example for metaphase spread, see Fig. 5a). For further investigation, cells with an overall chromosome content of n=40 were passaged several times and then used for DAPI staining. FACS analysis was performed (by Richard Friedl) to check for aneuploidy or polyploidy of the cells. For an internal control, chicken erythrocytes were added to the cell suspensions. As a result, a distinct cell cycle profile as expected for euploid cells could be observed for the Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$- as well as for different Hey-triple-KO-clones (Fig. 5b). Differentiation of these cells could be achieved repeatedly, without high rates of cell death in the progeny, so that the karyotype of these cells can be regarded as stable over time.

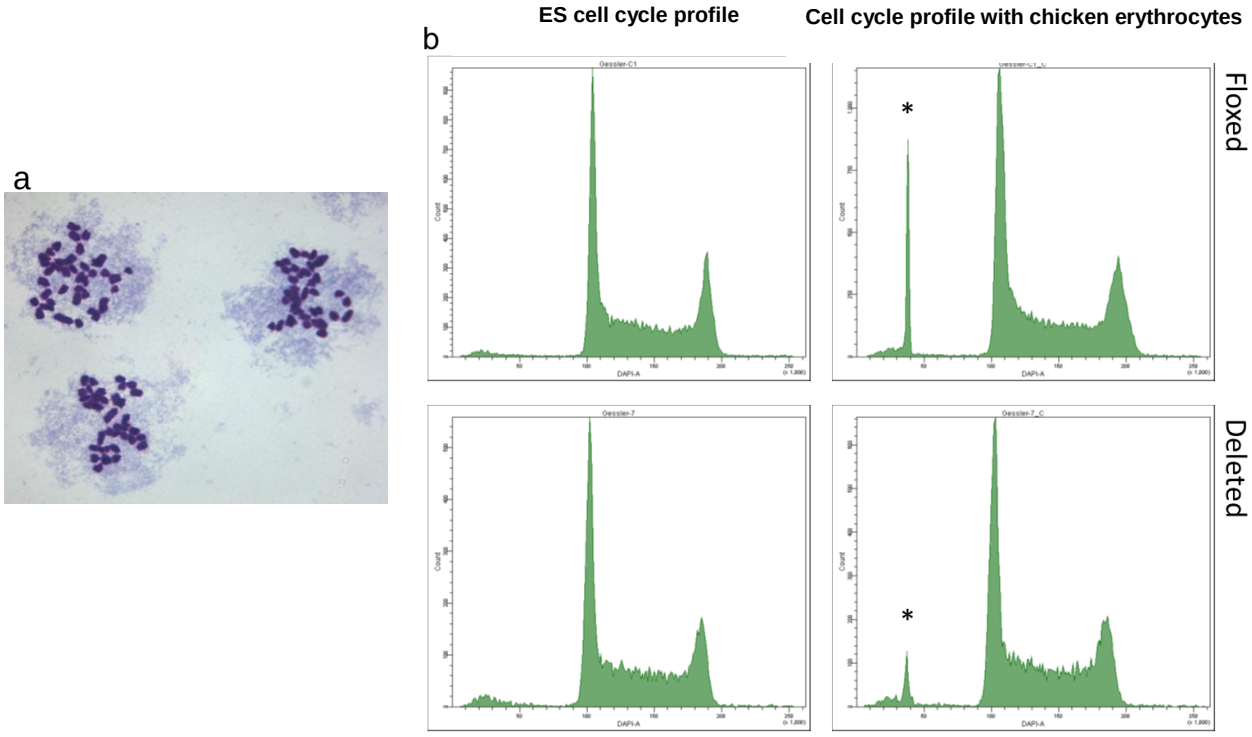


Fig. 5 Karyotype stability of floxed and triple KO-ESC
(a) At least 50 nuclei of metaphase spreads were analysed for chromosomal content and only ES cell clones with a vast majority of cells with n=40 were further used. (b) FACS cell cycle analysis (performed by Richard Friedl) confirmed that neither an aneuploid nor a polyploid subpopulation was present in the analysed clones; the peak marked with an asterisk represents the peak obtained for chicken erythrocytes.

4.3 Pluripotency of Hey-triple KO-ES cells

To assess whether Hey-triple KO-ES cells are *bona fide* ES cells and display pluripotency features, two analyses were performed. First, the ES cells ($Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$ - as well as Hey-triple-KO- clones) were tested for Alkaline Phosphatase (ALP) activity and second, pluripotency marker gene expression was tested by realtime-RT-PCR. In both cases it could be demonstrated that $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$ as well as Hey-less ES cells fulfilled stemness criteria: The ALP staining was positive, resulting in a red staining of all cell colonies (see Fig. 6 a) and the pluripotency marker genes Oct4 and Nanog were highly expressed (Fig. 6 b).

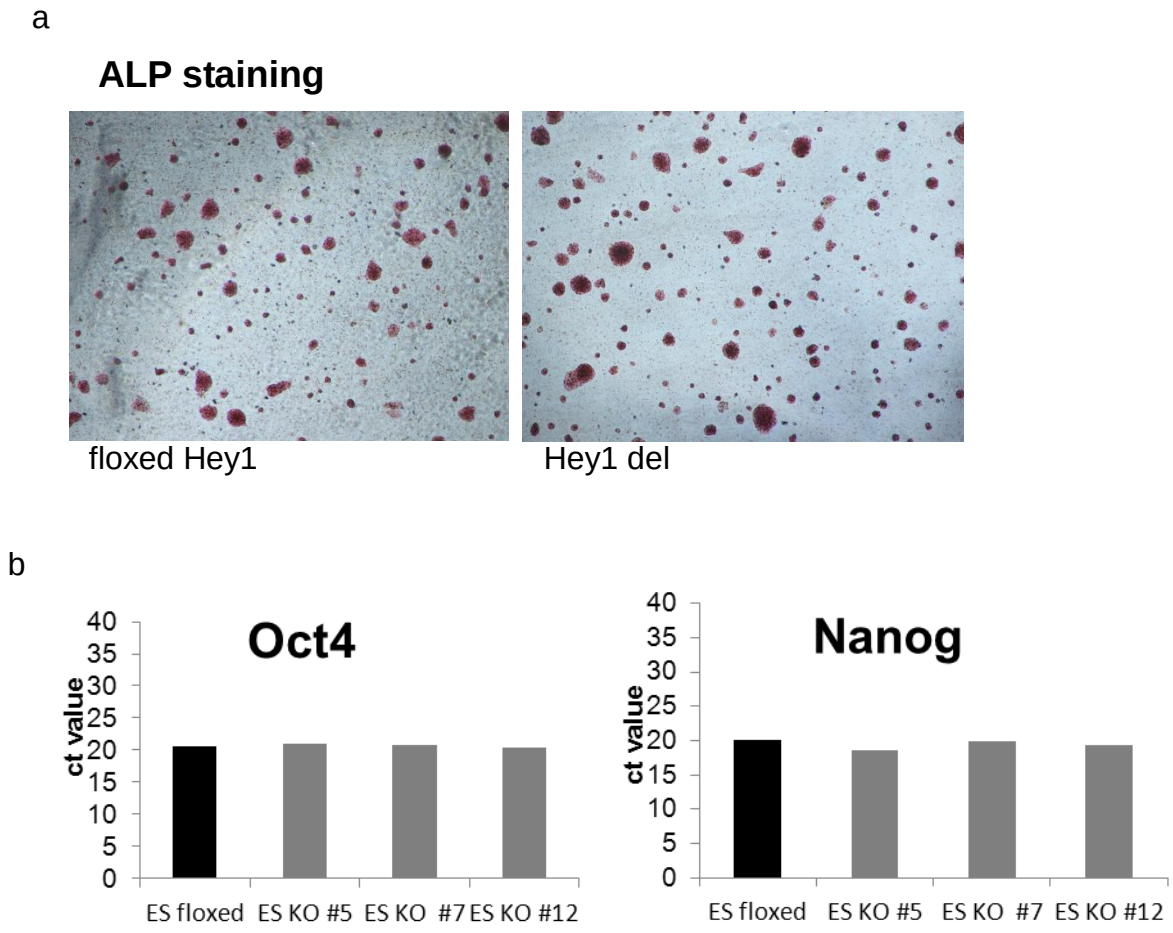


Fig. 6 Pluripotency of $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$- and Hey-triple-KO-ES cells
(a) Positive ALP staining of $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$- and Hey-triple-KO-ES cells
(b) high Oct4 and Nanog marker gene expression in realtime-RT-PCR analysis can be regarded as a sign for the successful generation of bona fide embryonic stem cells.

4.4 Differentiation of Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$- and Hey-triple-KO-ES cells

Besides the above described pluripotency marker gene expression and ALP activity, ES cells are only regarded as pluripotent if they can give rise to cells originating from all three germ layers (i.e. endoderm, mesoderm and ectoderm). To test for this, ES cells were differentiated as EBs for 10 days and RNA was harvested from ES cells and after 2, 4, 7 and 10 days of differentiation, respectively (i.e. EB d2, EB d4, EB d7 and EB d10). The formation of EBs is depicted in Fig. 7. The pictures below were chosen arbitrary: In all cases, small and big EBs were formed without any preference for the formation of small or big EBs in any of the differentiated clones. However, in the deleted clone 12, a higher number of EBs was formed in the beginning, followed by a more extensive decay and debris formation afterwards compared to the three other clones. The harvested RNA was transcribed into cDNA. This was followed by realtime-RT-PCR (performed by Anja Winkler) for marker gene expression.

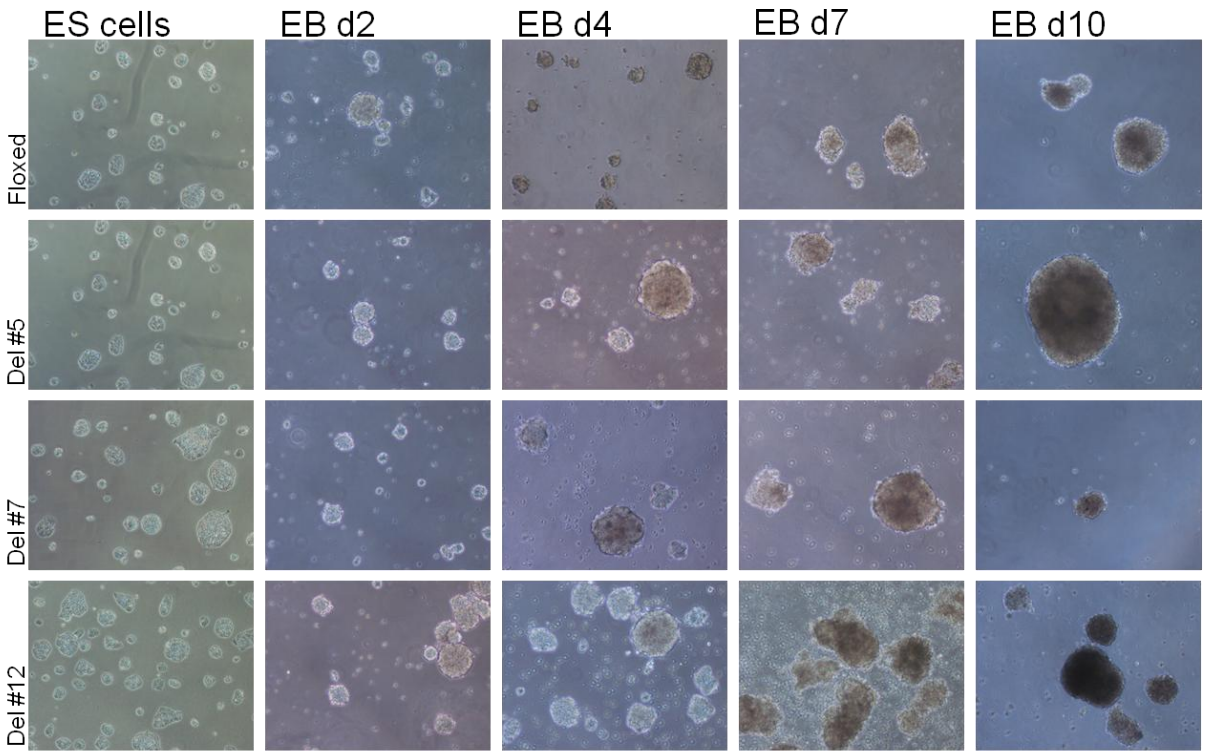


Fig. 7 Differentiation of ES cells as EBs
For the induction of ES cell differentiation, cells were grown in suspension culture on bacterial dishes on a rotary shaker without the addition of LIF. Embryoid bodies formed well in all clones analysed and were harvested for further analysis by realtime-RT-PCR at the indicated time points.

Three Hey-triple-KO clones (i.e. Del #5, 7 and 12) were compared to the Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$-clone from which they were derived. The genes analysed are summarised in Table 1. Genes were only regarded as being regulated if the delta cycle

threshold (ct) value (i.e. the PCR cycle at which background fluorescence is exceeded in the reaction for the first time) of floxed – deleted samples was more than -/+ 1.5 (regulated genes are shown in Fig. 8). Samples were normalised to the average expression determined for the Hprt and Gapdh house keeping genes.

As expected, neither Hey2 nor HeyL expression could be detected in any of the clones analysed. This means that the knock out of the genes was confirmed not only on the basis of genomic DNA as tested by the genotyping PCR, but also on mRNA levels. Furthermore, in none of the Hey-triple-KO-ES cell clones, Hey1 expression could be detected, thus these cells can really be regarded as Hey-less ES cells. In the floxed clone, however, Hey1 expression was easily detectable from day 4 on (in ES cells, only very low basal Hey1 expression could be detected) and the expression persisted and increased until day 7 and peaked on day 10.

Table 1 **Marker genes analysed by realtime-RT-PCR**
Hey family members
Hey1, Hey2 , HeyL
Pluripotency marker
Oct 4
Germ layer markers
AFP, Brachyury, Nestin
Endothelial markers
VE-Cadherin, Tie1
Neural markers
Neurog2, Mash1 ,Olig2, Dat1, Tubb3
Myogenic markers
Myogenin (MyoG), Myotilin (MyoT)
Other markers
Gata4, Gata6, Snail, Bre, Nkx2.5

Consistent with an efficient differentiation of the ES cells, Oct4 pluripotency marker gene expression was strongly down-regulated during the course of differentiation. An increase in the expression of all three germ layer marker genes (AFP as an endodermal, Brachyury as a mesodermal and Nestin as an ectodermal marker) could be seen following differentiation.
For Brachyury, no difference between Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$- and Hey-triple-KO-cells could be seen (data not shown). However, a differential regulation in two of the three deleted clones compared to their floxed Hey1 counterpart could be observed in the case of AFP and Nestin marker gene expression: #7 and 12 showed a higher expression of AFP and a lower expression of Nestin on EB d10 compared to the floxed control. Consistent with the latter observation, most of the neural marker genes analysed showed a down-regulation in these two clones, too. Dat1, the only neuronal marker, which increased in expression in Hey-less clones compared to the floxed Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$- clone was regulated in all three clones to the same extent. For the myogenic marker Myogenin (MyoG), an up-regulation for all three Hey-triple-KO-clones could be found. Myotilin (MyoT) on the contrary, is again only regulated in #7 and 12 in that it showed a diminished expression compared to the floxed clone. Besides these genes, an up-regulation of Gata4 and Gata6 could be observed in clones 7 and 12 (for differential marker gene regulation compare Fig. 8).

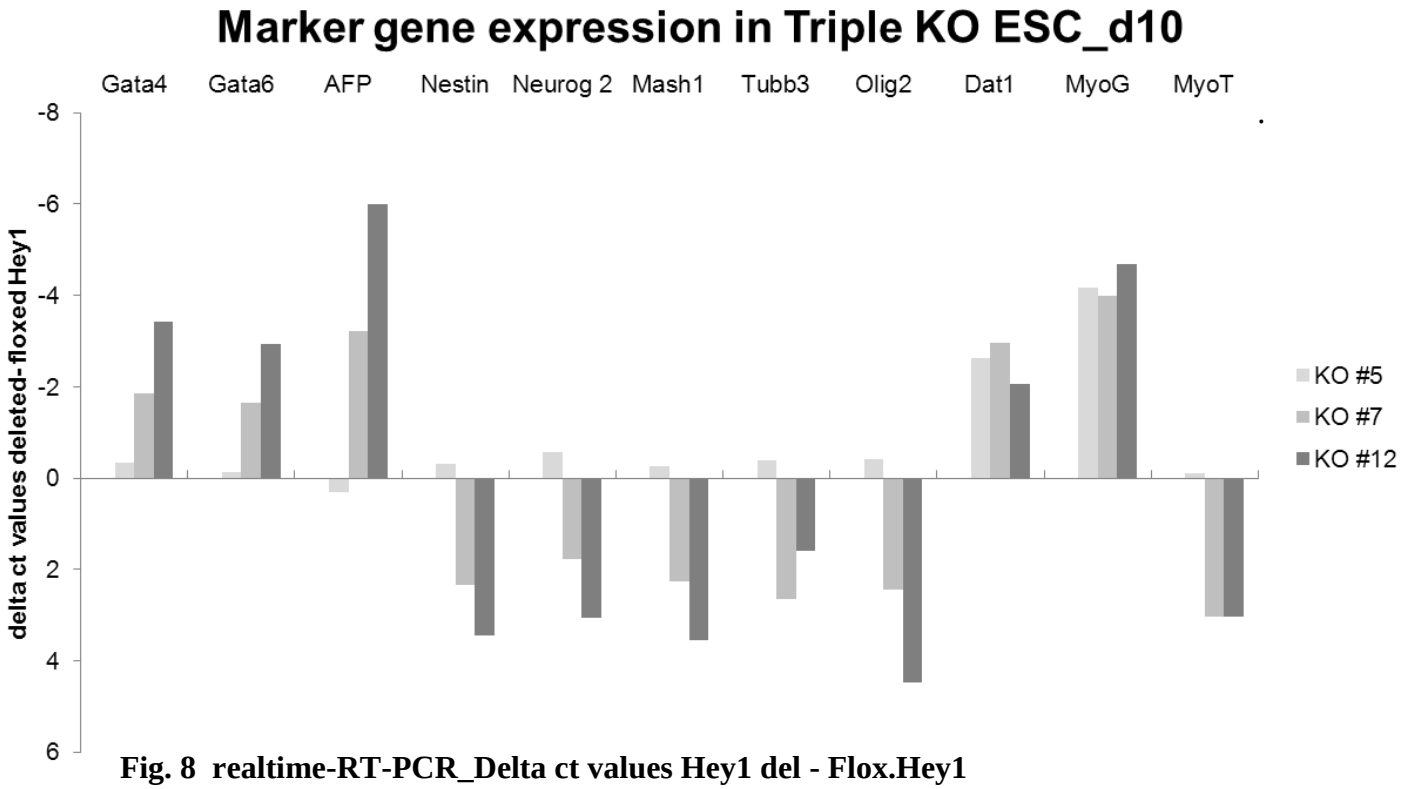


Fig. 8 realtime-RT-PCR_Delta ct values Hey1 del - Flox.Hey1
Graphical overview of up- and down-regulated genes in triple-KO-ES cells compared to the Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$- clone
For #5, only Dat1 and MyoG expression showed differential regulation (i.e. a delta ct value of more than -/+ 1.5) compared to the floxed Hey1 clone.

For all the other genes tested (i.e.VE-Cadherin, Tie1, Snail, Bre, Nkx2.5) no expression differences between Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$- and Hey-triple-KO-clones could be observed. Notably, any differential gene regulation could only be observed on EB day10, as at all earlier time points no significant differences between floxed and deleted clones were detectable.

4.5 Hey-triple-KO-ES cells can be genetically manipulated

The realtime-RT-PCR results described above revealed potential Hey1 target genes. For Hey2 and HeyL, however, further manipulation of the Hey-triple-KO-ES cells would be needed so that one could also investigate their functions during differentiation. Furthermore, validation of the potential Hey1 target genes by rescue experiments with exogenous Hey1 would be necessary. This can be done by introducing stable or inducible constructs encoding for one of the Hey genes and by comparing these cells to control-transduced/control-transfected or non-induced cells, respectively.

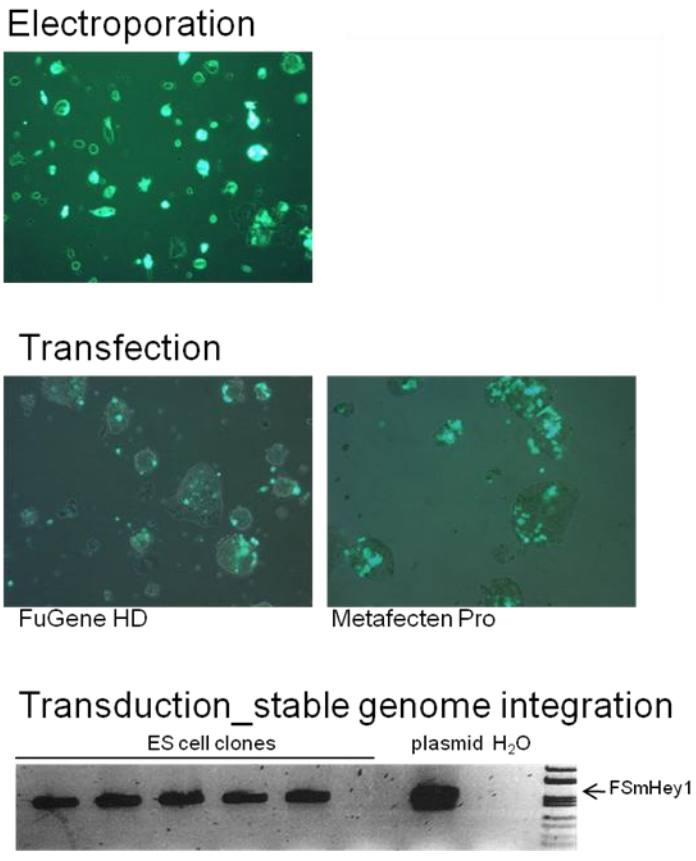


Fig. 9 Successful manipulation of triple KO-ES cells
Different methods and agents were used to either express GFP (a,b) or to obtain stably integrated DNA in the ES cell genome (c).
(a) electroporation of ES cells with the Lonza nucleofector; (b) transfection of ES cells by FuGene HD and Metafecten Pro; (c) stable DNA integration in ES cells after viral transduction as confirmed by genotyping PCR

To determine methods for the efficient targeting of ES cells, it was necessary to test whether the obtained ES cells can be manipulated by electroporation, transfection or transduction. Therefore, ES cells were either electroporated or transfected with different transfection reagents. As a test plasmid, pLL3.7 (encoding for GFP) was introduced into the cells, so that successful uptake of the construct could be monitored via GFP expression. Electroporation as well as two of different transfection reagents tested resulted in a good GFP expression (Fig. 9 a and 9 b). For the transduction of ES cells, diverse viral constructs encoding for inducible Hey constructs were used. Only in the case of the EF1TA3p-FSmHey1 construct, clones were obtained, which were further tested for protein expression by Westernblot. Even though these clones could not be used for an efficient and continuous induction of Hey1 protein expression (data not shown), PCR with genomic DNA from these cells as a template revealed that at least a 291bp fragment spanning the Flag Strep-tag and a N-terminal part of Hey1 was stably integrated into the ES cell genome. Thus, the Hey-triple-KO-ES cells can in general also be targeted and manipulated by viral transduction. This means that the ES cells described above could be used for the generation of stable or inducible Hey-expressing cells by any of the three methods. Hence, these cells represent a valuable tool for the future investigation of Hey function.

4.6 Bre is a Hey interaction partner

In an attempt to identify novel Hey binding partners, a yeast-two-hybrid screen was performed with a Hey1 C-terminal bait spanning aa 266-299. In this screen, the Bre protein was found to be an interaction partner of Hey1. This could be confirmed in co-immunoprecipitation experiments. Furthermore, an interaction of Bre with Hey2 and HeyL as well as with the Hes5 protein could be shown. Hes1, another bHLH downstream factor of the Notch signalling pathway, however, did not bind to Bre. This allowed the construction of Hes1-Hey1 chimeric proteins to narrow down the binding domain in Hey1 responsible for the interaction: The N-terminal part of Hes1 was fused to the C-terminus of Hey1. In doing so, a protein now capable of Bre binding was generated. Further deletion and mutation of the Hey1 C-terminus in the chimeric protein revealed that the region necessary for efficient Bre binding consists of a seven amino acid sequence. Mutation of two phenylalanine residues in the Hey1 AFPFSFS motif into alanine resulted in a loss of Bre binding (all the experiments described above were performed by Andreas Fischer; data not shown; for the binding motif compare Fig. 10).

```
Hey1:    263 A F P F S F S 269
Hey2:        S F P L S F A
mut Hey1:    A A P F S A S
```

Fig. 10 Amino acid motif responsible for efficient Hey1 and Bre interaction
A seven amino acid sequence in the Hey1 C-terminus is sufficient for the interaction with the Bre protein; mutation of two phenylalanine amino acid residues into alanine (=F264/8A mutant) abolishes the interaction.

The aim of this work was to confirm the interaction between Hey1 and Bre and to determine the domain of Bre, which is crucial for the interaction with Hey1. Bre consists of two UEV-domains, which have both been shown to be involved in the interaction with other proteins. Therefore, Bre deletion mutants were constructed and cloned into the pCS2p-HA-Cherry vector. For this, the Bre-protein was subdivided into three parts containing either one of the UEV-domains (Bre-UEV1 = aa1-138, Bre-UEV2 = aa 230-383) or the region spanning the middle part of Bre, which lacks any known domain (Bre-mid = aa 138-228). For a schematic overview, see Fig. 11. Before the constructs were used for co-immunoprecipitation experiments, the sub-cellular localisation of the respective proteins was observed by fluorescence microscopy. All of the proteins still showed nuclear localisation as it can be observed for the HA-Cherry-fulllength-Bre (compare Fig. 11). However, the proteins

consisting of the UEV1-domain and the Bre-middle part also had a prominent cytoplasmic fraction (yet the cytoplasmic signal was still more pronounced for the HA-Cherry protein without any Bre fusion), whereas the construct spanning the UEV2 mostly localised to the nucleus like the full-length Bre protein.

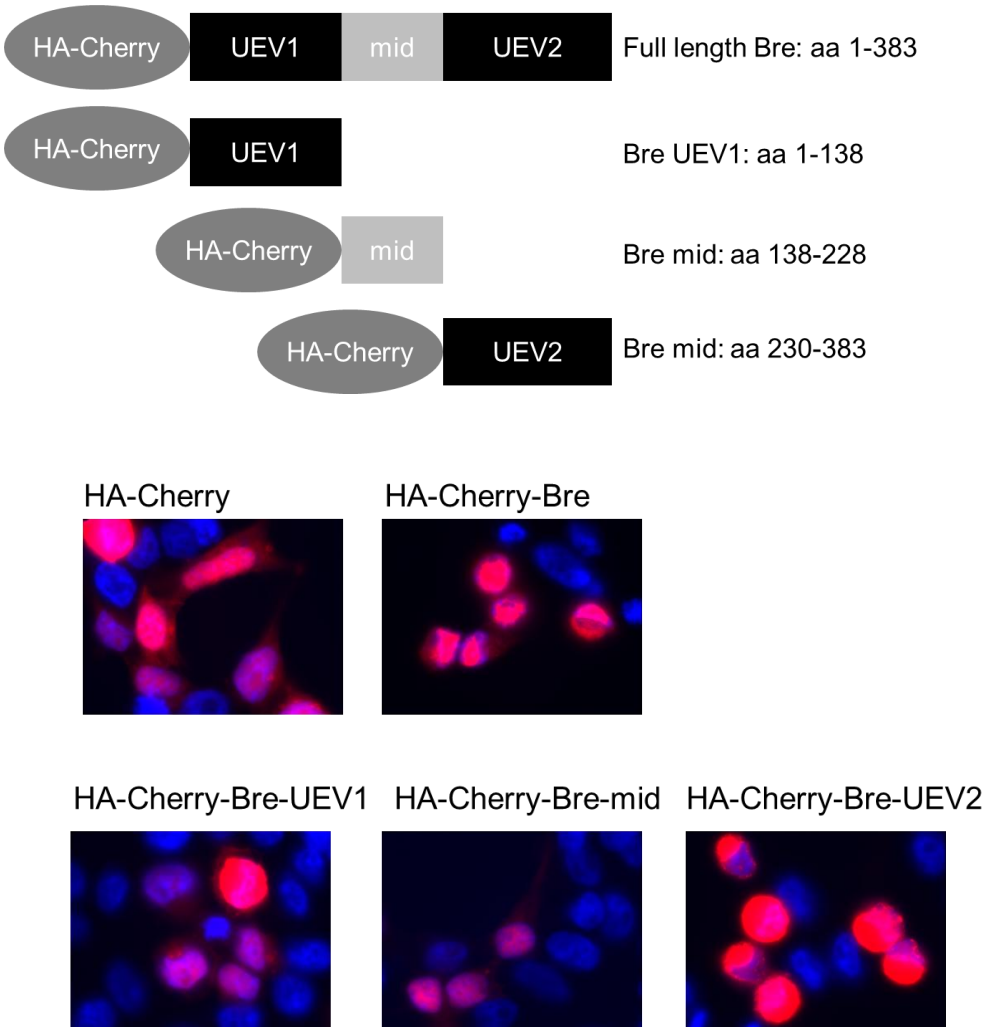


Fig. 11 Localisation of the different HA-Cherry-Bre fusion proteins
HEK 293T cells were transfected with HA-Cherry, HA-Cherry full-length-Bre or different HA-Cherry-Bre fusion proteins (i.e.HA-Cherry-Bre- UEV1, -mid,- UEV2). 48h after transfection, cell nuclei were counterstained with Hoechst 33342 and cells were observed under a fluorescence microscope.

After the determination of the sub-cellular localisation of the different Bre fusion proteins following over-expression, it was tested in co-immunoprecipitation experiments whether all of the fusion proteins still interacted with Flag-Hey1 or whether there is a specificity of Flag-Hey1 towards one of the fusion proteins. Therefore, HEK293 T cells were transfected with Flag-Hey1 and the different HA-Cherry-Bre constructs. Furthermore, HA-Cherry was included as a negative control and HA-Cherry-Bre as a positive control for the interaction

with Flag-Hey1. Samples were analysed by Westernblot with mouse αFlag and mouse αHA antibodies as primary antibodies and αmouse HRP as the secondary antibody. As expected, HA-Cherry-Bre showed clear binding to Flag-Hey1 (confirming the findings by Andreas Fischer), whereas HA-Cherry was not pulled down, when a Flag-IP was performed (Fig. 12).

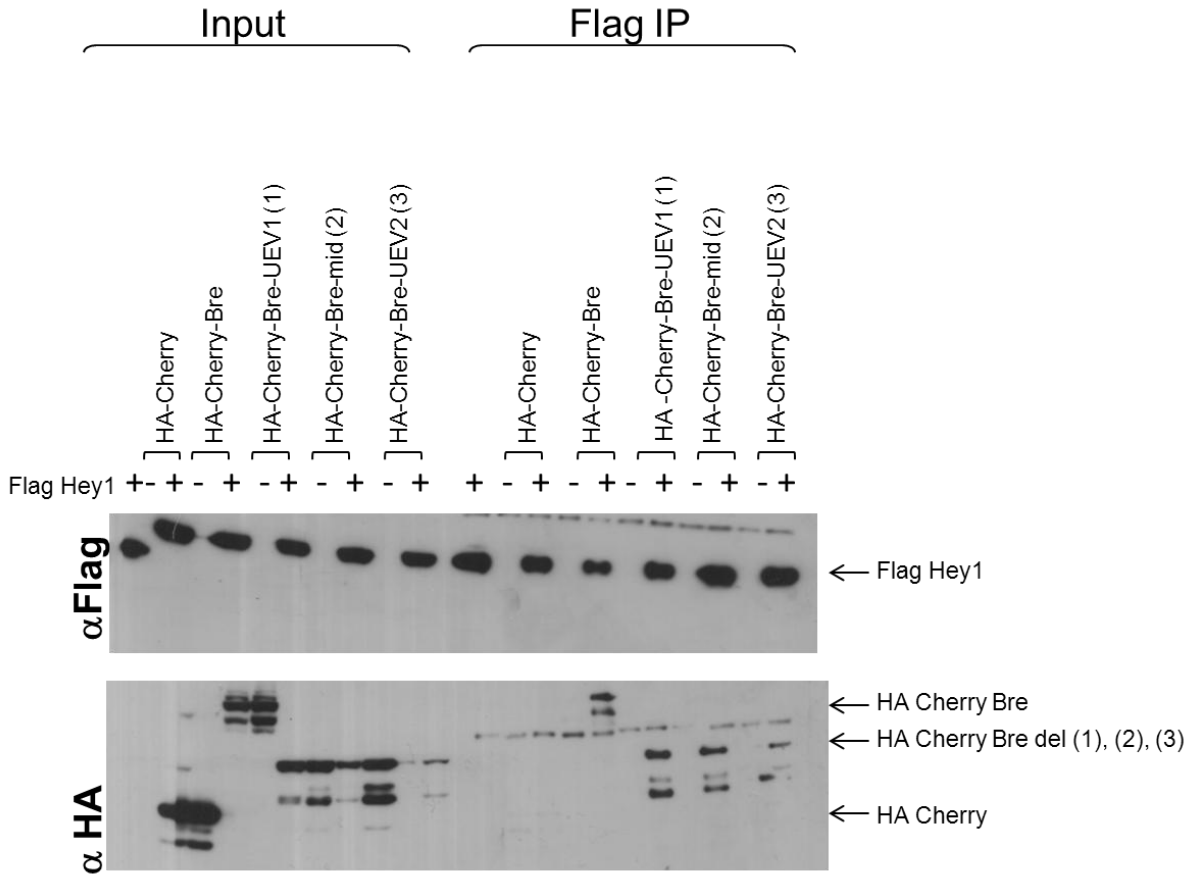


Fig. 12 Hey interacts with all Bre fusion proteins
HEK 293T cells were transfected with Flag-Hey1 and HA-Cherry, HA-Cherry-Bre or HA-Cherry- Bre fusion constructs: (1) HA-Cherry-Bre-UEV1; (2) HA-Cherry-Bre-mid; (3) HA-Cherry-Bre-UEV2. 48h after transfection, cells were harvested and a Flag-IP was performed. Samples were analysed in Westernblot and probed with mouse- αFlag and αHA antibodies and αmouse HRP. Full-length HA-Cherry-Bre and all HA-Cherry-Bre fusion proteins, but not HA-Cherry interacted with Flag-Hey1.

The interaction of Flag-Hey1 with all of the three HA-Cherry-Bre fusion proteins was quite unexpected. However, these interactions must be regarded as specific, because the HA-Cherry protein alone was not pulled down in the co-immunoprecipitation experiment. Furthermore, the Hey1 F264/8A Bre-binding mutant (in the following referred to as "Hey1-mut") did not interact with any of the HA-Cherry- Bre fusion proteins nor with the full length Bre protein, respectively (Fig. 13). Note that the co-immunoprecipitation experiments shown in Fig. 12

and Fig. 13 were performed as Flag-IPs. However, all of the experiments have also been carried out as HA-IPs and revealed the same results (data not shown).

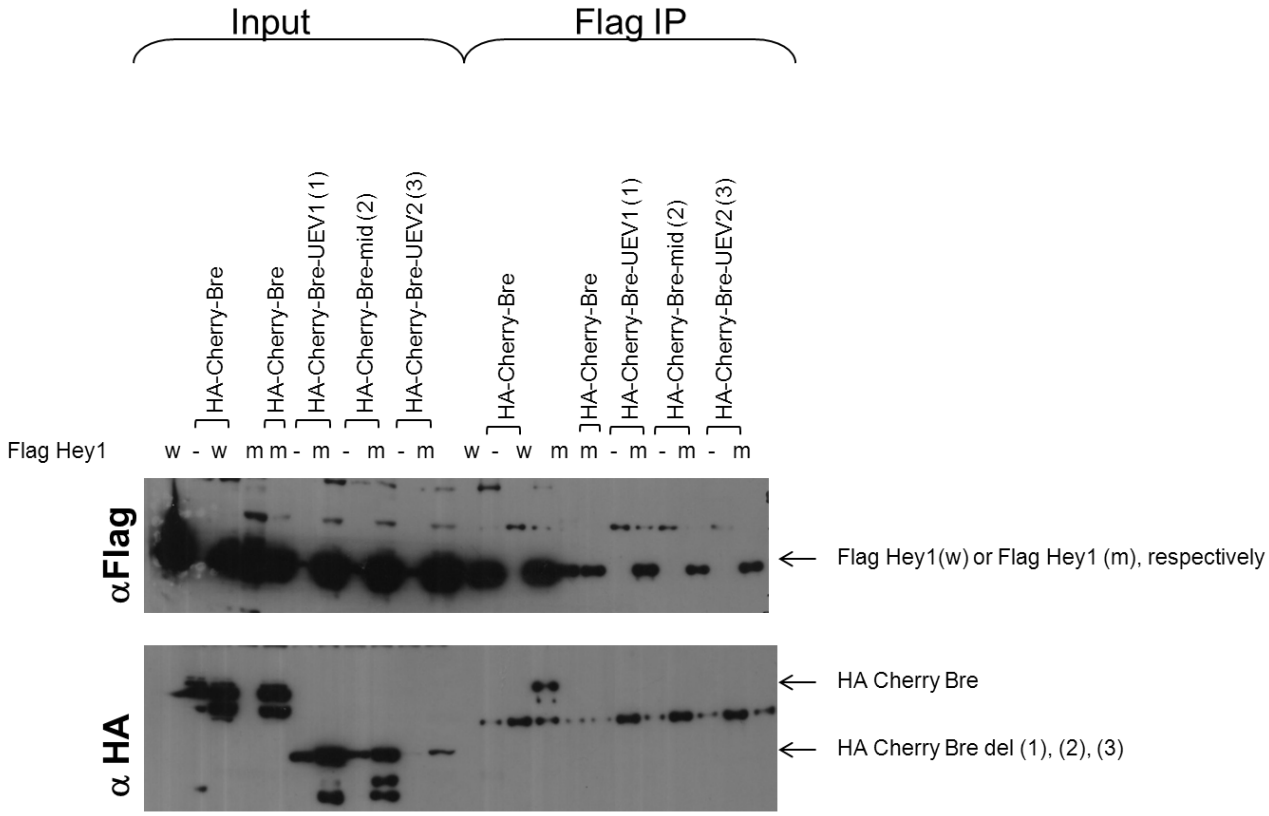


Fig. 13 Hey interacts with all Bre deletion constructs
HEK 293T cells were transfected with wild type Flag-Hey1 (w) or Flag-Hey1-mut (m) and with HA-Cherry-Bre or HA-Cherry-Bre fusion constructs: (1) HA-Cherry-Bre-UEV1; (2) HA-Cherry-Bre-mid; (3) HA-Cherry-Bre-UEV2. 48h after transfection, cells were harvested and a Flag-IP was performed. Samples were analysed by Western blot with αFlag and αHA antibodies and αmouse HRP secondary antibody. Full-length HA-Cherry-Bre interacted with wild type Flag-Hey1, but not with the Flag-Hey1-mut protein. Furthermore, none of the Bre fusion proteins showed binding to Flag-Hey1-mut.

The results obtained in the CoIPs with the HA-Cherry-Bre fusion proteins suggest that Hey1 either binds to more than one site in the Bre protein or that the Hey1 protein does not interact directly with all of the fusion proteins, but rather with endogenous Bre or another yet unknown partner, which functions as a bridge for the interaction. Thus, it was tested whether the Bre protein is able to interact with full-length Bre and/or diverse Bre fusion proteins. First, Flag tagged-Bre was used for co-expression with HA-Citrine-Bre in HEK 293T cells, followed by a Flag-IP. In Westernblot analyses performed with mouse αFlag or mouse αHA antibodies (followed by αmouse HRP) it could be seen that Bre is indeed able to bind to itself (Fig. 14). Next, diverse non-overlapping HA-Cherry-Bre fusion constructs were transfected

into HEK 293T cells together with Flag-Bre for CoIP experiments, followed by αFlag or αHA/ αmouse HRP Westernblot analysis. All of the fusion proteins showed binding to Flag-Bre (compare Table 2). Hence, endogenous Bre present in the HEK 293T cells might function as a linker for the interaction of Flag-Hey1 with the different HA-Cherry-Bre fusion proteins. Bre-Bre interactions have been confirmed by Flag-IP (Fig. 14) and by HA-IP experiments (data not shown).

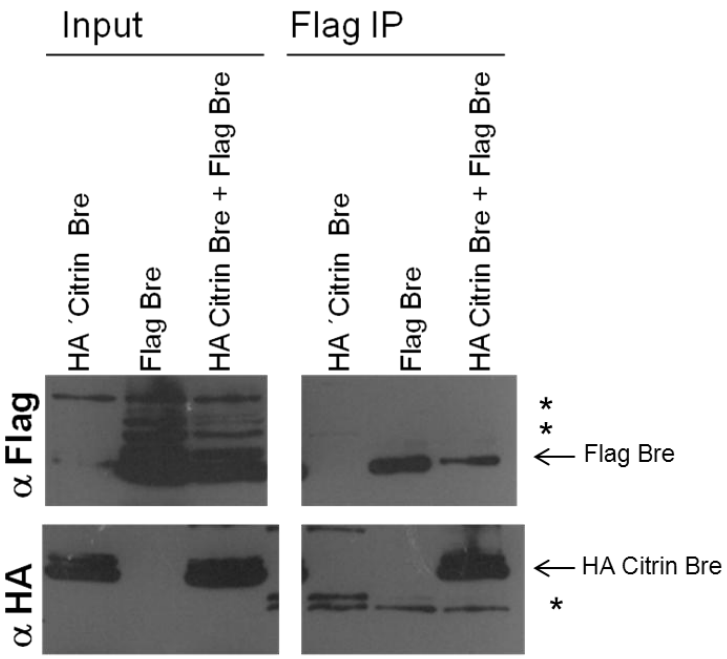


Fig. 14 Bre interacts with itself
HEK 293T cells were transfected with Flag-Bre and HA-Citrine-Bre. 48h after transfection, a Flag-IP was performed followed by αFlag and αHA/ αmouse HRP Westernblot analysis. HA-Citrine-Bre can be efficiently pulled down by Flag-Bre and hence Bre can bind to itself. Unspecific bands are marked with an asterisk.

Table 2 **Interaction partners of Flag-Bre**
HA-Cherry-Bre aa 1-110
HA-Cherry-Bre aa 1-226
HA-Cherry-Bre aa 138-383
HA-Cherry-Bre aa 1-383

4.7 Hey1 over-expression does not interfere with Bre localisation

After the confirmation of Hey1 and Bre interaction, it was of interest to investigate what the functional consequence for their binding to each other might be. First, it was analysed whether the interaction between these two proteins interferes with their sub-cellular localisation.

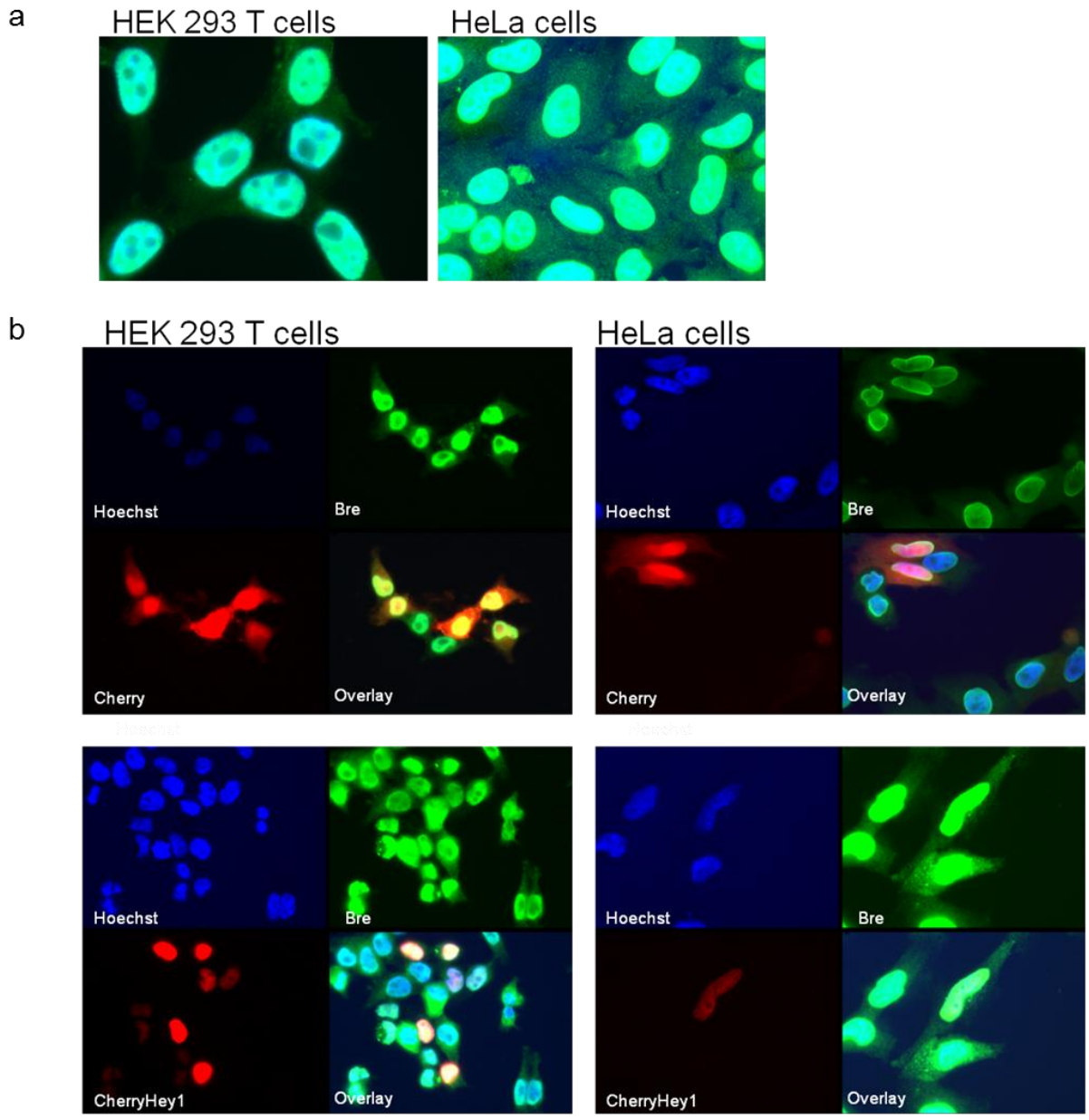


Fig. 15 Sub-cellular localisation of the Bre protein with and without Hey1 over-expression
Bre immunofluorescence staining in HEK 293T cells and HeLa cells was performed with a monoclonal rabbit Bre antibody, followed by Alexa goat-anti rabbit 488 secondary antibody. Nuclei were counter-stained with Hoechst 33342. (a) Endogenous Bre (green signal) displayed a very prominent nuclear staining, but there was also a cytoplasmic yet less intense signal. (b) Bre localisation (green signal) is unaffected by the over-expression of a pmCherry or pmCherry-Hey1 protein (red signal). Note that pmCherry was distributed in nucleus and cytoplasm whereas pmCherry-Hey1 expression could only be found in the nucleus.

As there is no working commercially available antibody for the detection of endogenous Hey1, only endogenous Bre localisation could be analysed in HEK 293T and HeLa cells using a monoclonal rabbit Bre specific antibody in immunofluorescence staining, followed by Alexa goat-anti rabbit 488 secondary antibody staining. In both cell lines analysed, Bre staining is very prominent in the nucleus (stained by Hoechst 33342), whereas there is only very faint but also a clear staining in the cytoplasm (Fig. 15a). To assess, whether Hey1 affects Bre sub-cellular localisation, pmCherry-Hey1 (or pmCherry as a control) was over-expressed (0.6µg/24well) for 18h in HEK 293T cells or HeLa cells, respectively, in two independent experiments. Note that whereas pmCherry-Hey1 over-expression resulted in an exclusively nuclear localisation of the protein, pmCherry expression could be found in the whole cell (Fig. 15b). Cells were fixed and stained for endogenous Bre expression using a rabbit monoclonal Bre antibody and an Alexa goat-anti rabbit 488 as secondary antibody. When analysing the HEK 293T and HeLa cells for Bre sub-cellular localisation, neither an overall change in nuclear staining intensity nor an increase or complete loss of cytoplasmic staining could be detected after pmCherry-Hey1 or pmCherry over-expression (Fig. 15b). Thus, Hey1 did not interfere with overall Bre sub-cellular localisation at the time point analysed.

4.8 Ubiquitination assay with Bre and Hey1

After the analysis whether Hey1 interferes with the localisation of Bre and hence possibly with Bre function, the impact of Bre over-expression on Hey1 function should be analysed. The description of Bre as a component of a complex with E3-ligase activity (Dong *et al.* 2003), led to the assumption that Bre might act as an E3-ligase for Hey1. To test this idea, HEK 293T cells were used for an ubiquitination assay (Fig.16). Cells were transfected with Flag-Hey1 (3µg) and HA-tagged ubiquitin (6µg) alone or in combination with HA-Cherry or HA-Cherry-Bre (3µg each). Two days after transfection, cells were either left untreated or incubated with MG132 to increase the amount of ubiquinated proteins. After 6h, cells were harvested (besides the usual PMSF and proteinase inhibitor cocktail, 20mM of NEM inhibitor were used for the stabilisation of modifications via ubiquitination) and a Flag-IP was performed. Samples were probed with αFlag and αHA antibodies in a Westernblot analysis; αmouse HRP was used as secondary antibody. In none of the combinations, ubiquinated Hey1 protein species were detectable.

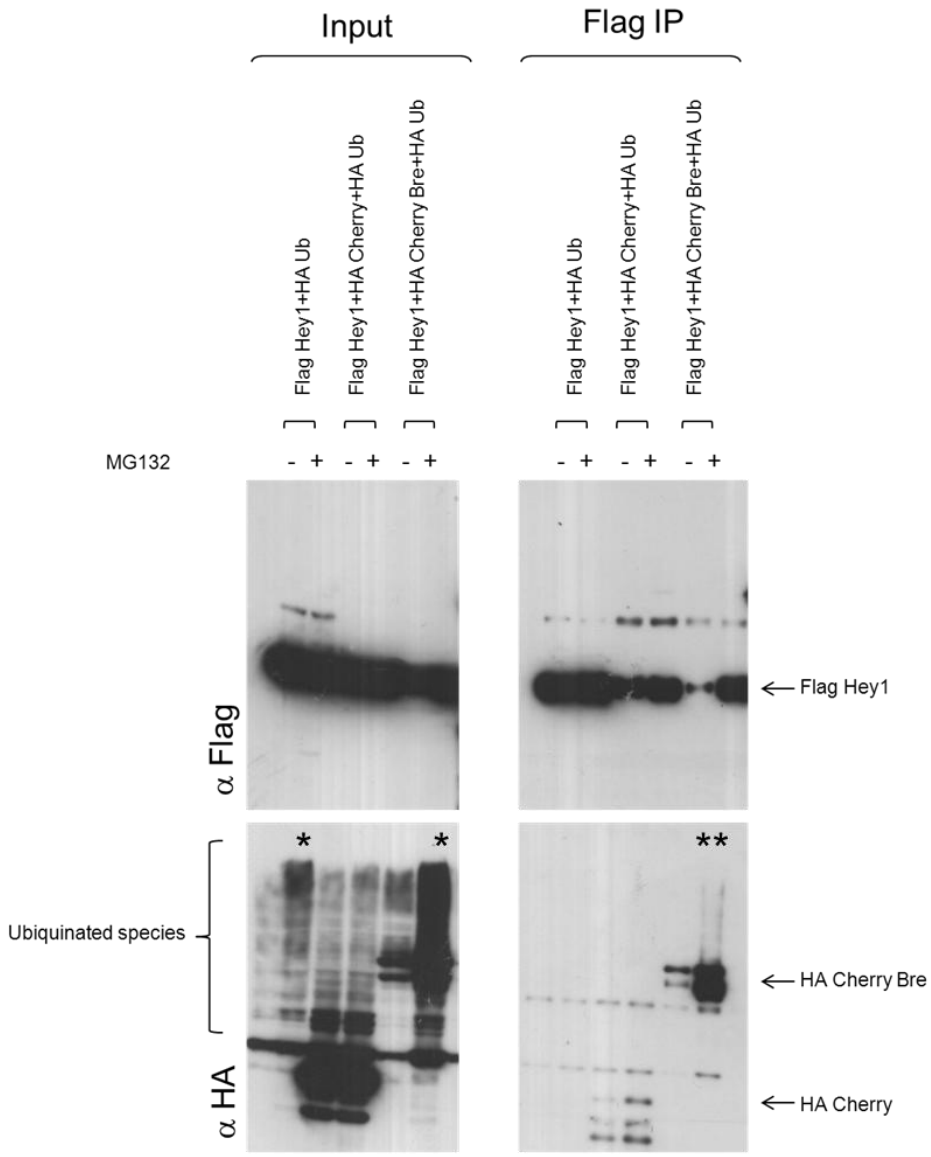


Fig. 16 Hey1 ubiquitination assay after Bre over-expression
*Flag-Hey1 was co-transfected with HA-ubiquitin with or without additional HA-Cherry or HA-Cherry-Bre. 48h after transfection, samples were either treated with MG132 or left untreated. In the following, a Flag-IP was performed. Westernblot analyses with αFlag and αHA antibodies (secondary antibody: αmouse HRP) did not detect ubiquinated species for Hey1. *: increase of ubiquinated proteins after treatment with MG132; **: faint smear of higher molecular weight proteins above the unmodified HA-Cherry-Bre signal. Note the loading differences between Flag-Hey1, HA-Cherry-Bre, HA-ubiquitin with and without MG132 treatment.*

If Hey1 was properly ubiquinated, a ladder-like signal or at least a shift to higher molecular weight band should be obtained in the Flag-Westernblot above the standard (i.e. unmodified) Hey1 signal. Furthermore, a positive (higher molecular weight) signal in the HA-Westernblot would be expected for a ubiquinated Hey1 reflecting the incorporation of HA-ubiquitin into Flag-Hey1. However, none of these observations could be made. Nevertheless, HA-ubiquitin must have been successfully incorporated into diverse cellular proteins as there is a smear in the HA-Westernblot for all input samples and as the intensity of the signal even increased

after addition of the proteasome inhibitor MG132 especially in the case of Flag-Hey1/HA-ubiquitin and Flag-Hey1/HA-ubiquitin/HA-Cherry-Bre (Fig. 16: compare lanes marked with an asterisk). Yet, this finding is less obvious in the case when Flag-Hey1, HA-ubiquitin and HA-Cherry were co-expressed. Furthermore, HA-Cherry-Bre seems to be ubiquinated or to highly efficiently bind to other ubiquinated proteins, as there is a strong signal or shift of bands (probably representing ubiquitinated species) above the standard HA-Cherry-Bre signal after MG132 treatment. Moreover, after treatment with MG132 there is even still a very faint signal of higher molecular weight protein species above the unmodified HA-Cherry-Bre signal after the Flag-IP (Fig. 16, marked with two asterisks): Hence, Flag-Hey1 either bound to unmodified as well as to ubiquinated HA-Cherry-Bre or Flag-Hey1 led to the pull-down of other ubiquinated proteins together with HA-Cherry-Bre.

4.9 Bacterially expressed Bre protein does not interact with ubiquitinated species

For the test if the Bre-UEVs represent functional ubiquitin binding domains and might hence be able to bind to ubiquitinated species under the IP conditions used in this work, an ubiquitin binding assay was performed with bacterially expressed and purified MBP-Bre protein. Purified MBP-protein served as a control. Coomassie staining of MBP- and MBP-Bre on amylose beads is depicted in Fig. 17 a. Both, MBP- and MBP-Bre protein were incubated with cellular lysates from HEK 293T cells, which were either left untreated or incubated with MG132 to obtain a high amount of ubiquinated proteins. Moreover, HEK 293T cells were transfected in parallel with Flag-Hey1 as a positive control for the interaction with MBP-Bre. 6h after addition of the proteasome inhibitor MG132, cellular lysates were harvested, pre-cleared (with amylose-beads+MBP protein) and added to amylose beads, which had been pre-coupled with MBP- and MBP-Bre protein, respectively. After further 2h, beads were washed extensively and resuspended in SDS loading buffer. Samples were used for SDS-PAGE and Western blot analysis with mouse αFlag and mouse αubiquitin antibodies as well as αmouse HRP as secondary antibody. Whereas Flag-Hey1 bound to the bacterially expressed MBP-Bre protein (but not to the MBP-protein, Fig. 17 b) thereby confirming a direct interaction between Hey1 and Bre, there was no interaction detectable between MBP-Bre and ubiquinated proteins (see Fig. 17 c). Thus, in this setting the Bre UEV-domains were not able to mediate binding of Bre to any ubiquitinated protein species.

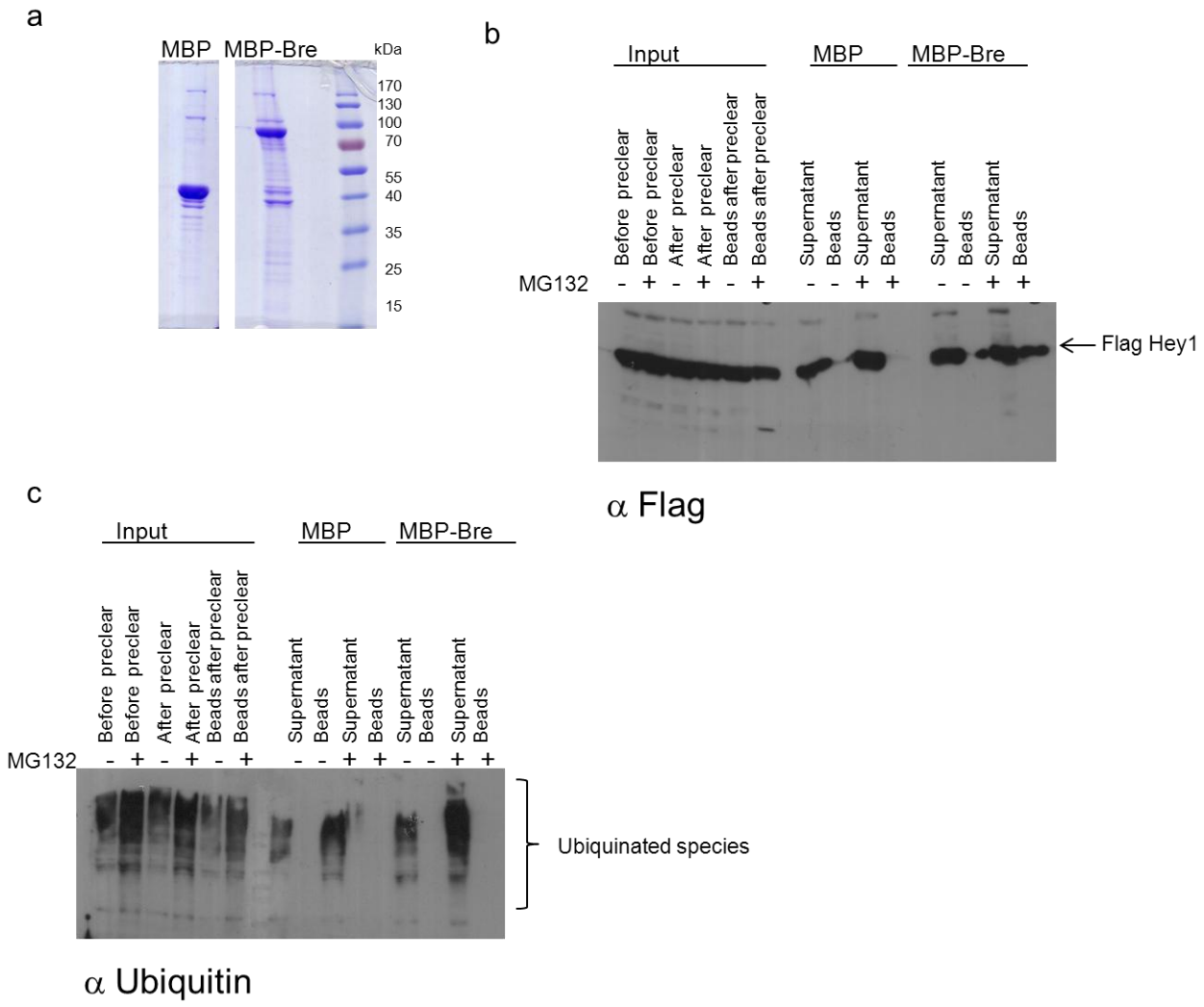


Fig. 17 Test for functionality of the Bre UEV-domains under standard IP conditions
Bacterially expressed MBP- or MBP-Bre protein was incubated with cellular lysates of HEK 293T cells transfected with Flag-Hey1 (-/+MG132) on amylose beads. (a) Expression of bacterially purified proteins was confirmed by Coomassie staining; (b) Interaction of bacterially expressed MBP-Bre and Flag-Hey1 could be demonstrated in a mouse αFlag/αmouse HRP Westernblot; (c) No interaction of MBP-Bre with ubiquinated proteins could be seen in a mouse αubiquitin/αmouse HRP Westernblot.

4.10 Determination of Hey1 stability after Bre over-expression

Even though the above described experiments did not support the notion that Bre might bind to ubiquitin or function as an E3-ligase for Hey1, Cycloheximide (Chx) assays were performed to analyse whether Bre over-expression affects Hey1 turnover. For this, 293tet-FS-mHey1 cells were used. These cells express a Flag and Strep tagged mHey1 construct, which is only expressed after the addition of Doxycyclin. Cells were stimulated with 100ng/ml Doxycylin for 48h for an efficient induction of FS-mHey1 expression. Then, cells were transfected with low amounts of HA-Citrine-Bre or HA-Citrine (2µg/10cm dish).

Transfection efficiencies were comparable as observed under the fluorescence microscope (data not shown). 24h after transfection, cells were treated with either Chx alone or in combination with MG132 (for the blocking of proteasomal degradation) and cells were harvested after 0, 0.5, 1, 1.5, 2, 3, 4 and 6 hours, respectively. Westernblot analysis was performed, using an αFlag antibody for FS-mHey1 detection, an αHA antibody for the detection of HA-Citrine and HA-Citrine-Bre, respectively, and an αβTubulin antibody for the loading control. In all cases, αmouse HRP was used as the secondary antibody. It was not easy to interpret the results obtained from different experiments using different controls (transfected with different DNA concentrations ranging from 2-8µg). Without transfection of the cells, Hey1 expression was highly reduced after 90 minutes following Chx treatment (data not shown). Depending on the control, there was either no influence on Hey1 expression at all (leading to the same turnover rates as in untransfected cells, for example after the transfection of 8µg of pLL3.7 plasmid; data not shown) or a stabilisation of the Hey1 protein compared to untransfected cells could be observed (for example after 8µg of HA-Cherry or HA-Citrine transfection: data not shown). Therefore it was not feasible to calculate the correct half life times for Hey1 in control transfected cells. Cells with Bre over-expression either showed a comparable or even clearly stabilised Hey1 expression compared to the control transfected cells. This was true for all Bre constructs tested and for all DNA concentrations applied (i.e. Flag-Bre, HA-Citrine-Bre, HA-Cherry-Bre; 2µg-8µg). Hence, it could at least definitely be excluded that Bre over-expression led to an enhanced Hey1 turnover (also compare Fig. 18).

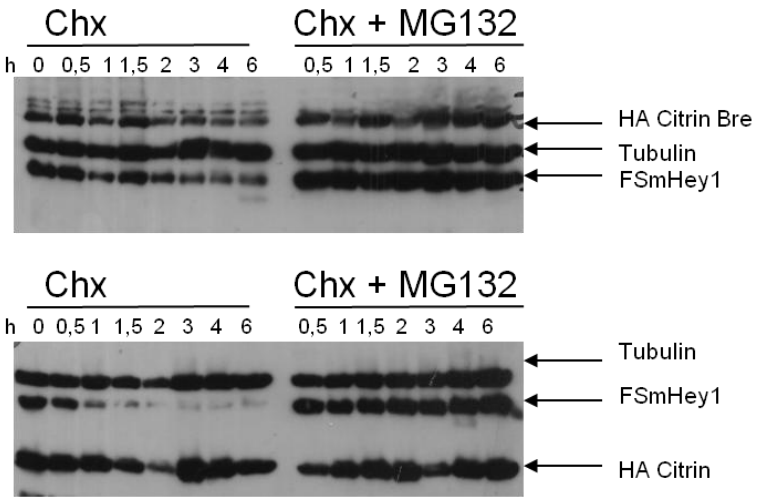


Fig. 18 Bre over-expression did not diminish Hey1 stability
293tet-FS-mHey1 were induced with 100ng/ml Doxycyclin for 72h. 48h after induction, cells were co-transfected with HA-Citrine-Bre or HA-Citrine. 24h after transfection, cells were then used for a Chx assay; MG132 inhibitor was added to block proteasomal degradation. HA-Citrine-Bre over-expression did not diminish FS-mHey1 halftime compared to the control transfected cells and rather even led to a slight stabilisation of the FS-mHey1 protein. MG132 treatment of the cells efficiently blocked proteasomal degradation of the Hey1 protein.

4.11 Diminished repression potential of the Flag-Hey1 F264/8A mutant in luciferase assay

One of the main characteristics of Hey proteins is their function as transcriptional repressors. It was thus analysed whether Bre interferes with Hey1 target promoter repression in luciferase assay experiments. For this, HEK 293T cells (24well format) were transfected with diverse luciferase promoter constructs (0.25µg each) and further plasmids encoding for Flag-Hey1 (0.05µg) or Flag-Hey1-mut (0.05µg) together with or without Flag-Bre (0.05µg-0.5µg). 48h after transfection, cells were harvested and analysed. The luciferase activity of Hey1, Dll4, Gata6 and Jagged1 promoters was measured after Flag-Hey1 or Flag-Hey1-mut over-expression. Additional effects after Bre co-expression (with different amounts of Flag-Bre) as well as after Flag-Bre expression alone were also determined. Luminescence measured for the respective promoter and Flag-protein alone was set to 100%.

Three of the four promoters analysed showed a better repression by wild type Flag-Hey1 than by the Flag-Hey1-mutant. Wild type Flag-Hey1 led to a repression of the Hey1 promoter down to 20.9%, whereas the mutant only led to a repression down to 41.0%. The same tendency could be observed for the Dll4 (13.5%/38.6%) and Gata6 (4.5%/12.3%) promoters. Yet, when analysing the Jagged1 promoter, repression by wild type Flag-Hey1 and by the Flag-Hey1-mut was comparable (36.0%/44.6%). In the case of the three former promoters, however, it can be concluded that Bre binding enhanced or modulated Hey1 repressive function as wild type Hey1 performed better than the Bre binding mutant. After Flag-Bre co-expression, the down-modulation of luciferase activity could be observed for both, the wild type and the mutant Flag-Hey1 (Fig. 19 a). Flag-Bre protein expression alone resulted in a highly significant promoter repression of the Hey1 (residual luciferase activity of 41.6%), the Dll4 (49.5%) and the Gata6 promoter (36.1%), but not in Jagged1 promoter repression (92.8%; see Fig. 19 b). To exclude that the diminished repression potential of Flag-Hey1-mut lies in a disturbed nuclear localisation, nuclear-cytoplasmic fractions of HEK 293T cells either transfected with wild type Flag-Hey1 or with Flag-Hey1-mut were prepared and Westernblot analysis with mouse-αFlag/ αmouse HRP antibody was performed. Here, it could be seen that wild type as well as mutant Flag-Hey1 could be found in both compartments in similar amounts. Hence, it can be excluded that the diminished repression potential of the Bre binding mutant is due to a reduced nuclear localisation (Fig. 19 c).

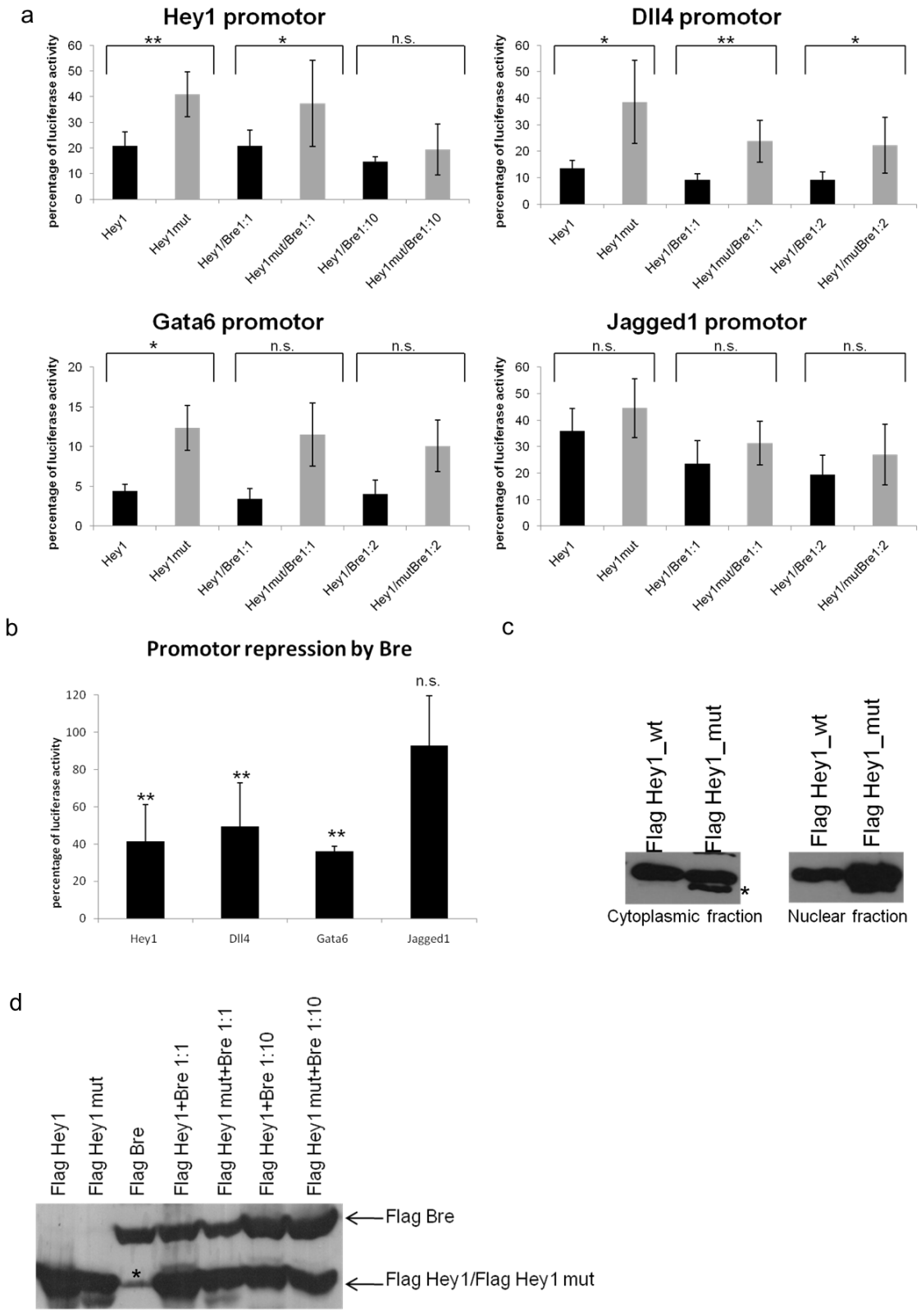


Fig.19 Influence of Bre on the repressive function of Hey1

Luciferase assays with Hey1, Dll4, Gata6, and Jagged1 promoters

(a) Comparison of Flag-Hey1 and Flag-Hey1-mut repressive function with or without Flag-Bre co-expression; (b) Promoter repression activity by Flag-Bre protein alone; (c) Nuclear and cytoplasmic fractions without differences in wild type (wt) and mutant (mut) Flag-Hey1 sub-cellular localisation; (d) Comparison of Flag-Bre to Flag-Hey1 and Flag-Hey1-mut expression levels in HEK 293T cells; (c), (d): αFlag/ αmouse HRP Westernblot analyses: Asterisks mark unspecific bands in the Westernblot analyses.

Furthermore, Western blot analysis demonstrated that even if 10-fold of Bre plasmid was used for transfection compared to Flag-Hey1/Flag-Hey1-mut plasmid concentrations, there was no impairment of Flag-Hey1 or Flag-Hey1-mut protein expression. Bre levels were rather comparable to Flag-Hey1/Flag-Hey1-mut levels only after a high over-expression. Thus, any unspecific effects by artificially high Flag-Bre over-expression can be excluded. Moreover, Flag-Hey1 wild type and Flag-Hey1-mut expression levels were comparable. Thus, it can be excluded that the diminished repression potential of the Flag-Hey1-mut was due to lower overall expression levels compared to wild type Flag-Hey1 (Fig. 19 d).

4.12 Generation of Bre LacZ gene trap-ES cells

The modulation of Hey1 repressive function by Bre over-expression is a first indication for a possible function, which Bre might exert in combination with Hey1. However, further information about Bre biology is necessary to understand this finding and to reveal other Bre functions. A suitable model for gene function analysis is the generation of KO cells. Hence, it was decided to generate Bre KO-ES-cells, as they would later on allow the analysis of Bre function in an *in vitro* differentiation model system. For the generation of Bre KO-ES cells, Bre heterozygous ES cells were obtained from EUCOMM. For a scheme of the genomic arrangement compare Fig. 20.

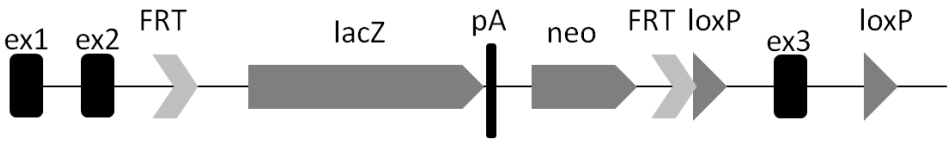


Fig. 20 Schematic depiction of Bre LacZ gene trap-ES cell genetic organisation
In Bre heterozygous ES cells, one Bre wild type allele is left, whereas in the other allele a LacZ cassette is inserted in between exon 2 and 3. Furthermore, exon 3 is flanked by loxP sites allowing the exicision of this exon by addition of Cre recombinase. Moreover, the LacZ cassette can be deleted via the FRT sites following Flp recombinase treatment.

Homozygous clones were generated in that the heterozygous Bre LacZ ES cell clone was subjected to a high selection pressure with G418. Several clones survived this procedure, but most of the clones still displayed a heterozygous genotype. Nevertheless, four homozygous Bre LacZ KO clones, which had undergone spontaneous LOH (loss of heterozygosity) were also obtained after selection with 1.5mg/ml of G418. It was demonstrated by genotyping PCR that only the KO band was present in genomic DNA isolated from these clones (Fig. 21).

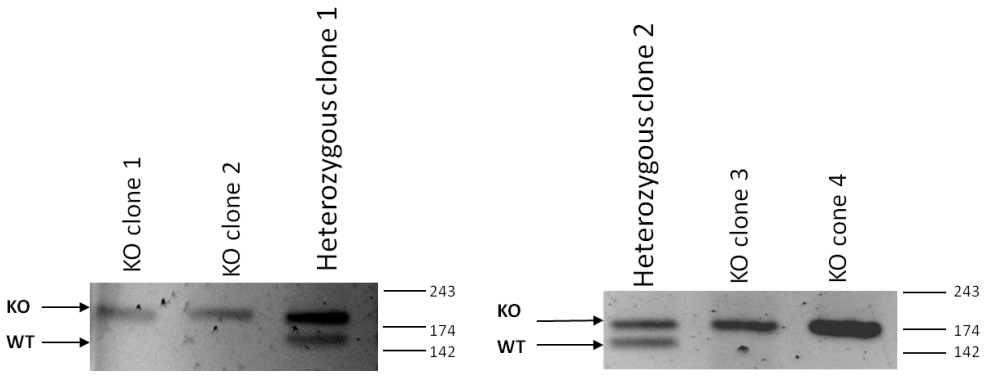


Fig. 21 Genotyping of ES cell clones after high G418 selection
Bre LacZ heterozygous ES cells were selected under high G418 selection pressure. The obtained clones were tested for a spontaneous LOH resulting in the loss of the wild type Bre allele and hence the generation of homozygous Bre LacZ-KO-ES cells. All in all, four clones, which were only positive for the KO band were obtained as tested in the genotyping PCR.

4.13 Differentiation of Bre LacZ-ES cells

After the successful establishment of homozygous Bre LacZ-KO-ES cells, the cells were tested for their ability to give rise to all three germ layers after differentiation as embryoid bodies (EBs). Marker gene expression in Bre LacZ KO-ES cells was compared to heterozygous ES cells, which had also undergone the selection process. Heterozygous and homozygous ES cells could both be efficiently differentiated and cultivated as EBs. RNA was harvested from ES cells, EB d 2, d 4, d 6 and d 10 and reverse-transcribed into cDNA. In the following, realtime-RT-PCR analysis was performed.

All in all, four KO and two heterozygous clones were analysed and their average ct values were determined after normalisation to Hprt expression. After the normalisation, the delta ct values between ES cells and differentiated cells (i.e. EB d 2, d 4, d 6 and d 10) were determined and the fold change induction of marker gene expression was calculated (expression in ES cells was set to 1). Here, 1 cycle difference was defined as equivalent to a fold change of factor 2. Only a difference in the fold change induction of a factor higher than 2.5 would have been regarded as a regulation. However, when comparing homozygous and heterozygous clones, no differences could be detected except for Brachyury induction at EB d10. Yet, this finding must probably be regarded as an artefact, because expression levels between the different KO clones displayed high differences with two of the clones displaying Brachyury expression levels comparable to the heterozygous clones. In summary, it can be concluded that all three germ layer markers were induced properly and at the same time points in heterozygous and homozygous cells (endoderm = AFP; mesoderm = Brachyury; ectoderm

= Nestin; see Fig. 22). This means that differentiation of Bre LacZ KO-ES cells did not negatively interfere with germ layer marker induction.

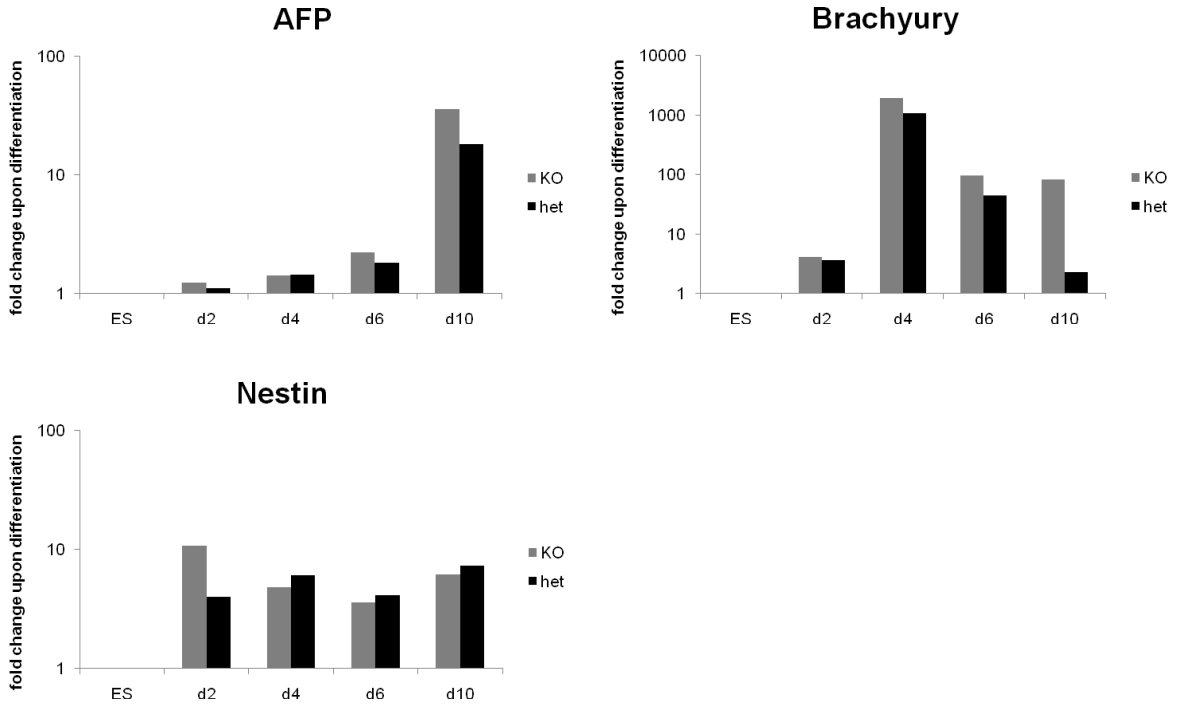


Fig. 22 Realtime-RT-PCR analysis of germ layer marker genes after Bre LacZ ES cell differentiation

Bre KO and Bre heterozygous LacZ ES cells were compared for their ability to differentiate into all three germ layers during an EB in vitro differentiation. An induction of all three germ layer markers could be observed for KO and heterozygous ES cells after differentiation (AFP: endodermal, Brachyury T: mesodermal and Nestin: ectodermal marker). No difference between KO and heterozygous clones was detectable.

4.14 Inefficient splicing in Bre LacZ-cells

The cDNA samples of the ES cells and differentiated cells described above were also used to check for a successful loss of Bre expression in the Bre LacZ KO-ES cells on mRNA level. This was done by the use of a primer combination spanning exon 2 and 4. These primers should only result in a detectable realtime-RT-PCR product in Bre-wild type or Bre-heterozygous cells. In Bre LacZ homozygous KO cells no product should be obtained because of the LacZ cassette inserted in between exons 2 and 3 (compare Fig. 20). However, the Bre ex2_4 product could still be detected in Bre LacZ homozygous KO cells to a high degree. This can also be seen in the delta ct values for Bre expression determined for KO and heterozygous samples. Here, a maximal difference of 2.7 cycles could be found between KO and heterozygous samples during the course of the EB differentiation after normalisation to

Hprt (compare Table 3). These findings would imply that there must be an inefficient splicing of the LacZ cassette in Bre LacZ KO cells resulting in a skipping of the LacZ cassette. Hence, the generation of an intact exon 2-4 structure would still be possible in these cells.

Table 3 **Delta ct values_KO Bre - het (#2)**			
Bre #3_ES	2.2	Bre #4_ES	2.7
Bre #3_d2	1.1	Bre #4_d2	0.7
Bre #3_d4	1.3	Bre #4_d4	1.4
Bre #3_d6	1.5	Bre #4_d6	1.9
Bre #3_d8	1.9	Bre #4_d8	2.1
Bre #3_d10	1.8	Bre #4_d10	2.4

4.15 Generation of Bre LacZ-KO-mice

In parallel to the Bre LacZ-KO-ES cells, Bre LacZ KO-mice were generated from the heterozygous Bre LacZ-ES cell clone. Heterozygous animals were obtained from EUCOMM and mated to homozygosity. The resulting KO animals were viable and fertile and did not show any obvious phenotype. As for the Bre LacZ-KO-ES cells, cells derived from Bre KO-LacZ mice should also be tested for Bre knock out on mRNA level. For this, MEFs were generated from Bre LacZ homozygous and heterozygous mice. In the following, RNA was harvested from these cells, reverse-transcribed into cDNA and the Bre ex2_4 expression was measured by realtime-RT-PCR. Even more striking than observed in the Bre LacZ-KO-ES cells, there was not any difference at all in the ct values obtained for cells derived from KO or heterozygous animals (data not shown).

4.16 Detection of Bre expressing organs in Bre LacZ mice by β-galactosidase staining

Further primer combinations were chosen for the detection of the expression levels of the LacZ cassette product (i.e. β-galactosidase) in E11.5 heterozygous Bre LacZ embryos. Primers were chosen in such a way that they were generating a product from the Bre exon 2 into the LacZ cassette. With this combination it could be demonstrated that there was only weak β-galactosidase expression in the Bre LacZ mice compared to Hprt (data not shown). However, β-galactosidase expression levels were nevertheless sufficient for the generation of

positive signals in a β-galactosidase assay. In this assay, Bre expression sites in mouse embryos can be visualised: The LacZ cassette is under the control of the Bre promoter; hence only organs which would express Bre also express β-galactosidase, the product of the LacZ cassette. β-galactosidase activity leads to a conversion of the X-Gal substrate into a blue dye. Hence, a blue staining can be detected in Bre expressing organs by the β-galactosidase assay. Bre expression could be observed in diverse embryonic structures. All in all, Bre expression was rather low and was only visible after over night staining. Thus, it cannot be excluded that only organs with really strong Bre expression were detected as positive and that structures with lower Bre expression levels could not be visualised by this method in these mice. Bre expression patterns were analysed in mouse embryos from E9.5-E12.5, but a positive signal was only detectable from E10.5 on. Positive staining could be obtained in the developing limb bud on E10.5, in the brain (from E10.5 on), in the eye (from E10.5 on) and in the liver (from E11.5 on). Furthermore, a diffuse signal in the neural tube region was detectable from E10.5 on (Fig. 23).

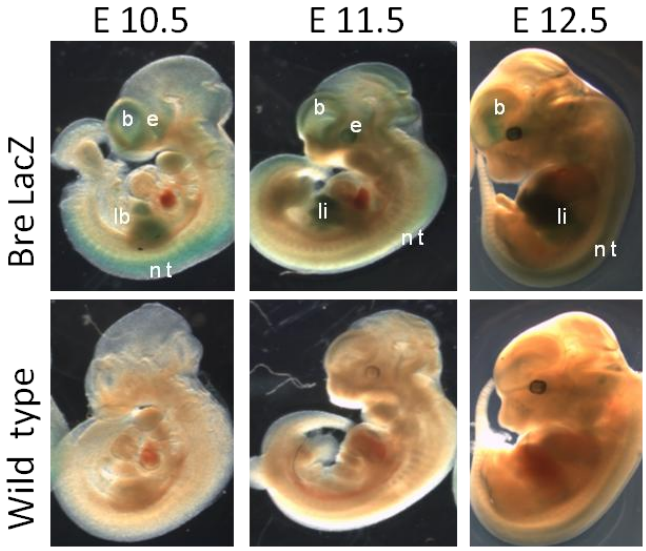


Fig.23 β-galactosidase assay with Bre LacZ embryos
Bre LacZ embryos were analysed for organs with β-galactosidase activity and hence Bre expression at different developmental time points (E10.5-E12.5; in E9.5 embryos no positive staining was obtained, data not shown). A clear Bre signal i.e. blue staining could be detected in the brain (b), the eye (e), the limb bud (lb), the neural tube (nt) and the liver (li).

4.17 Generation of Bre del mice by deleting the exon 3

As homozygous cells originating from Bre LacZ mice did not result in the generation of a robust Bre knock-out, Bre LacZ mice were further crossed to Cre deleter- and Flp recombinase-mice, which resulted in the excision of the floxed exon 3 with or without the

additional deletion of the LacZ cassette. Efficient loss of exon 3 could be demonstrated in realtime-RT-PCR analysis by use of the exon2_4 primer combination: In MEFs derived from homozygous deleted animals, a shift to a smaller product size (del: 177bp; wt: 236bp) could be seen (Fig. 24).

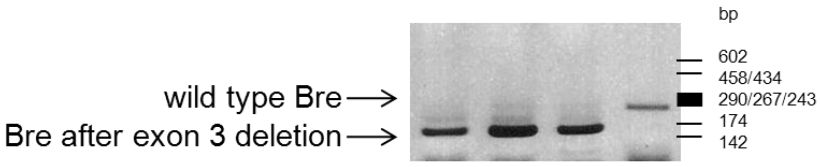


Fig. 24 Realtime-RT-PCR of Bre mice with exon 3 deletion
Realtime-RT PCR was performed on cDNA derived from homozygous Bre del-MEFs, which should have a deletion of exon 3 according to genotyping PCR (data not shown). Loss of exon 3 on mRNA levels could be seen by the resulting smaller product (177bp instead of 236bp, compare HaeIII standard) with the exon2_4 primer combination.

Furthermore the "classical" realtime-RT primer combination for exon11_12 led to a much weaker signal in Bre del- cells compared to wild type cells (Fig. 25: depiction of the fold change difference between Bre wt and Bre del-MEFs) probably due to non-sense mediated decay and degradation of the aberrant Bre del- mRNA after the loss of exon 3. Therefore, one can deduce that mice homozygous for exon 3 deletion (and the cells derived from these mice) show a Bre deficiency. In the following, they will be referred to as "Bre del"- mice or cells, respectively.

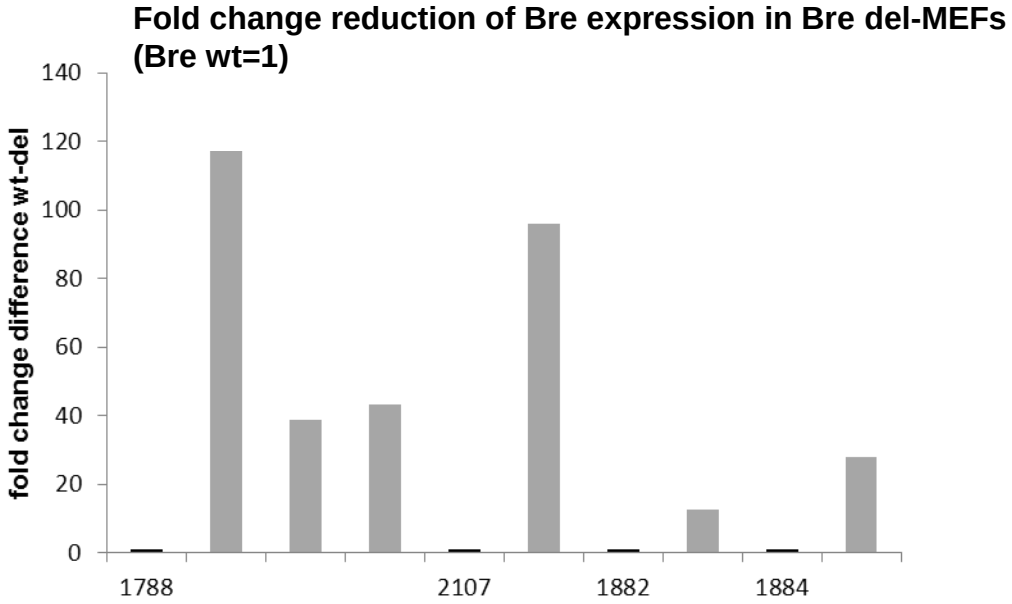


Fig. 25 realtime-RT-PCR for Bre expression in Bre mice with or without exon 3 deletion
Bre expression levels in Bre del-MEFs (grey bars) were highly reduced compared to Bre wt-MEFs (black bars). Expression levels in Bre wt-MEFs were set to 1 and the fold change difference (i.e. reduction) of Bre expression was determined for the corresponding Bre del-MEFs.

Bre mice with a deleted exon 3 were viable and mating of homozygous animals also led to viable offspring. Furthermore, no obvious defect such as disturbed organ structures, reduced size/weight or shorter life span was observed.

4.18 MTT assay in Bre MEFs under normal and stress conditions

As the Bre del- animals did not have any obvious deficiencies, MEFs isolated from these animals should be used for a further and more detailed characterisation. First, cell proliferation was investigated. For this, Bre MEFs were seeded in 96 well plates in triplicates. In total, 6 Bre del- (i.e. 1882.3, 1884.2, 2107.2, 2221.10, 3171.4, 3171.6) and 5 wt-clones (i.e. 1882.6, 1884.5, 2107.7, 2221.6, 3171.5) were analysed and their average proliferation was calculated. Cells were stained 6h after plating (after they had settled down). This was set as the day 0 time point. In the following, MTT measurement was performed every 24h until day 4. There was no difference detectable in the average proliferation rates of Bre del- and wt-cells (Fig. 26 a).

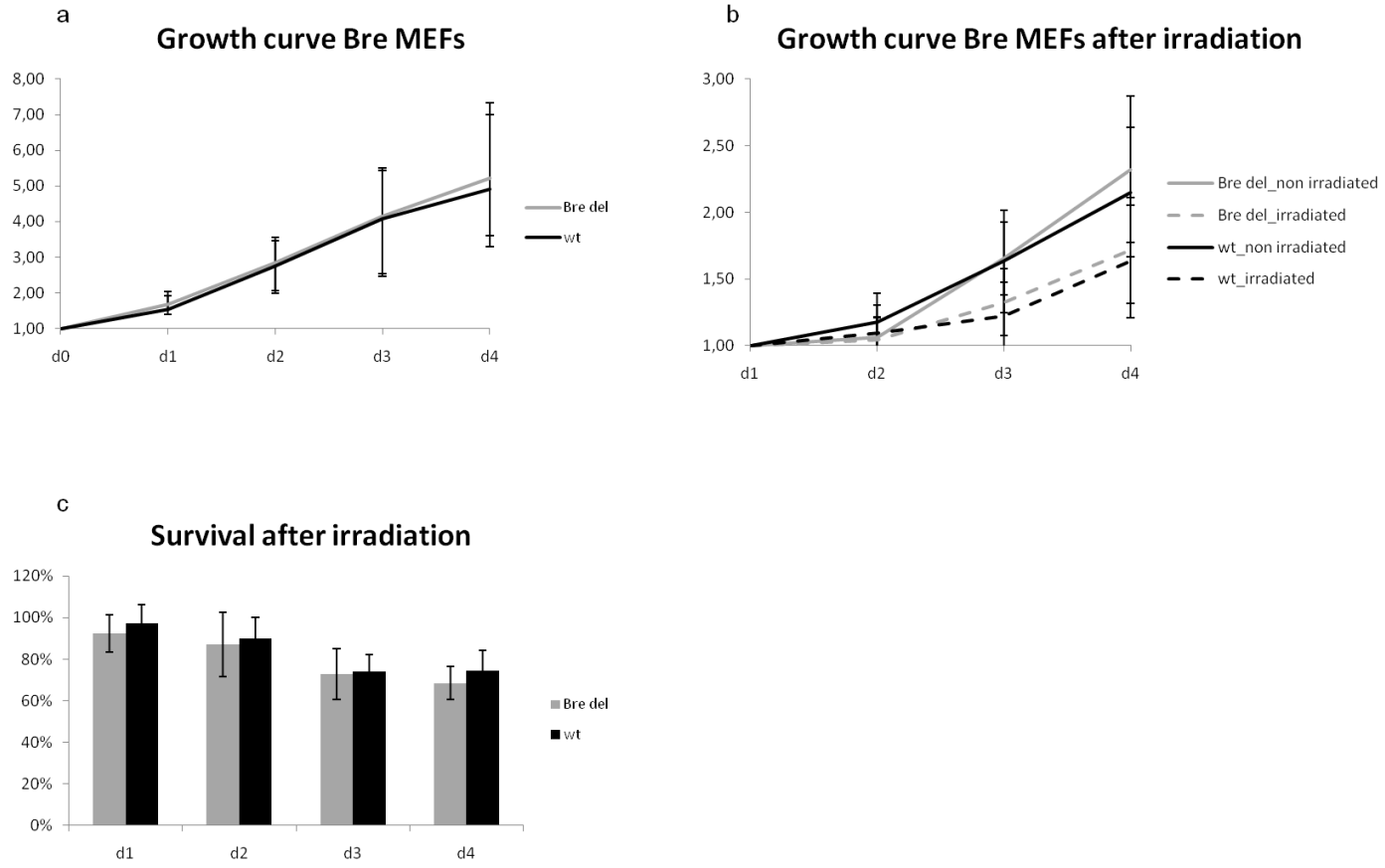


Fig.26 MTT proliferation assay and survival of Bre wt and Bre del-MEFs
(a) Cell growth under standard cell culture conditions was monitored for four days by MTT assay. No change in proliferation rates between Bre del and wt cells was detectable; (b) MTT assay under stress conditions (i.e. after irradiation of the cells with 5 Gy) did not result in proliferation alterations, either; (c) Survival rates of the cells after irradiation are comparable between Bre del and wt cells.

As loss of Bre might be only with an effect under stress conditions such as it is the case for example for Brcc36 (Chen *et al.* 2006), Bre MEFs should be stressed by the introduction of DNA damage. For the proliferation assay under stress conditions, Bre MEFs were detached from their plates and irradiated in suspension with 5 Gy; control cells (i.e. non-irradiated cells) were also in suspension until all cells could be re-plated. The first measurement was performed 24h hours after the plating of the cells and then every 24h for the 3 ensuing days. In this setting, there was no significant difference between non-irradiated Bre del- and wt-cells either. Furthermore, irradiated cells also displayed similar proliferation behaviour independent of their genotype (Fig. 26 b). Consistent with this, there was no clear difference between the survival rates of Bre del- and wt-MEFs (calculated as the ratio of the absorption of irradiated versus non-irradiated cells; compare Fig. 26 c). Hence, loss of Bre expression did not interfere with MEF proliferation under normal or stress conditions. Furthermore, Bre del-cells did not have an increased IR sensitivity, because the survival after irradiation was not altered at least until 4 days after irradiation.

4.19 DNA damage induction and immunofluorescence analysis in Bre MEFs

Bre as a member of the so called BRCA1-A complex has been shown to participate in BRCA1 recruitment to sites of DNA damage. Thus, Bre del-MEFs were analysed for possible alterations in BRCA1 localisation under DNA damage conditions. Bre del- and Bre wt-MEFs were irradiated with 5 Gy and fixed after 15 and 80 minutes, respectively. After double staining with rabbit-γH2AX (secondary antibody: Alexa 488, chicken-anti rabbit, i.e. green staining) and goat-BRCA1 antibodies (secondary antibody: Alexa 568 donkey-anti goat, i.e. red staining), positive nuclear foci were counted using the ImageJ counter window tool. Nuclei were visualised by Hoechst 33342 staining. In non-treated cells no or only very low and evenly distributed nuclear staining with γH2AX and BRCA1, respectively, could be detected (Fig. 27 a). γH2AX positive foci formation could only be observed after irradiation; these foci were regarded as indicative of DNA damage induction (as defined by Paull *et al.* 2000). The total number of γH2AX positive foci was determined, as well as the number of foci, which were also positive for BRCA1 staining: A yellow signal was obtained for double positive foci when the green (γH2AX) and red (BRCA1) channels were merged (representative nuclei, compare Fig. 27 b).

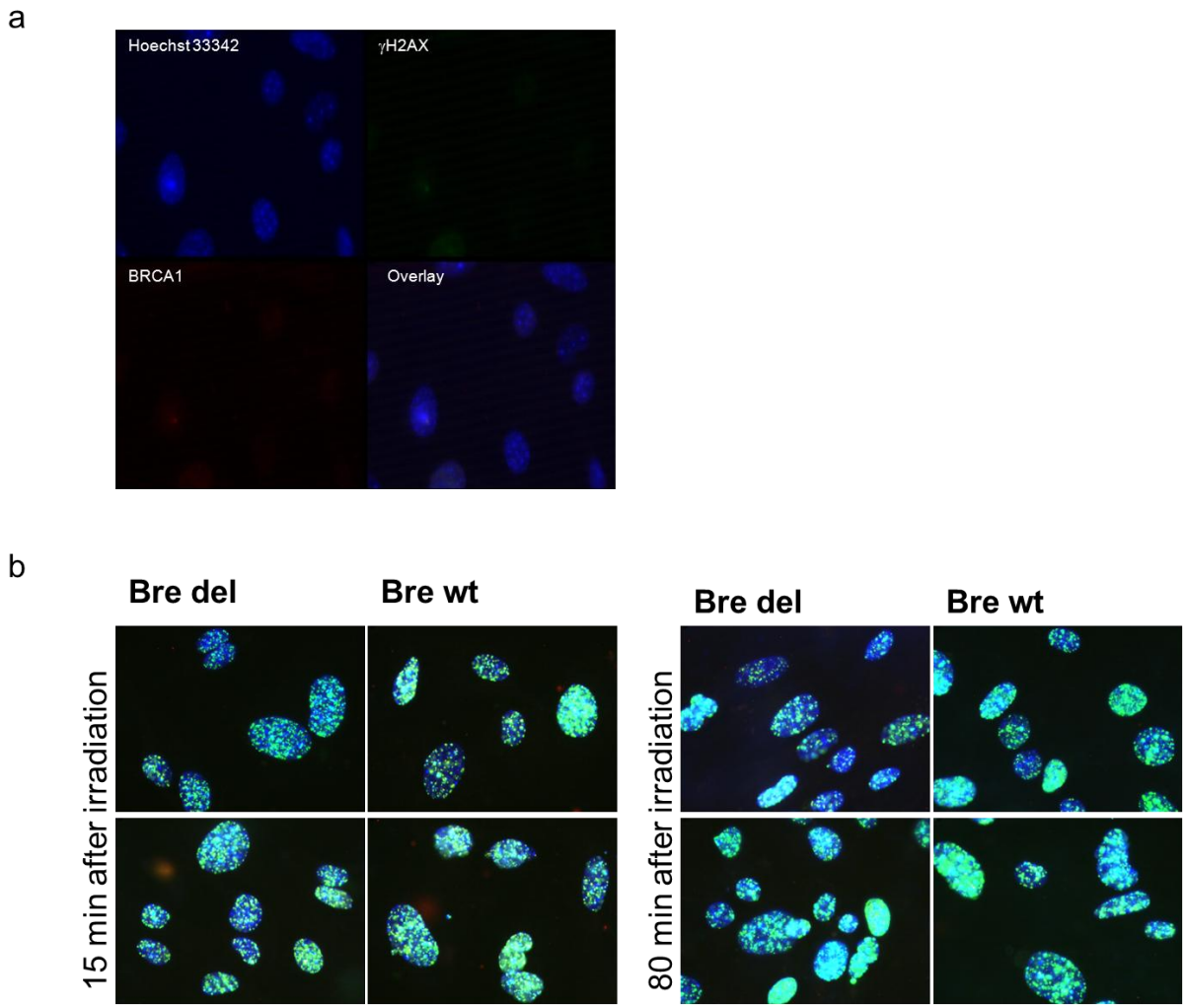


Fig.27 Immunofluorescence staining of irradiated Bre MEFs
(a) Representative picture of non-irradiated cells. Only a diffuse signal for γH2AX or BRCA1 was obtained (b) Bre del and wt-MEFs were irradiated with 5Gy, fixed at the indicated time points and stained with γH2AX and BRCA1 antibodies and their respective secondary antibodies (Alexa 488 chicken anti-rabbit and Alexa 568 donkey anti-goat, respectively). Nuclei were counterstained with Hoechst 33342. The number of γH2AX positive (i.e. green foci) and the number of γH2AX/BRCA1 double positive foci (i.e. yellow signal in the overlay) was determined for the quantification of the BRCA1 DNA damage response (compare Fig. 27).

In total, 2 wild type- (1788.9 and 1884.5) and 4 Bre del- (1788.4, 1788.5, 1788.6 and 1884.2) cell lines were analysed. 227 Bre del- nuclei and 107 wt- nuclei were counted for the 15 minute time point, and 347 Bre del- and 133 wt- nuclei for the 80 minute time point.

15 minutes after irradiation, a highly significant difference in the number of BRCA1 positive nuclei between Bre del- and wt-MEFs (68.7% compared to 93.5%) could be found, whereas after 80 minutes this difference was no longer significant (75.2% positive nuclei in Bre del- and 69.9% positive nuclei in wt-cells). When only nuclei with a strong BRCA1 signal (only nuclei with at least 10 BRCA1 positive foci) were analysed, the difference between wild type and Bre del-cells 15 min after irradiation was even more striking with 68.2% nuclei in wild

type cells compared to 33.9% in the Bre del situation. After 80 minutes, the difference is less obvious, yet still significant (wt-cells: 32.3%; Bre del-cells: 22.2%). Furthermore, the percentage of BRCA1 and γH2AX double positive foci compared to the overall number of γH2AX positive foci was determined: 15 minutes after irradiation, the number of BRCA1/γH2AX double positive nuclei is higher in wt-cells (16.2%) than in Bre del-cells (9.1%). 80 minutes after irradiation, however, the percentage of BRCA1 positive nuclei is more similar between the two genotypes: for the wt: 5.8% and for the Bre del-cells 7.6%, respectively (compare Fig. 28).

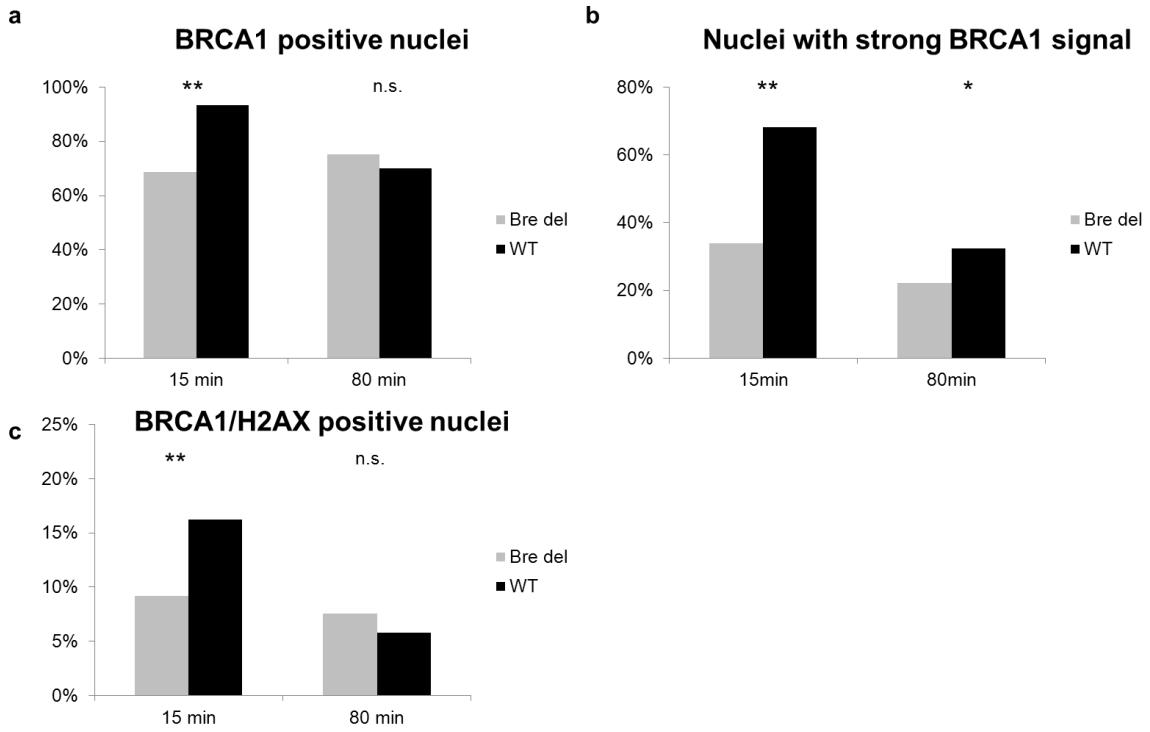


Fig. 28 Quantification of DNA damage induction
Induction of DNA damage after irradiation of Bre del- and wt-MEFs was detected by immunofluorescence staining of γH2AX positive foci; γH2AX positive foci were counted 15 and 80 minutes after irradiation.
(a) Number of nuclei with a BRCA1/ γH2AX double positive signal; (b) Nuclei with more than 10 BRCA1/γH2AX double positive foci (classified as nuclei with a strong BRCA1 signal); (c) Percentage of γH2AX positive foci, which were also positive for BRCA1.

4.20 Stripe assay: DNA damage induction in Bre MEFs by UV laser treatment

Irradiation of cells with γ-rays leads to a very efficient induction of DNA damage; however, with this method it is not possible to observe DNA damage induction at a specific site, because the foci formation occurs spontaneously at sites where DNA double strand breaks have randomly been introduced. Therefore, under these conditions a targeted BRCA1

recruitment to a defined locus cannot be detected. To overcome this problem, specific stripes of DNA damage were introduced into nuclei from Bre MEF cells (pre-stained with Hoechst 33258) by UV treatment using the confocal microscope laser system (at 405nm with about 75nW/pixel for 25 scans). The successful induction of stripes could be observed by fluorescence microscopy after staining with γH2AX (secondary antibody: Alexa 488 chicken-anti rabbit; compare Fig. 29). Furthermore, cells were also stained for BRCA1 (secondary antibody: Alexa 568 donkey-anti goat) to test whether BRCA1 localisation to the induced stripes was possible in Bre wt- as well as in Bre del- cells.

When comparing Bre del- and Bre wt-MEFs, it could be seen that there was a recruitment of BRCA1 to the γH2AX positive stripes in cell nuclei of both genotypes (see Fig. 29). This means that loss of Bre in Bre del-MEFs did not block BRCA1 recruitment to sites of DNA damage. Yet, as this method was very time-consuming (note that Bre del- cells were fixed after 2.5h and the wild type cells after 2h) and did not always lead to proper DNA damage induction, it did not allow for quantification and comparison of the BRCA1 signals in Bre del- and wt-MEFs. Nevertheless, with this assay it could be demonstrated that a targeted recruitment of BRCA1 to sites of DNA damage was still possible after the loss of Bre expression.

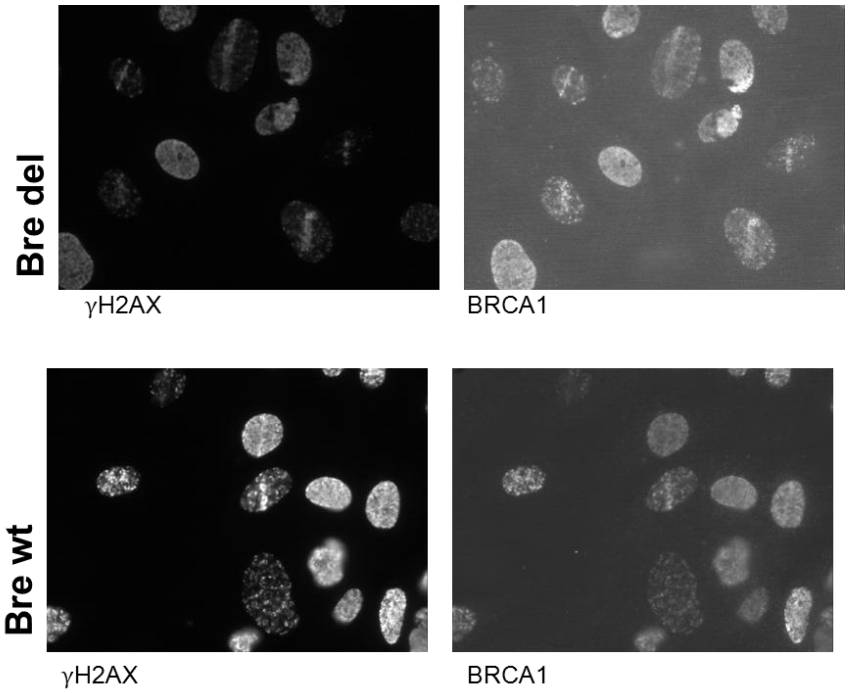


Fig. 29 Stripe assay in Bre MEFs

A confocal microscope laser was used at 405nm to induce a stripe pattern (with 25 scans) into nuclei pre-stained with Hoechst 33258. Bre del- and wt-MEFs were fixed after 2.5h and 2h, respectively and stained for γH2AX and BRCA1 (secondary antibodies: Alexa 488 chicken-anti-rabbit and Alexa 568 donkey-anti-goat). BRCA1 positive stripes were found in Bre del- and Bre wt-MEFs. Pictures are depicted in grey scale for a better contrast of the signals.

4.21 Cytotoxicity assay

After the analysis whether loss of Bre negatively interferes with BRCA1 DNA damage recruitment; it should be investigated whether Bre deficiency also correlates with a change in the apoptotic response, because it has been shown in different cell lines, that Bre regulates cellular survival. Hence, it should be tested whether loss of Bre resulted in an altered sensitivity towards TNFα or FasL stimulation in Bre del-MEFs. For this, a cytotoxicity assay was performed. Cells were first sensitised with Chx and then TNFα or FasL were titrated each in 1:3 dilution steps (in triplicates), starting from a concentration of 1µg/ml. 16h later, cells were quantified by MTT assay. Whereas TNFα stimulation did not induce obvious cell death at all (not even after more than 24h; data not shown), FasL treatment resulted in a proper dose-dependent killing of the cells. All in all, two Bre del- (1884.2 and 2107.2) and the two corresponding wild type cell lines (i.e. 1884.5 and 2107.7) were compared in three independent experiments. In five of the six Bre wt-del comparisons, the wild type cells showed a slightly better survival than their Bre del- counterparts. For one set, however, there was no difference detectable at all. The average survival curves for Bre del- and wt-MEFs were determined and are depicted in Fig. 30. Moreover, the LD_{50} for FasL treatment was calculated, which was reached at a concentration of 0.24ng/ml in Bre del- cells and at a concentration of 0.41ng/ml in wild type cells.

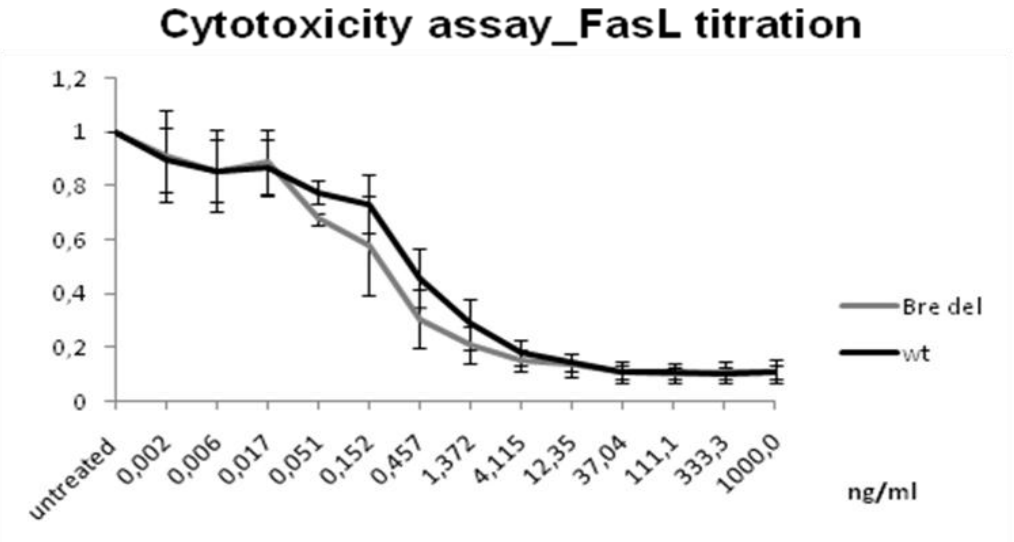


Fig. 30 Cytotoxicity assay
Bre MEFs were sensitised by Chx treatment and then titrated with FasL in 1:3 dilution steps starting from a concentration of 1µg/ml. 16h later, cell survival was measured by MTT assay. Here, it could be seen that the cells showed a dose-dependent sensitivity towards FasL treatment. Bre del-cells were only marginally more sensitive than wt-MEFs with a calculated LD of 0.24ng/ml compared to 0.41ng/ml in wt cells.

The findings above suggest that there is a difference in the FasL sensitivity between wt- and Bre del- cells; however, there was only a small shift (with less than one dilution step) towards an increased cell death after the loss of Bre. Hence, the obtained results point to an only minor protective effect of Bre on FasL induced cell death.

4.22 Increase of poly-K63-ubiquitinated protein species in Bre MEF cytoplasmic fractions

Besides participation of Bre in the nuclear DNA damage response, Bre is also a member of the cytoplasmic BRCC complex, which displays a de-ubiquitinase (DUB) function. The DUB activity of this complex is specific for poly-K63-ubiquinated protein species, leading only to the cleavage of poly-ubiquitin chains linked via lysine 63. Loss of Bre might lead to a reduction of the DUB function in the BRISC complex. Hence, it was tested whether there is a change in poly-Ub K63 levels in Bre del-MEFs compared to their wt-counterparts.

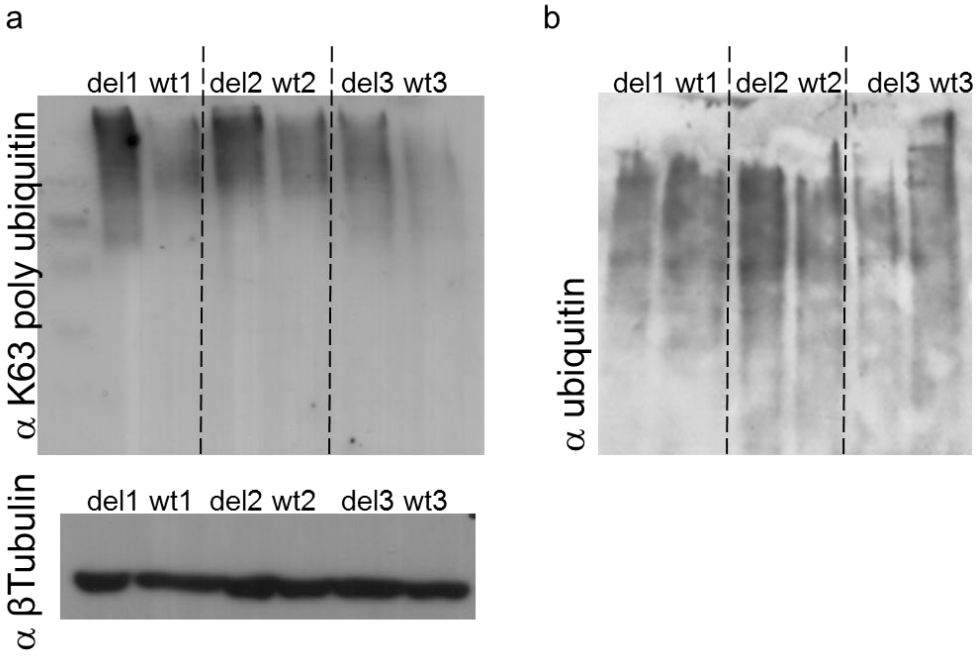


Fig. 31 K63 poly-Ub species in Bre del and Bre wt-MEFs
Cytoplasmic fractions derived from Bre del and Bre wt-MEFs were analysed in SDS-PAGE and Westernblot. (a) αK63 polyubiquitin antibody (secondary antibody: human HRP); (b) αubiquitin antibody (secondary antibody: αmouse HRP). As a loading control αβTubulin antibody was used.

To do so, nuclear and cytoplasmic fractions of Bre del- and wt-MEFs were prepared and both fractions were analysed for the content of K63-poly-ubiquinated protein species by Westernblot analysis with human αK63-poly Ub (secondary antibody: anti-human HRP). However, there was only a clear K63-poly Ub signal in the cytoplasmic fraction detectable,

whereas in all nuclear fractions only a very faint signal was obtained (data not shown). In the cytoplasmic fractions, αβTubulin antibody (secondary antibody: αmouse HRP) was used as a loading control. Two Bre del- (1884.2 and 2107.2) and the two corresponding wt- (1884.5 and 2107.7) cell lines were analysed for their K63-poly-Ub content. In all cases, cytoplasmic fractions derived from Bre del-MEFs seemed to contain slightly more poly-K63-Ub protein species than the fractions obtained from the corresponding wild type counterparts when compared to the αβtubulin loading control (Fig.31 a). In contrast, total ubiquitin levels were not affected (Fig. 31b). Therefore, loss of Bre might result in a reduced DUB activity of the BRISC complex and thus to less degradation of poly-K63-Ub-chains in Bre del-MEFs.

5. Discussion

5.1 Hey genes are dispensable for ES cell maintenance

Hey-triple-KO-ES cells were established in this work for the investigation of Hey function during differentiation. The use of KO SR 2i medium (as described by Batlle-Morera *et al.* 2008; Ying *et al.* 2008; Gertsenstein *et al.* 2010) and a temporary culture on feeder cells allowed the generation of ES cells with a stable karyotype from $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$ - murine blastocysts. As Hey2/HeyL double KO-ES cells were generated in high numbers, it can be deduced that these two genes are not essential for the formation of pluripotent inner cell masses. The floxed Hey1 alleles are still present in the blastocyst and only deleted after the establishment of the ES cells, therefore it cannot be completely excluded that this gene is involved in the process of ES cell formation. However, this is rather unlikely as Hey1 single KO animals do not show any defects at all and as Hey1/Hey2- or Hey1/HeyL-double-KO animals develop until at least E10.5 (Fischer *et al.* 2004b; Kokubo *et al.* 2005; Fischer *et al.* 2007). The established Hey-triple-KO-ES cells display a stem cell character, as they are positive for ALP staining and express the pluripotency marker genes Oct4 and Nanog. Thus, none of the Hey genes is involved in the maintenance of the embryonic stem cell status. Consistent with this observation, the maintenance of murine or human embryonic stem cells was not disturbed when upstream Notch was blocked, either. Disruption of Notch signalling was achieved by the deletion (or reduction) of *O*-fucosyltransferase1-, Notch1-, Notch 2- or RBPJ- expression or by addition of γ-secretase inhibitor (Shi *et al.* 2005; Nemir *et al.* 2006; Noggle *et al.* 2006; Fox *et al.* 2008; Jang *et al.* 2008; Yu, X. *et al.* 2008). Hence, Notch signalling and its Hey down-stream effectors are dispensable for the maintenance of the pluripotency state in ES cells.

5.2 The onset of differentiation and the induction of germ layer markers are not altered in Hey-triple-KO- vs. $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$ -ES cells

When $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$ - and Hey-triple- KO-ES cells were differentiated as EBs, they gave rise to cells of all three germ layers, which could be assessed by the induction of germ layer marker gene expression of AFP, Brachyury and Nestin. Furthermore, an increase in expression levels was observed for all three germ layer markers from day 4 on with similar dynamics. Thus, Hey1 deficient ES cells did not show a premature or delayed onset or longer persistence of any of these marker genes. Yet, it must be mentioned, that all of the marker genes analysed in this work are only compared between Hey-triple-KO- and Hey2/HeyL-

double KO-ES cells. Hence, differences after loss of Hey1 in comparison to ES cells without any Hey deficiency cannot be excluded.

For the onset of differentiation, there was no obvious difference between Hey-triple-KO- and $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$ -ES cells, either. Notch1 has been demonstrated to be necessary for an efficient induction of ES cell differentiation: on the one hand, NICD over-expression in murine ES cells resulted in an earlier onset of neural progenitor determination and on the other hand loss of Notch in human ES cells led to a reduced differentiation potential (Lowell *et al.* 2006; Noggle *et al.* 2006; Yu, X. *et al.* 2008). However, this effect must be exerted by Notch down-stream effectors other than Hey1, because down-regulation of the pluripotency marker Oct4 is not delayed after loss of Hey1, for instance. This correlates with the fact that Hey1 expression (which is very low in ES cells) is induced only from EB d4 on when the differentiation process has already begun.

Regarding the induction of germ layer marker gene expression, there is no difference in the expression levels for Brachyury between $Hey1^{fl/fl}/Hey2^{-/-}/HeyL^{-/-}$- and Hey-triple-KO-ES cells at any given point of time. In Notch1 deficient ES cells, however, the mesodermal markers Brachyury and Fgf8 are up-regulated (Nemir *et al.* 2006). In line with this, mesodermal gene expression is barely detectable when Notch signalling is activated by NICD over-expression in ES cells. The same holds true for endodermal markers (Lowell *et al.* 2006). In two of the analysed Hey-triple-KO-ES cells, AFP expression is elevated on EBd10, suggesting that Hey1 is involved in the regulation of endodermal differentiation by Notch signalling.

Furthermore, neural marker gene expression is increased in NICD-overexpressing ES cells (Lowell *et al.* 2006). Hey1 might be a down-stream effector responsible for this observation, because a decrease in neural marker gene expression could be detected in two of three Hey1 deleted clones. However, this is only true at a very late time-point (at EB d10), so that Hey genes probably are not the main driving force for neural cell formation. Another very important feature during development and also of EB differentiation is the process of EMT (epithelial-to-mesenchymal transition). The involvement of Hey1, Hey2 and HeyL in this process could be shown in KO-mice and in cellular models (Kokubo *et al.* 2005; Fischer *et al.* 2007). However, during the course of EB differentiation, EMT marker gene expression (i.e. Snail) was not altered after loss of Hey1.

5.3 Neural marker gene expression is reduced in Hey-triple-KO-ES cells

Hey gene over-expression leads to the repression of proneural genes (Sakamoto *et al.* 2003). Thus, loss of Hey1 in Hey-triple-KO-ES cells should result in a de-repression and eventually in a higher neural marker gene expression compared to the floxed Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$ -clone. However, the only neuronal marker, with an elevated expression in Hey-triple-KO-ES cells compared to the Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$-control cells, is the Dat1 gene, as shown in all three Hey1 deleted clones. This regulation correlates with the studies of Fuke et al, in which it was found that loss of Hey1 leads to an up-regulation of this marker (Fuke *et al.* 2005; Fuke *et al.* 2006). For all the other markers analysed, there is a lower expression in two of the Hey-triple-KO-ES cell clones compared to the Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$-control cells (i.e. Neurog2, Mash1, Tubb3, Olig2). An explanation for a lower instead of an expected higher marker gene expression could be found in a disturbed neural stem cell maintenance after loss of all three Hey genes. A precocious loss of the neural stem cell pool would then result in overall fewer neural progeny. Similar observations were made in Hes1/Hes5 double KO animals (Ohtsuka *et al.* 1999; Hatakeyama *et al.* 2004) supporting the notion that loss of Hey1 and Hey 2 might have the same effects, because Hey1/Hey2 mis-expression also leads to similar results as Hes1/Hes5 mis-expression does (Ohtsuka *et al.* 2001; Sakamoto *et al.* 2003). Furthermore, RBPJκ deficient ES cells reflected the findings from KO animals with a disturbed neural maintenance (Hitoshi *et al.* 2002). Hence, the Hey-triple-KO-ES cells might also mimic such an effect. Yet, further experiments need to be done in order to determine and quantify neural stem cell numbers and the resulting neural progeny after ES cell differentiation.

5.4 No change in cardiovascular marker gene expression after loss of Hey1

Besides the differential gene regulation of neural marker genes, loss of Hey1 also resulted in an altered regulation of the Gata 4 and Gata 6 genes. This correlates with the findings obtained for Hey1$^{fl/fl}$/Hey2$^{-/-}$ vs. Hey1$^{-/-}$/Hey2$^{-/-}$-ES cells. Here, these two Gata factors were also up-regulated after loss of the floxed Hey1 alleles (Fischer *et al.* 2005). It could be demonstrated that Hey1 represses the Gata4 promoter as well as promoters of Gata 4 and Gata 6 target genes, which eventually leads to modifications in gene expression during heart development (Kathiriya *et al.* 2004; Shirvani *et al.* 2006; Xiang *et al.* 2006; Koibuchi and Chin 2007; Liu, Y. *et al.* 2010). However, in KO animals it could be observed that the loss of Hey genes did not result in defects in cardiomyocyte development *per se*, but only led to structural deformations (Fischer *et al.* 2002; Gessler *et al.* 2002; Kokubo *et al.* 2004; Kokubo

et al. 2005; Fischer *et al.* 2007; Kokubo *et al.* 2007). Consistent with this, there is no change in the expression of the early cardiac marker Nkx2.5 in Hey-triple-KO-ES cells (note that this marker is only weakly expressed in the Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$- as well as in the Hey-triple-KO-clones).

Loss of Notch1 expression in murine or human embryonic stem cells enhanced cardiomyocyte formation (Nemir *et al.* 2006; Jang *et al.* 2008); the same is true in the case of RBPJκ deficiency (Schroeder *et al.* 2003). However, no beating cardiomyocytes were found in Hey-triple-KO or in Hey1$^{fl/fl}$/Hey2$^{-/-}$/HeyL$^{-/-}$- differentiated ES cells until EB d10 precluding analysis of cardiomyocyte numbers. Yet, this is a time-point at which first beating clusters should already have occurred under ideal culture conditions (Zweigerdt *et al.* 2003). Nevertheless, it can be excluded that loss of Hey gene expression resulted in enhanced or even overabundant cardiomyocyte differentiation.

Vascular marker gene expression (i.e. Tie1 and VE-cadherin) is not altered, either. Yet, this correlates with the findings from Hey1/Hey2 double KO animals, where the formation of vascular structures *per se* is not disturbed, but only the remodelling afterwards (Fischer *et al.* 2004b).

5.5 Alterations in muscle marker gene expression after loss of Hey1

Hey1 is an efficient suppressor of the Myogenin promoter and leads to a decrease in Myogenin expression as has been demonstrated in different studies (Sun *et al.* 2001; Buas *et al.* 2009; Buas *et al.* 2010; Wen *et al.* 2012). Consistent with this, loss of Hey1 in the Hey-triple-KO-ES cells resulted in a higher Myogenin expression after differentiation at EB d10. For Myotilin, an opposing regulation was observed with a lower expression after loss of Hey1 at EB d10. However, the expression of Myotilin during mouse embryonic development has been demonstrated not to be restricted to the developing muscle, but to be present in many other tissues and only to be mainly expressed in muscle later in development (Mologni *et al.* 2001). Therefore, it is hard to determine why loss of Hey1 leads to a down-regulation of this marker. The EB differentiation and analysis of marker gene expression by realtime-RT-PCR cannot answer the question whether Myogenin marker gene expression is elevated after loss of Hey1 due to the formation of a higher number of mature myogenic cells or due to a premature loss (i.e. increased differentiation) of progenitor cells as described for the satellite cells in Hey1 and HeyL double KO animals (Fukada *et al.* 2011). As for the neural stem cell to neural progeny differentiation system it would also be necessary to include further

quantification studies to answer this question concerning the role of Hey genes during muscle cell differentiation.

5.6 Time-and context dependency of Notch signalling

Meier-Stiegen and co-workers proposed a model, in which Notch signalling ensures stem cell differentiation in a highly coordinated context- and time-dependent manner: according to this model, Notch induces lineage specific transcription factors in early phases leading to the formation of certain lineage progenitors, whereas later in development Notch signalling inhibits further differentiation by Hey and Hes genes (Meier-Stiegen *et al.* 2010). Consistent with this idea, activation of Notch at early time points induced the neural fate by suppression of the mesodermal and endodermal fate, but later on active Notch signalling suppressed further neuronal differentiation by the induction of its down-stream effector genes (Lowell *et al.* 2006) Time-dependency of Notch effects could also be observed for Notch 4-NICD over-expression: at early time-points, the re-specification of a hemangioblast to a cardiac precursor cell phenotype was induced, whereas at later time points cardiomyocyte differentiation was blocked (Chen, V. C. *et al.* 2008). NICD-induction at different developmental stages of mesodermal development also had different effects ranging from a complete block of mesodermal differentiation in the beginning to a preference for the formation of mesodermally derived mural cells over cardiac muscle/endothelial cells/hematopoietic cells at later stages (Schroeder *et al.* 2006). Interestingly, NICD-over-expression at different time-points during neural differentiation of ES cells only resulted in Cyclin D1 induction if NICD was administered on day 3, but at no other time point (Das *et al.* 2010).

With these findings in mind, it would be interesting to establish Hey-inducible ES cell lines from the Hey-triple-KO-ES cells and to analyse in further experiments whether switching Hey function on or off at defined time-points leads to alterations in the transcriptional programme and in the obtained cell progeny after differentiation. Such cell lines could also help to investigate whether Hey gene expression is a prerequisite for the maintenance of different multipotent stem cells such as neural stem cells or muscle satellite cells. Furthermore, Hey inducible ES cell lines would represent a valuable tool for the identification of novel Hey target genes during differentiation. The generation of stable cell lines with inducible Hey constructs should in general be possible, as Hey-triple-KO-ES cells can be targeted by transfection, electroporation and viral transduction.

5.7 Hey1 interacts with different Bre domains and both proteins are partially co-expressed during mouse embryonic development

The discovery of Hey target genes/interaction partners would allow to shed further light onto Hey function and/or its regulation. Bre has been found to be such a Hey1 interacting protein in a yeast-two-hybrid screen by Andreas Fischer (see PhD thesis: http://opus.bibliothek.uni-wuerzburg.de/volltexte/2003/608/). In the present work, it could be corroborated that Hey1 and Bre represent binding partners and that mutation of only two amino acids in the Hey1 C-terminal sequence abolished the interaction between these two proteins. First, co-immunoprecipitation experiments with full-length proteins verified the findings by Andreas Fischer and second, bacterially expressed and purified MBP-Bre protein also interacted with Hey1. Further known binding partners of Bre are the TNFα receptor and the Fas receptor (Gu *et al.* 1998; Li, Q. *et al.* 2004) as well as the different BRCC and BRISC complex proteins. For the death receptors, no further investigation of the domains of Bre involved in the binding were performed, but in the case of the BRCC- and BRISC- complex partners, deletion studies were carried out. Here it could be shown, that Bre consists of two UEV-domains, which are both involved in the interaction with the different BRCC and BRISC complex proteins. The N-terminal part comprising the UEV1-domain was sufficient for Brcc36 and ABRO binding, whereas the C-terminal UEV2 alone mediated the interaction with Merit40. For RAP80 and Abraxas, either both UEV-domains or at least constructs spanning great parts of both UEV-domains were necessary for an efficient interaction with Bre. Mutation of conserved motifs in both UEV-domains (WPN motif in the UEV1; YSP motif in the UEV2) abolished Abraxas, Brcc36 and RAP80 binding and Merit40 binding, respectively (Feng *et al.* 2009; Patterson-Fortin *et al.* 2010; Hu, X. *et al.* 2011). These findings imply on the one hand that different proteins need different interactions sites (of different length) inside the Bre protein and on the other hand that this binding is mainly mediated by the UEV-domains. To determine whether Hey1 shows a binding behaviour comparable to Brcc36 or Merit40 (i.e. the binding to only one of the two UEV-domains), three non-overlapping HA-Cherry-Bre fusion constructs were generated (spanning the UEV1, a middle part without any annotated domain and the UEV2). Surprisingly, each of these proteins was able to interact with Flag-Hey1. However, none of the fusion proteins bound to Flag-Hey1-mut, confirming the findings for the interaction with HA-Cherry-full length-Bre protein. The co-immunoprecipitation results might suggest a slight preference for Hey1 binding to the UEV2-domain. The protein comprising the Bre-UEV2 also showed a sub-cellular localisation more or less identical to full-length Bre: both proteins were nearly exclusively found in the nucleus, whereas the Bre-mid and the Bre-UEV1 containing

proteins also often showed an additional cytoplasmic staining. Yet, these findings were not quantified. The binding of Flag-Hey1 and the different HA-Cherry-Bre fusion proteins may be either direct or mediated by further proteins (for example by binding of Flag-Hey1 or HA-Cherry-Bre to other Bre complex partners or to the endogenous Bre protein). So far, evidence is lacking that Hey1 (directly or indirectly) interacts with BRCC- or BRISC-complex members other than Bre, however. Yet, the endogenous Bre (which is highly expressed in HEK 293T cells) might act as a bridge for Flag-Hey1 and HA-Cherry-Bre fusion-protein interaction, because Flag-Bre was able to bind to itself and also to non-overlapping HA-Cherry-Bre fusion proteins.

The interaction studies described above were performed in a cell culture system with over-expressed proteins. Therefore, the binding of the two proteins to each other could merely be an *in vitro* artefact. To investigate whether both proteins are co-expressed during embryonic development and hence could also interact *in vivo*, Bre LacZ mice were analysed by β-galactosidase staining. The obtained expression pattern was compared to the findings from whole mount *in situ* hybridisation for Hey1, Hey2 and HeyL expression (Leimeister *et al.* 1999; Kokubo *et al.* 1999; Leimeister *et al.* 2000b). Strong and overlapping signals for Hey and Bre proteins could particularly be found in the brain and the developing limb bud. Therefore, Hey-Bre interaction could in principle be possible in these regions during murine embryonic development.

5.8 Sub-cellular localisation of Bre is not affected by Hey1 over-expression

Bre expression cannot only be found during embryonic development, but also in the adult mouse in many organs. Here, the sub-cellular localisation of Bre (i.e. in the nucleus or in the cytoplasm) is dependent on the cell type analysed. It could for example be observed in the liver that Bre expression is mainly localised in the cytoplasm in hepatocytes, whereas it is mostly found in the nucleus in cells from the bile duct and in Kupffer cells (Chan, B. C. *et al.* 2008). Both nuclear and cytoplasmic localisation was also found in other studies by immunohistochemistry as well as by sub-cellular fractionation experiments. In most of these analyses, Bre protein was described to be essentially located to the cytoplasm (Miao *et al.* 2001; Li, Q. *et al.* 2004; Tang, M. K. *et al.* 2009; Hu, X. *et al.* 2011). However, prominent nuclear localisation could also be found, especially in tumour cells where the Bre nuclear signal is enhanced compared to cells from normal surrounding tissue (Chan, B. C. *et al.* 2008; Chen, H. B. *et al.* 2008; Tang, M. K. *et al.* 2009). In this work, Bre localisation was analysed by immunofluorescence staining in HEK 293T cells and in HeLa cells. In both cell lines, Bre

expression was predominantly found in the nucleus with a uniform staining pattern, but a faint cytoplasmic signal was also detectable.

Interestingly, sub-cellular localisation of Brcc36, one of the complex partners of Bre in the BRCA1-A- and BRISC-complexes, was affected by alterations in Abraxas or ABRO protein levels, eventually leading to changes in IRIF formation or DUB activity, respectively (Feng *et al.* 2009; Patterson-Fortin *et al.* 2010). The same observations (i.e. a shift towards the nuclear or cytoplasmic compartment) should hold true for Bre localisation, as it represents a core component of the BRCA1-A- and the BRISC-complexes. As a change in Bre sub-cellular localisation might interfere with its function, it was of interest to analyse whether Hey1 over-expression would alter Bre sub-cellular localisation. However, when a pmCherry-Hey1 construct was overexpressed, alterations in Bre sub-cellular localisation such as an obvious translocation of endogenous Bre to the nucleus or a shift towards a more pronounced cytoplasmic localisation of Bre were not detecable. From this it must be concluded that Hey1 does not interfere with Bre sub-cellular localisation. Furthermore, preliminary nuclear-cytoplasmic CoIP results found an interaction of Flag-Hey1 and HA-Cherry-Bre in the nucleus as well as in the cytoplasm (data not shown), suggesting that sub-cellular localisation probably does not interfere with the binding capacity of the two proteins. These first findings do not suggest that Hey1 is involved in the regulation of Bre sub-cellular localisation or in the formation of Bre sub-cellular BRISC- or BRCA1-A complexes.

5.9 Bre does not serve as an E3-ligase for Hey1 and shows no binding to ubiquitin

The first description of Bre as a member of a higher molecular weight complex (i.e. of the BRCA1/BARD1 containing complex) was by Dong and co-workers (Dong *et al.* 2003). The BRCA1/BARD1 heterodimer has been shown to have an intrinsic E3-ligase activity (Lorick *et al.* 1999; Hashizume *et al.* 2001; Mallery *et al.* 2002), which has been demonstrated to be further enhanced by Bre and Brcc36, because Bre and Brcc36 co-expression together with the BRCA1/BARD1 heterodimer led to an increased ubiquitination of the p53 substrate (Dong *et al.* 2003). As Hey1 has been shown to be ubiquinated by RTA E3-ligase activity (Gould *et al.* 2009), it should be investigated whether Bre functions as an E3-ligase for Hey1. However, in a ubiquitination assay for Flag-Hey1, no ubiquitin attachment could be detected after HA-Cherry-Bre over-expression. Ubiquinated proteins were not even found after 18h of MG132 treatment (data not shown), which resulted in efficient Hey1 ubiquitination by the RTA-protein as described by Gould et al. Yet, ubiquitination assays are often performed as mere *in*

vitro reactions, which represent a more sensitive system. Here all components involved in the generation of poly-ubiquitin chains (i.e. substrate, ubiquitin, E1, E2 and E3) are added to the reaction. Even though endogenous E1 and E2 as well as BRCA1/BARD1 should be expressed in HEK 293T cells, it cannot be excluded that Bre (in complex with BRCA1/BARD1) may serve as an E3-ligase for the Hey1 substrate in another, more sensitive system. When the Hey1 ubiquitination assay was performed, a strong signal of higher molecular weight bands above the unmodified HA-Cherry-Bre signal was obtained. This could be either due to ubiquitination or even due to auto-ubiquitination of the HA-Cherry-Bre protein: the BRCA1/BARD1 E3-ligase has been shown to have an auto-ubiquitination activity (Chen, A. *et al.* 2002; Mallery *et al.* 2002). Another possibility for the obtained higher molecular weight signal might be that these bands do not represent a modified version of the Bre-protein, but other ubiquitinated proteins, which were bound to Bre via one of its UEV-domains. To account for the latter possibility, it was tested whether the UEV-domains of the Bre protein can bind to ubiquitinated proteins from cellular lysates pre-treated with MG132. A bacterially purified MBP-Bre protein was used for the test, but no binding to ubiquitin could be demonstrated in SDS-PAGE and Western blot analyses, whereas the MBP-Bre protein efficiently interacted with a Flag-Hey1 protein. Hence, the higher molecular weight signal obtained for Bre in the ubiquitination assay is probably rather due to ubiquitination of the Bre-protein instead of binding to ubiquinated proteins by Bre.

Bre, Abraxas, ABRO and Brcc36 have been shown to bind efficiently to K63- as well as to K48-poly-ubiquitin chains (Wang, B. *et al.* 2009). The reason why in this work no binding of Bre to any ubiquinated proteins could be demonstrated may lie in the fact that UEV-domains only show low affinity towards ubiquitin binding compared to other ubiquitin-binding domains as assessed for the Tsg101 protein (Hurley *et al.* 2006). Moreover, the conditions used in this work (i.e. the analysis of cellular lysates) are less ideal than those found in the *in vitro* systems applied by Wang et al (Wang, B. *et al.* 2009). In line with this, only very faint binding of Bre to ubiquinated proteins could be shown in HeLa cells by Li and co-workers under normal conditions. An interaction was only detectable to a high degree when the cells were treated with TNFα or FasL, which then led to an increase of proteins modified by ubiquitination (Li, Q. *et al.* 2004). Thus, stress or apotosis induction prior to ubiquitin binding studies might also be necessary to allow the detection of Bre binding to ubiquitin with the IP-conditions applied in this work.

5.10 Bre over-expression does not lead to an enhanced Hey1 turnover

Consistent with the observation that Bre over-expression did not lead to the formation of any ubiquinated Hey1-protein under the applied experimental conditions, over-expression of HA-Citrine-Bre did not enhance FS-mHey1 turnover, either. This would be the case if Bre acted as a K48-Ubiquitin E3-ligase for Hey1: increase in poly K48-ubiquitination of Hey1 would normally provoke enhanced degradation of the protein by the proteasome (rev. by Hershko and Ciechanover 1998). However, even if Bre acted as an E3-ligase for Hey1, it would first have to be determined whether the modification then would consist of a poly-K48 signal, because the BRCA1 E3-ligase has particularly been shown to have a preference to form K6-linked ubiquitin chains. However, only contradictory results have been obtained so far for the potential of the BRCA1/BARD1 heterodimer to form K48- or K63- linked chains (Chen, A. *et al.* 2002; Wu-Baer *et al.* 2003; Xia *et al.* 2003; Morris and Solomon 2004; Nishikawa *et al.* 2004; Starita *et al.* 2005).

Depending on the control, Bre over-expression sometimes seemed to lead to a slight or even to a clear stabilisation of Hey1 expression compared to control-transfected cells. Even though a quantification of this observation was not possible, this finding would correlate well with the stabilising Bre function on BRCC- and BRISC-complex members observed by Hu and co-workers: after knockdown of Bre, protein expression of all complex partners is (nearly) lost. This effect can be rescued by Bre over-expression (Hu, X. *et al.* 2011). Hence, it could for example also be worth to check for Hey1 protein levels in Bre KO-cells. However, Feng and co-workers did not observe a loss in protein expression of any of the complex members after Bre knockdown (Feng *et al.* 2009).

Even though no ubiquitination of Hey1 could be determined by Bre over-expression, it is nevertheless noteworthy that K6 poly-ubiquitination of the BRCA1/BARD1 substrate nucleophosmin resulted in a stabilisation of this protein (Sato, K. *et al.* 2004). It might be possible that Bre also leads to a stablisisation of the Hey1 protein by a comparable modification.

5.11 Bre modulates Hey1 repressive function

Alteration of Hey1 stability might also interfere with Hey1 function, because a prolonged Hey1 expression would allow Hey1 to exert its actions for a longer time. One of the best characterised Hey1 features is its repression of target gene promoters. In this work, luciferase assays were performed with promoters, which are efficiently repressed by Hey1 to investigate

whether binding to Bre interferes with Hey1 repressive function. When analysing the repression of luciferase activity, it could be observed that there was a significantly enhanced repressor activity of wild type Flag-Hey1 compared to the Bre-binding-mutant Flag-Hey1-mut at three of the promoters chosen (i.e. the Hey1, Dll4 and Gata6 promoters). Moreover, Flag-Bre protein expression alone was sufficient for the repression of these promoters. Bre has already been shown to be able to exert a suppressive function on a luciferase promoter construct, namely in the case of a NFκB reporter construct. However, this was only true under conditions in which Bre associates with the TNFR (i.e. after TNFα stimulation: Gu et al, 1998). Bre repression of Hey1 target promoters might also be dependent on a direct interaction with Hey1. For this, the overexpressed Flag-Bre protein might bind to the endogenous Hey1 protein found in the HEK 293T cells, which then in turn would lead to target promoter repression together with Bre. Interaction of Bre with endogenous Hey1 may also account for the increase in promoter repression observed for the Flag-Hey1-mut, when Flag-Bre was added in increasing amount.

For the Jagged 1 promoter, no significant differences in repression could be seen when comparing wild type Flag-Hey1 to the Flag-Hey1-mut and there was no promoter repression at all when Flag-Bre protein alone was overexpressed. The Jagged1 promoter is the only one of the promoters analysed, which has been demonstrated to be dependent on an intact E box for an efficient suppression by Hey1 (Heisig *et al.* 2012). May be this promoter is affected by Hey1 in a different (i.e. Bre independent) mode of function. Different domains in the Hey1 protein have been demonstrated to display a repressive function, but so far the recruitment of co-repressors has only been shown for the Hey bHLH domain (Iso *et al.* 2001b; Takata and Ishikawa 2003). Interestingly, Bre interacts with the C-terminal domain of Hey1: First, a Hey1 C-terminal bait was sufficient to pull down Bre in the yeast-two-hybrid screen and second the amino acids, which are necessary for Bre binding (F264/268) are also located in the C-terminal part of the protein. Thus, Bre may be involved in the modulation of the repressive function that has been ascribed to the Hey1 C-terminus (Sun *et al.* 2001; Belandia *et al.* 2005; Holderfield *et al.* 2006). However, how Bre might account for this still remains elusive.

5.12 Bre LacZ KO-ES cells and mice and Bre del- mice are viable, fertile and without any obvious defect

The generation of Bre LacZ gene trap-ES cells did not lead to a successful loss of Bre expression in these cells on mRNA levels; thus, it is not surprising that there was no change in germ layer marker gene expression between Bre heterozygous and Bre homozygous LacZ ES cells during EB differentiation. In homozygous Bre LacZ mice, which were generated by crossing of heterozygous Bre LacZ mice (obtained from EUCOMM), a knock out of Bre could not be observed on mRNA level, either (data not shown). Therefore, these animals were not suitable for the analysis of Bre function, but only for the detection of Bre expressing embryonic tissues by β-gal staining.

Additionally to the integration of the LacZ cassette, the exon 3 of Bre is floxed in the Bre LacZ-ES cell construct allowing the excision of this exon by crossing Bre LacZ-animals to Cre delete-mice. Loss of exon 3 should result in a frameshift and premature stop of the protein and could be confirmed on mRNA levels in MEFs derived from homozygous deleted animals. Bre del- mice were viable after birth and did not show any defect into adulthood such as shorter lifespan or premature aging. Furthermore, Bre del-MEFs did not display a senescence morphology at early passages. These are all finding obtained for the loss or truncation of the Bre binding partner BRCA1 (Ludwig *et al.* 2001; Cao *et al.* 2003). Homozygous BRCA1 deficiency even resulted in embryonic lethality (Evers and Jonkers 2006). However, BRCA1 is not only a member of the Bre containing BRCA1-A complex, but amongst others also of the BRCA1-B and BRCA1-C complexes (rev. by Wang, B. 2012), so that loss of this gene has certainly more negative down-stream effects. In BRCA1 animals, there is also spontaneous tumour development, for instance. Bre del-mice did not show any obvious increase in tumour development compared to their wild type littermates; however, the mice have not been investigated histologically in detail so far. Yet, even for BRCA1 deficiency, tumour development was only observed after long latency and could be significantly enhanced by intercrossing to p53 deficient animals (rev. by Deng and Scott 2000). Hence, mating of Bre del- mice with known tumour model mice might also result in enhanced tumour formation.

Bre is highly expressed in ovary and testes and takes part in the regulation of steroidogenesis. Therefore it was postulated that loss of Bre might negatively interfere with fertility (Li, L. *et al.* 1995; Miao *et al.* 2001; Poon *et al.* 2004; Miao *et al.* 2005; Chan, B. C. *et al.* 2008). Yet, both female and male Bre deficient mice give rise to viable offspring even if both parents

were of homozygous deleted genotype. Loss of BRCA1 has also a more dramatic phenotype in this case in that male mice are infertile depending on the genetic background (Ludwig *et al.* 2001; Xu 2003). Besides the phenotypes observed after BRCA1 deficiency, the other Bre complex partners Brcc36 and ABRO are also involved in disease development: Brcc36 mutation is associated with Moyamoya syndrome (a cerebrovasclular angiopathy) in humans and led to a disturbed vascular remodelling in the trunk of zebrafish embryos (Miskinyte *et al.* 2011). For ABRO1 a cardioprotective effect has been found: following myocardial infarction, ABRO1 levels increased leading to the de-ubiquitination of poly-K63-ubiquinated proteins (Cilenti *et al.* 2011). As Bre function is closely related to BRCC/BRISC function it cannot be excluded that subtle yet undiscovered changes also occurred in the murine embryos. Furthermore, it might also be worth to test for the effects after loss of Bre under stress conditions such as myocardial infarction models.

5.13 Bre del-MEFs do not show proliferation defects and no increased IR sensitivity

Bre del- mice were used for the generation of MEFs to gain further insight into Bre biology by analysis of these cells. First, Bre del-MEFs were compared to the corresponding cells derived from wild type littermates with regard to their proliferation potential. Bre del-MEFs did not show alterations in cellular growth under normal culture conditions. This is in contrast to a study performed by Tang and co-workers, who found that Bre deficiency after siRNA knock down led to a slight but significant increase in the proliferation of C2C12 cells. Accordingly, Bre over-expression led to a decrease of cell growth in D122 cells (Chan, B. C. *et al.* 2005; Tang, M. K. *et al.* 2006). However, Bre knock down in Chang liver cells resulted in no change of proliferation as it has been observed for the Bre del-MEFs analysed in this work. Of note, Chan and co-workers monitored proliferation only for 48h, which is a relatively short timespan (Chan, B. C. *et al.* 2005). Nevertheless, it might be possible that the effects of Bre on proliferation are cell type dependent.

After Brcc36 knock down in breast cancer cells, differences in cell numbers were only observed after irradiation, but not under normal conditions (Chen, X. *et al.* 2006). Hence, stress conditions could also lead to a change in proliferation and an increased IR sensitivity in Bre del-cells. However, this was not the case when comparing the growth curves of Bre del- and Bre wild type MEFs after irradiation with 5 Gy.

Loss of the BRCA1-A members RAP80, Abraxas, Brcc36, Merit40 led to a strong IR sensitivity (Kim *et al.* 2007a; Kim *et al.* 2007b; Sobhian *et al.* 2007; Wang, B. *et al.* 2007;

Yan *et al.* 2007; Shao *et al.* 2009; Wang, B. *et al.* 2009; Hu, X. *et al.* 2011). The IR sensitivity of Merit40 was found to be dependent on Bre as only a Merit40 construct capable of Bre binding could restore cell viability in a rescue experiment after Merit40 knockdown (Hu, X. *et al.* 2011). Yet, consistent with no apparent overall change in cellular proliferation, Bre del-MEFs did not display an increased IR sensitivity, either, as assessed by the number of surviving cells after irradiation compared to non-irradiated cells. Contrary to these findings, there is a strong IR sensitivity in HeLa cells treated with Bre siRNA: here, the cell survival is dramatically decreased in a dose-dependent manner (Dong *et al.* 2003). In this work, Bre MEFs were irradiated with 5Gy; this is a dose which efficiently induced DNA double strand breaks (as marked by efficient γH2AX foci formation, compare Fig. 27); yet it cannot be excluded that higher doses might have had a more pronounced effect on Bre del-MEF survival compared to their wild type counterparts. Furthermore, cell growth was only monitored for four days after plating. At the fourth day, a slight decrease in the percentage of surviving cells of Bre del-MEFs compared to wild type cells was observed. Monitoring of cell growth for a longer time point might therefore reveal more striking differences. However, the results obtained in the present work exclude a major role of Bre in the modulation of cell proliferation and survival after stress induction, because if this was the case at least minor changes should already be detectable at early time points.

5. 14 Bre del-MEFs show a reduced early BRCA1 response

Even though loss of Bre did not result in an increased IR sensitivity in Bre del-MEFs compared to wt-MEFs, these cells were investigated for potential defects in DNA damage response, because loss of Bre (Feng *et al.* 2009; Shao *et al.* 2009; Wang, B. *et al.* 2009) or of any of the other BRCA1-A members resulted in a strong reduction of BRCA1 IRIF formation as assessed in diverse studies (Chen, X. *et al.* 2006; Kim *et al.* 2007a; Kim *et al.* 2007b; Liu *et al.* 2007; Sobhian *et al.* 2007; Wang, B. and Elledge 2007; Wang, B. *et al.* 2007; Shao *et al.* 2009; Wang, B. *et al.* 2009; Feng *et al.* 2010; Hu, X. *et al.* 2011; Hu, Y. *et al.* 2011). To test, whether this holds also true for Bre del- and wt-MEFs, these cells were analysed for their BRCA1 response following irradiation. Quantification of the total number of BRCA1/γH2AX double positive nuclei as well as of the nuclei with a strong BRCA1 response (i.e. >10 double positive nuclei) showed that there is a highly significant difference especially 15 minutes after irradiation between Bre del- and wt-MEFs in that Bre deficiency resulted in a reduced BRCA1 signal. Differences are less striking 80 minutes after irradiation and can only be observed for cells with a strong BRCA1 response. These findings would at least suggest a

delay in early BRCA1 response in Bre deficient MEFs. The percentage of Bre del- vs. wt-cells with a strong BRCA1 signal after 15 minutes are comparable to the percentage of BRCA1 positive cells in Bre knock down- vs. wt-cells obtained by Feng and co-workers (Feng *et al.* 2009): In this work 68% vs. 34% BRCA1 positive nuclei were obtained and in the study of Feng et al 75% vs. 28%. However, in the study performed by Feng and co-workers (and in most of the other studies for BRCA1 quantification), cells were fixed at a much later time-point (i.e. 4h after irradiation). Yet, this is a time point at which BRCA1 as well as γH2AX were already diminished in Bre MEFs as determined in a preliminary irradiation test followed by γH2AX immunofluorescence staining (data not shown). Furthermore, the overall maximal percentage of BRCA1/γH2AX positive in Bre MEFs is rather low with less than 20%. It is however noteworthy, that the dynamics for the recruitment of BRCA1-A components or of BRCA1 are subject to a high degree of variance between different studies (see for example Hu, Y. *et al.* 2011)). In line with the observation that BRCA1 recruitment to sites of DNA damage was only reduced but not abolished after loss of Bre, BRCA1 still localised to the laser-induced stripes (i.e. sites where DNA damage had been introduced) in the confocal stripe-assay. This correlates well with the findings obtained in studies with knock down of Merit40, RAP80 or Abraxas. Here, BRCA1 recruitment to the sites of DNA damage was still possible, however the signal obtained for BRCA1 intensity was significantly lower (Sobhian *et al.* 2007; Shao *et al.* 2009; Patterson-Fortin *et al.* 2010). Unfortunately, it was not possible to compare signal intensities between Bre del- and wt-MEFs, because the time span between the fixation steps was too long. In conclusion, even though Bre MEFs showed a comparatively weak response towards irradiation (which is also consistent with the finding that Bre del-MEFs did not have an increased IR sensitivity), the results obtained suggest a modulatory role of Bre in BRCA1 recruitment to sites of DNA damage.

5.15 Bre deficiency only has minor impact on FasL cytotoxicity

Irradiation of Bre MEFs did not lead to an increase in cell death after loss of Bre, thus, it was analysed whether Bre del-MEFS showed alterations in apoptosis rates after stimulation with FasL or TNFα, because Bre has also been ascribed a role in apoptosis. For instance, FasL and TNFα treatment of Bre overexpressing cells has been demonstrated to result in a reduced apoptotic answer. Concomitantly, reduction of Bre levels led to enhanced cell death after TNFα stimulation (Li, Q. *et al.* 2004; Chan, B. C. *et al.* 2008). Therefore, Bre del- and Bre wt-MEFs were used for cytotoxicity assays to test whether loss of Bre results in an enhanced sensitivity of these cells towards death receptor ligand stimulation. Here, only FasL treatment

led to a killing of the cells, whereas TNFα did not display negative effects on cell survival. Even though Bre del- cells displayed in average a slightly enhanced dying of the cells after FasL treatment, the effect observed was not dramatic with LD_{50} values of 0.24ng/ml (Bre del-MEFs) and 0.41ng/ml (Bre wt-MEFs), respectively. However, this finding nevertheless correlates with the notion of Bre as a protein with anti-apoptotic properties. The differences in the percentage of viable cells obtained by Li and co-workers after reduction of Bre levels and TNFα treatment were also only moderate (Li, Q. *et al.* 2004). Hence, the anti-apoptotic effect exerted by Bre after over-expression might be much more relevant (for example for the process of tumour development) than the loss of Bre. Consistent with this, the mere reduction of Bre levels did not result in spontaneous apoptosis, but cell death was only increased after stimulation of Bre-deficient cells with stress inducing agents (Li, Q. *et al.* 2004). Of note, different results were obtained for Bre over-expression in HeLa cells with respect to cell survival. Whereas Gu and co-workers claimed that Bre over-expression *per se* resulted in enhanced cell death (Gu *et al.* 1998), Li et al observed a protective effect of Bre over-expression in cells stimulated with TNFα (Li, Q. *et al.* 2004). In this work, over-expression of Bre in HeLa cells (both by transient and stable expression in cell lines) did not affect programmed cell death (data not shown). Therefore, even though Bre has been shown to have properties of an anti-apoptotic protein, further investigation will be necessary to substantiate these findings and to shed light onto the cellular contexts in which Bre function plays a decisive role.

5.16 Bre does not lead to major changes in Poly-K63-Ub levels

Besides the investigation of Bre function in the nuclear BRCA1-A complex by the DNA damage assays, the effect of loss of Bre on the cytoplasmic BRISC complex (Cooper *et al.* 2009) was assessed. For this, cytoplasmic fractions from Bre del- and wt-MEFs were probed with αpoly-K63-ubiquitin antibody after SDS-PAGE and Western blotting. Samples derived from Bre wt-MEFs yielded a slightly less intense signal for poly-K63-ubiquitin-modified proteins compared to Bre del-MEFs. The increase in poly-K63 Ubiquitin modified proteins in Bre del-MEFs might suggest a reduction of DUB activity in the BRISC complex after the loss of the Bre protein. The fact that only minor differences were observed between the Bre del- and wt-MEF samples might be explained by the observation that the BRISC core activity is already found within the Brcc36 and ABRO heterodimer yet with reduced activity after loss of Bre (Feng *et al.* 2009; Cooper *et al.* 2010; Patterson-Fortin *et al.* 2010). Furthermore, the quantification of an increase of proteins modified via K63-poly Ub in cellular lysates after

loss of Bre is certainly not as accurate as the de-ubiquitination activity determined in DUB assays with *in vitro* generated poly-K63-ubiquitin chains. Hence, it would be worth to include DUB assays with cellular lysates derived from Bre del- and wt-MEFs, respectively and to compare their efficiency in cleaving poly-Ub chains. Moreover, the DUB activity intrinsic to the nuclear DUB complex seems to be more dependent on Bre function (Feng *et al.* 2009; Patterson-Fortin *et al.* 2010). However, an analysis of nuclear extracts from Bre MEFs only resulted in very faint to non-detectable poly-K63-ubiquitin levels, thereby precluding further analyses. Hence, it would also be helpful to investigate nuclear fractions in *in vitro* assays. The de-ubiquitinase activity within the BRISC is specific for K63-linked-poly-Ub chains; all other ubiquitin linkages are unaffected (Cooper *et al.* 2009; Cooper *et al.* 2010; Patterson-Fortin *et al.* 2010). In line with this, total ubiquitin levels in the cytoplasmic fractions were not affected by the loss of Bre. As K48-poly-ubiquitinated proteins are the most abundant form in total ubiquitin it can be deduced that loss of Bre in Bre del-MEFs only interfered with poly-K63-ubiquitin levels, but not with poly-K48-ubiquitin levels. For all other ubiquitin modifications, further experiments (with the respective specific antibodies) will be necessary to detect the effect of the loss of Bre on their abundance.

5.17 Relevance of Hey and Bre binding remains elusive

The functional relevance of Hey1 and Bre interaction still remains elusive. Even though first evidence exists that Hey1 and Bre might work together during gene transcription regulation (as demonstrated by the enhanced promoter repression when Hey1 and Bre were co-expressed in luciferase assays), the mode of action is yet unknown. Both proteins could either interact with each other prior to DNA binding or one of the two factors might already be bound to DNA, whereupon the other protein is recruited to the DNA. Moreover, Bre might just modulate Hey1 so that its repressive function is enhanced (for example by stabilisation of the protein as suggested in Fig. 18). Hey1 is a well-established transcription factor and has been shown to bind to thousands of regions in the genome by ChIP-Seq analysis (Heisig *et al.* 2012). For Bre no DNA binding capabilities could be demonstrated, so far. Even though there is no evidence so far one might speculate that Bre might be bound to DNA via its interaction partner BRCA1. BRCA1 has been proposed to "build a bridge to transcription", as it has intrinsic chromatin remodelling activity (Bochar *et al.* 2000; Ye *et al.* 2001), is part of the (processive) RNA polII and associates with RNA helicase A (Scully *et al.* 1997; Anderson *et al.* 1998; Krum *et al.* 2003). Furthermore, BRCA1 can recruit p300/CBP histone acetylases as well as histone de-acetylases (Yarden and Brody 1999; Pao *et al.* 2000) and finally can also

lead to gene activation (Somasundaram *et al.* 1997; Zhang *et al.* 1998; MacLachlan *et al.* 2002; Williamson *et al.* 2002) as well as gene repression (Wang, Q. *et al.* 1998). Interestingly, association with BARD1 reduces BRCA1 activation of the NFκB promoter (Benezra *et al.* 2003). Maybe association with Bre (together with or without Hey proteins) might have the same impact on BRCA1 gene activation ability. Of note, BRCA1 induces gene transcription of factors, which are repressed by Hey proteins such as p21 or p27Kip1 (Zhang *et al.* 1998; Williamson *et al.* 2002; Wang, W. *et al.* 2003; Havrda *et al.* 2006). Yet so far, no evidence exists pointing towards a crosstalk between BRCA1 and Notch signalling. Interestingly, Notch signalling and BRCA1 have both been shown to regulate apoptosis in neural progenitors during brain development in a p53 dependent manner (Yang *et al.* 2004; Pulvers and Huttner 2009). Whereas NICD (i.e. active Notch signalling) induces apoptosis, BRCA1 inhibits this process during development. Thus, one might speculate that there could be an interference with each other. Furthermore, both Hey1 and Bre might be involved in this regulation, because both proteins are highly expressed in the brain during development (Leimeister *et al.* 1999; Fig. 23; Kokubo *et al.* 1999).

Even though Hey1- and Bre- single KO-mice are without any obvious defect, a combined loss of Hey1 and Bre might result in an obvious phenotype as it has also been shown for the combination of Hey1 and HeyL KO, where the single KO-mice were also without obvious defects (Fischer *et al*, 2007; Fukada *et al*, 2011). Hey1/Bre double KO as well as Hey1/Hey2/Bre triple KO-mice are currently being generated. Maybe these animals show deficiencies, which will allow further insight into the functional relevance of Hey and Bre interaction.

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7. Abbreviations

ALP	alkaline phosphatase
APS	ammonium peroxydisulfate
bp	base pairs
BSA	bovine serum albumin
°C	degree Celsius
cDNA	complementary DNA
C. elegans	Caenorhabditis elegans
CHIR 99021	glycogen synthase kinase GSK-3 inhibitor
Chx	cycloheximid
Cm	centimetre
Co-IP	co-immunoprecipitation
ct value	cycle threshold value
d0, 2, 4, 7,10	0, 2, 4, 7,10 days (after differentiation)
DABCO	1,4-diazobicyclo-2.2.2-octane
DAPI	4'6-diamidino-2-phenylindole, dihydrochloride
ddH_2O	double distilled water
del	deleted
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside-triphosphate
DTT	dithiothreitol
E3.5, E13.5	embryonic day 3.5, 13.5
EB	embryoid body
EDTA	ethylenediamine-tetraaceticacid
EGTA	ethylene glycol bis(2-aminoethyl) tetraacetic acid
ES cell	embryonic stem cell
FCS	fetal calf serum
Fig.	Figure
FITC	fluoresceinisothiocyanat
Fl.	Floxed
g	g force or gram
GFP	green fluorescent protein
Gy	Gray
h	hour
HEPES	N-2-Hydroxyethylpiperazin-N´-2-ethanoicsulfoacid
HRP	horseradish peroxidase
ICM	inner cell mass
KO	knock out
KO SR 2i	KO serum replacement medium with 2 inhibitors (i.e. CHIR 99021, PD 0325901)
LB	Lysogeny broth
LIF	leukemia inhibitory factor
LOH	loss of heterozygosity
M	molar
mA	milli Ampere
MEFs	murine embryonic fibroblasts

µg	microgram
mg	milligram
MG132	Z-Leu-Leu-Leu-CHO
min	minute
µl	micro litre
ml	millilitre
µm	micrometre
µM	micro molar
mM	milli molar
mRNA	messenger RNA
µs	micro second
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetr -azolium bromide
mut	mutant
NEAA	non essential amino acids
NEM	N-Ethylmaleimide
nm	Nanometre
nW	nano Watt
PAGE	poly acrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	PBS/Triton
PCR	polymerase chain reaction
pcv	packed cell volume
PD 0325901	Inhibitor of MEK 1
PEI	polyethylenimine
PFA	paraformaldehyde
PI	protease inhibitor
PMSF	phenylmethylsulfonyl fluoride
rpm	rounds per minute
SDS	sodium dodecylsulfate
RNA	ribonucleic acid
RT	room temperature
TEMED	tetramethylethylendiamine
Tris	Tris-(hydroxymethyl)-aminomethane
Triton	t-Octylphenoxypolyethoxyethanol
TM	Tris-maleat
U	Unit
Ub	ubiquitin
UV	ultra violet
V	Volt
wt	wild type

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Poster

03/2011 „Hey genes in ES cell differentiation and as Bre binding partners"
(Joint Meeting of the German and Japanese societies of developmental biologists, Dresden)

10/2009 „Hey genes – regulators of endothelial development and molecular binding partners"
(Molecular basis of organ development in vertebrates, Würzburg)

03/2009 „Hey genes in endothelial development and as molecular binding partners"
(4th Graduate Symposium, Würzburg)

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation „Establishment of Hey-triple-KO-ES cells and characterisation of Bre, a Hey binding partner“ selbstständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel durchgeführt habe.
Ich erkläre außerdem, dass diese Dissertation weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen hat.
Ich habe früher außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 30.7.2012

Traudel Schmidt