Characterisation and regulation of the Egfr/Egfr ligand system in fish models for melanoma

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Content

1. List of publications

Schartl, M., Wilde, B., Laisney, J. A., Taniguchi, Y., Takeda, S., Meierjohann, S. (2010). A mutated EGFR is sufficient to induce malignant melanoma with genetic background-dependent histopathologies. *J Invest Dermatol*. **130**, 249-58.

Laisney, J.A., Braasch, I., Walter, R.B., Meierjohann, S., Schartl, M. (2010). Lineage-specific co-evolution of the Egf receptor/ligand signaling system. *BMC Evol Biol*.**10**, 27.

Laisney, J.A., Schartl, M., Meierjohann, S. Dimerized and oncogenic EGFR variants can induce an autocrine loop to enhance EGFR activation.

2. Summary

Fish of the genus *Xiphophorus* belong to the oldest animal models in cancer research. The oncogene responsible for the generation of spontaneous aggressive melanoma encodes for a mutated epidermal growth factor receptor (Egfr) and is called *xmrk* for *Xiphophorus* melanoma receptor kinase. Xmrk constitutive activation mechanisms and subsequent signaling pathways have already been investigated and charaterized but it is still unknown if Egfr ligands may also play a role in Xmrk-driven melanoma formation.

To investigate the potential role of Egfr ligands in Xmrk-driven melanoma, I firstly analyzed the evolution of teleost and tetrapod Egfr/Egfr ligand systems. I especially focused on the analysis on the medaka fish, a closely related species to *Xiphophorus*, for which the whole genome has been sequenced. I could identify all seven Egfr ligands in medaka and could show that the two teleost-specific Egfr copies of medaka display dissimilar expression patterns in adult tissues together with differential expression of Egfr ligand subsets, arguing for subfunctionalization of receptor functions in this fish. Our phylogenetic and synteny analyses supported the hypothesis that only one gene in the chordate ancestor gave rise to the diversity of Egfr ligands found in vertebrate genomes today. I also could show that the Egfr extracellular subdomains implicated in ligand binding are not evolutionary conserved between tetrapods and teleosts, making the use of heterologous ligands in experiments with fish cells debatable.

Despite its well understood and straight-forward process, Xmrk-driven melanomagenesis in *Xiphophorus* is problematic to further investigate *in vivo*. Our laboratory recently established a new melanoma animal model by generating transgenic *mitf*::*xmrk* medaka fishes, a *Xiphophorus* closely related species offering many more advantages. These fishes express *xmrk* under the control of the pigment-cell specific Mitf promoter. During my PhD thesis, I participated in the molecular analysis of the stably transgenic medaka and could show that the Xmrk-induced signaling pathways are similar when comparing *Xiphophorus* with transgenic *mitf*::*xmrk* medaka. These data together with additional RNA expression, protein, and histology analyses showed that Xmrk expression under the control of a pigment cell-specific promoter is sufficient to induce melanoma in the transgenic medaka, which develop very stereotyped tumors, including uveal and extracutaneous melanoma, with early onset during larval stages.

To further investigate the potential role of Egfr ligands in Xmrk-driven melanoma, I made use of two model systems. One of them was the above mentioned *mitf*::*xmrk* medaka, the other was an *in-vitro* cell culture system, where the EGF-inducible Xmrk chimera HERmrk is

stably expressed in murine melanocytes. Here I could show that HERmrk activation strongly induced expression of amphiregulin (Areg) and heparin-binding EGF-like growth factor (Hbegf) in melanocytes. This regulation was dependent on the MAPK and SRC signaling pathways. Moreover, upregulation of *Adam10* and *Adam17*, the two major sheddases of Egfr ligands, was observed. I also could demonstrate the functionality of the growth factors by *in-vitro* analyses. Using the *mitf*::*xmrk* medaka model I could also show the upregulation of a subset of ligand genes, namely *egf*, *areg*, *betacellulin* (*btc*) and *epigen* (*epgn*) as well as upregulation of medaka *egfrb* in tumors from fish with metastatic melanoma. All these results converge to support an Xmrk-induced autocrine Egfr ligand loop. Interestingly, my in-vitro experiments with conditioned supernatant from medaka Egf- and Hbegf-producing cells revealed that not only *Xiphophorus* Egfrb, but also the pre-activated Xmrk could be further stimulated by the ligands.

Altogether, I could show with *in-vitro* and *in-vivo* experiments that Xmrk is capable of inducing a functional autocrine Egfr ligand loop. These data confirm the importance of autocrine loops in receptor tyrosine kinase (RTK)-dependent cancer development and show the possibility for a constitutively active RTK to strengthen its oncogenic signaling by ligand binding.

3. Zusammenfassung

Fische der Gattung *Xiphophorus* gehören zu den ältesten Tiermodellen für die Krebsforschung. Das im *Xiphophorus*-System für die Melanomentstehung verantwortliche Onkogen codiert für eine mutierte Version des epidermalen Wachstumsfaktorrezeptors (Egfr) und wird *xmrk* (für "*Xiphophorus* melanoma receptor kinase") genannt. Die konstitutiven Aktivierungsmechanismen dieses Rezeptors und die daraus resultierenden aktivierten Signalwege sind bereits gut untersucht und charakterisiert. Dennoch war bisher unbekannt, ob Egfr-Liganden auch eine Rolle bei der Xmrk-vermittelten Melanomentstehung spielen.

Um eine potenzielle Rolle dieser Egfr-Liganden im Xmrk-induzierten Melanom zu erforschen, habe ich zunächst die Evolution des Egfr/Egfr-Liganden-Systems in Teleostiern und Tetrapoden untersucht. Hierfür fokussierte ich mich im besonderen auf den Medaka-Fisch, der zum einen eine nahe evolutionäre Verwandtschaft zu *Xiphophorus* aufweist und zum anderen – im Gegensatz zu *Xiphophorus* - ein komplett sequenziertes und gut annotiertes Genom besitzt. Ich konnte alle sieben Egfr-Liganden in Medaka identifizieren und konnte weiterhin zeigen, dass die zwei Teleost-spezifischen Egfr-Kopien dieses Fisches ein unterschiedliches Expressionsmuster in adulten Geweben aufweisen, welches außerdem mit unterschiedlicher Egfr-Liganden-Expression einherging. Diese Daten sprechen für eine Subfunktionalisierung der Egfr-Funktionen in Medaka.

Unsere phylogenetischen und Syntenie-Analysen unterstützen die Hypothese, dass nur ein einziges Egfr-Liganden-Gen des Chordaten-Vorfahren der genetische Ursprung für die zahlreichen Egfr-Liganden-Gene, die in heutigen Vertrebraten zu finden sind, darstellt. Ich konnte weiterhin zeigen, dass die an der Ligandenbindung beteiligten Domänen des Egfr nicht zwischen Tetrapoden und Teleostiern konserviert sind. Diese Daten sprechen somit gegen die Verwendung heterologer Liganden in Zellkulturexperimenten mit Fischzellen.

Trotz der gut verstandenen Konsequenzen einer Xmrk-Expression auf die Pigmentzelle lässt sich die Xmrk-vermittelte Melanomentstehung in *Xiphophorus* relativ schwer *in vivo* untersuchen. In unserem Labor wurde daher kürzlich ein neues Tiermodell für Melanome entwickelt. Dabei handelt es sich um einen *mitf::xmrk*-transgenen Medaka. Diese Fische exprimieren *xmrk* unter der Kontrolle des Pigmentzell-spezifischen Mitf-Promoters. Während meiner Doktorarbeit trug ich zur molekularen Analyse der stabil transgenen Tiere bei und konnte zeigen, dass die Xmrk-vermittelte Signalgebung in *mitf::xmrk*-Medakas der von Xmrk-exprimierenden *Xiphophorus*-Fischen gleicht.

Diese Daten, zusammen mit weiteren RNA-Expressions-, Protein- und histologischen Analysen, zeigten, dass die Expression von *xmrk* unter der Kontrolle eines Pigmentzell-

spezifischen Promoters ausreichend für die Melanomentstehung in Medaka ist. Eine Besonderheit dieses Melanommodelles ist die auffallend stereotype Tumorentstehung. Der Beginn der Hyperpigmentierung wird bereits in frühen Larvenstadien sichtbar und führt – je nach Fischlinie – anschließend zuverlässig zu extrakutanen Pigmentzelltumoren oder invasiven bzw. uvealen Melanomen.

Um eine potenzielle Funktion der Egfr-Liganden für Xmrk-induzierte Melanome zu untersuchen, machte ich mir zwei Modellsysteme zunutze. Eines der beiden Modelle war der bereits oben erwähnte *mitf*::*xmrk*-transgene Medaka, das andere war ein *in-vitro*-Zellkultursystem, bei dem die EGF-induzierbare Xmrk-Chimäre HERmrk stabil in murinen Melanozyten exprimiert wird. Hier konnte ich zeigen, dass HERmrk-Aktivierung zu einer starken Genexpression der EGFR-Liganden Amphiregulin (Areg) und Heparin-binding EGF-like growth factor (Hbegf) in Melanozyten führte. Diese Regulierung war abhängig von den MAPK- und SRC-Signalwegen. Weiterhin wurde eine Induktion von *Adam10* und *Adam17*, den zwei bedeutsamsten Proteasen zur Freisetzung von EGFR-Liganden ("Sheddasen"), festgestellt. Ich konnte die Funktionalität der so sezernierten Liganden durch *in-vitro*-Experimente nachweisen. Anhand des *mitf*::*xmrk* Medaka-Modelles konnte ich ebenfalls zeigen, dass sowohl mehrere Egfr-Ligandengene, nämlich *egf*, *areg*, *betacellulin* (*btc*) und *epigen* (*epgn*), als auch *egfrb* in Tumoren von Medaka-Fischen mit metastatischen Melanomen heraufreguliert wurden. All diese Daten lassen auf einen durch Xmrk induzierten autokrinen EGFR-Liganden-Loop schließen. Interessanterweise zeigte sich durch *in-vitro*-Experimente mit konditioniertem Überstand von Medaka Egf- und Hbegf-produzierenden Zellen, dass nicht nur *Xiphophorus* Egfrb, sondern auch das bereits aktivierte Xmrk durch beide Liganden weiter stimuliert werden konnte.

Zusammengefasst zeigen meine *in-vitro*- und *in-vivo*-Daten, dass Xmrk in der Lage ist, einen funktionalen autokrinen Egfr-Liganden-Loop zu induzieren. Dieses Ergebnis unterstreicht die Bedeutung autokriner Loops in Rezeptortyrosinkinasen (RTK)-abhängiger Tumorentstehung und zeigt auf, dass selbst die onkogene Signalgebung prädimerisierter RTKs durch Ligandenbindung verstärkt werden kann.

4. Introduction

The epidermal growth factor receptor (EGFR) that plays central roles in invertebrate and vertebrate development and physiology is one of the most investigated members of the family of EGF receptors that also comprises three know homologous receptors: ErbB2 (also called Neu or HER2), ErbB3 (HER3) and ErbB4 (HER4). EGFR is a transmembrane tyrosine kinase receptor that functions normally in a ligand-dependent fashion forming homodimers, but also forming heterodimers with its three homologues (see for a review (Jorissen, Walker et al. 2003)). Recently, the EGFR has emerged as key target of molecular therapy for solid tumors since it also plays central roles in proliferation, angiogenesis, invasiveness, decreased apoptosis and loss of differentiation processes (see for a review (Takeuchi and Ito 2010)).

4.1. EGFR functions

Many functions of EGFR during development were revealed using invertebrate model organisms like the nematode *Caenorhabditis elegans* or the fruit fly *Drosophila melanogaster*, where extensive investigations of EGFR allowed its assignment in cardinal functions. In *C.elegans*, EGFR, also called LET-23, was demonstrated to be involved in vulval induction, viability, male spicule development, hermaphrodite ovulation and differentiation of ventral uterus and posterior ectoderm (Sundaram 2006). In *D.melanogaster*, EGFR or Drosophila Egf receptor (DER) has been assigned 30 distinct functions, among them cell fate induction, cell proliferation and cell migration (Shilo 2003). In these two invertebrate organisms, EGFR signaling was demonstrated to take place repeatedly with different functions during development.

During vertebrate embryogenesis EGFR and its ligands are expressed and functional whenever epithelial-mesenchymal interactions take place. The epithelial-to-mesenchymal transition (EMT) is a critical process in embryonic development and is considered to be the single most important mechanism regulating organ development in vertebrates (Gurdon 1992). Most organs are formed from epithelial and mesenchymal tissues and share common morphological features during early development (Thesleff, Vaahtokari et al. 1995). Moreover, the EMT process is also thought to be an important mechanism for promoting cancer invasion and metastasis (see for a review (Barr, Thomson et al. 2008)).

Knockout of Egfr in mice is lethal, and, depending on the genetic background, abnormalities affect skin, lung and gastrointestinal tract development, but also trigger neurodegeneration (Miettinen, Berger et al. 1995; Threadgill, Dlugosz et al. 1995; Sibilia, Steinbach et al. 1998).

In adult organs, EGFR signaling maintains cellular activities at equilibrium to preserve normal structure and function (homeostasis); this is particularly important for the skin.
In normal epidermis, a strict balance between proliferation of cells in the basal layer and loss of terminally differentiated cells from the surface has to be preserved. In humans, EGFR is mostly expressed in keratinocytes in the basal layer of the epidermis (Nanney, McKanna et al. 1984) where it stimulates cell cycle progression through autocrine and paracrine mechanisms (Jost, Kari et al. 2000). However, it also regulates programmed cell death in keratinocytes (Stoll, Benedict et al. 1998; Jost, Huggett et al. 2001). Most importantly, activation of the EGFR also appears to be crucial for regulation of keratinocyte migration (Gibbs, Silva Pinto et al. 2000). This is important for proper development and wound healing processes, but also can be responsible for metastasis formation in pathological states. Additionally, upregulation of EGFR is leading to autocrine growth of melanocytes which are singly situated between keratinocytes (Udart, Utikal et al. 2001).
Few works are documenting the role of the Egfr and the Egf-family ligands within fish species. In zebrafish, the Egfr was shown to promote oocyte maturation in vitro (Pang and Ge, 2002) and to have cardiovascular functions during development (Goishi et al, 2003). The full-length zebrafish Egfr and Egf have been cloned and their spatio-temporal pattern of expression in the ovary were investigated. Recombinant human EGF was tested on zebrafish ovarian follicle cells primary cultures and was shown to act as potential paracrine factor from the oocyte to regulate the activin/follistatin system in the follicle cells (Wang et al., 2004). Using recombinant EGF, betacellulin (BTC) and heparin-binding EGF-like growth factor (HB-EGF), it was also demonstrated that the Egf signaling network in the zebrafish ovarian follicle was self-regulated by members of the EGF family (Tse at al., 2008). In the goldfish *Carassius auratus*, use of recombinant EGF and TGFA showed that these growth factors may play a role in the regulation of ovarian functions (MacDougall and Van Der Kraak, 1998; Pati et al., 1996; Kumar Srivastava and Van Der Kraak, 1995). Finally, in the medaka *Oryzias latipes*, Egfr is present during development supporting the hypothesis that Egfr ligands also are important during cleavage, gastrulation and early organogenesis (Boomsma et al., 2001).

4.2. Function of EGFR ligands

To date, there are seven known ligands to bind the EGFR: epidermal growth factor (EGF), transforming growth factor-a (TGFA), amphiregulin (AREG), betacellulin (BTC), epiregulin (EREG), heparin-binding egf-like growth factor (HBEGF) and epigen (EPGN).

Although all EGFR ligands bind the EGFR within the receptor extracellular ligand-binding pocket, they are only partly redundant and their biological effects are astonishingly distinct (see for a review (Schneider, Werner et al. 2008)). It is somehow surprising that only the absence of HBEGF is lethal in mice due to heart and lung malformations (Iwamoto, Yamazaki et al. 2003; Jackson, Qiu et al. 2003). Indeed, HBEGF was later demonstrated to be involved in heart development and homeostasis (Iwamoto and Mekada 2006). Conversely, mice deficient for AREG or EGF (Luetteke, Qiu et al. 1999), for BTC (Jackson, Qiu et al. 2003), EREG (Mann, Fowler et al. 1993; Lee, Pearsall et al. 2004; Shirasawa, Sugiyama et al. 2004), TGFA (Luetteke, Qiu et al. 1993) and even triple null mice that lack AREG, EGF and TGFA (Luetteke, Qiu et al. 1999) are viable. These observations point out that some, but not all, EGFR ligands display functional redundancy.

To preserve epidermal homeostasis, four EGFR ligands are of special importance to regulate keratinocyte proliferation, differentiation and migration, namely EGF, TGFA, AREG and HBEGF (see for a review (Hashimoto 2000) and (Schneider, Werner et al. 2008)). Overexpression of EGFR ligands in kerationocytes conducts to different phenotypes depending on the ligand. For example, overexpression of AREG was shown to be responsible for hyperkeratosis/psoriasis-like phenotype (Cook, Brown et al. 2004), whereas increased levels of EGF cause hyperproliferation of basal cells and hair follicle developmental arrest (Mak and Chan 2003), BTC transgenic animals have a relatively mild skin phenotype with delay in hair cycle induction and increased angiogenesis at wound sites (Schneider, Werner et al. 2008). In contrast, overexpression of TGFA causes hyperproliferative tumor-prone skin (Vassar and Fuchs 1991; Dominey, Wang et al. 1993).

Epigen, the newest member of the EGFR ligands, is the least investigated EGF-family growth factor. One of its functions is the ability to promote growth of epithelial cells (Strachan, Murison et al. 2001), and lately, it was reported that EPGN transgenic mice develop enlarged sebaceous glands (Dahlhoff, Muller et al. 2009). Altogether, these data demonstrate overlapping and specific functions for the seven ligands in order to maintain epidermis homeostasis.

4.3. Egfr is conserved throughout evolution

The epidermal growth factor receptor (Egfr) as key regulator of fundamental functions in multicellular organism development is highly conserved, both in vertebrates and invertebrates, at least in triploblastic organisms (meaning all superior and intermediate

animals from human to worm that arise from a blastula that possesses three primary germ layers: ecto-, meso- and endoderm) (Stein and Staros 2006; van Kesteren, Gagatek et al. 2008). There is to date no evidence for presence of Egfr in simpler diploblastic animals, like jellyfish and corals, which arise from a blastula that possesses two primary germ layers: ectoderm and endoderm.

In coelomate organisms, Egfr was isolated in protostomian animals like the nematode worm *Caenorhabditis elegans* (Aroian, Koga et al. 1990), the bivalve mollusc *Mytilus galloprovincialis* (Canesi, Malatesta et al. 2000) and the gastropod mollusc *Lymnaea stagnalis* (van Kesteren, Gagatek et al. 2008). Within the non-chordate deuterostomes, Egfr was identified in insects (Lycett, Blass et al. 2001) (Gibson and Tolbert 2006) (Nakamura, Mito et al. 2008), including the fruit fly *Drosophila melanogaster* (Schejter, Segal et al. 1986). It was also isolated in the echinoderm *Paracentrototus lividus* (Romancino, Montana et al. 2008). In acoelomate organisms, Egfr was also identified in the flatworm *Schistosoma mansoni* (Ramachandran, Skelly et al. 1996).

Within the Chordata phylum, there is evidence for the presence of Egfr in bony fish, amphibians, reptiles, birds and mammals, as their sequenced genomes harbour well-defined Egfr encoding DNA (www.ensembl.org) (Hubbard, Aken et al. 2009). Noteworthy, two copies for the Egfr can be identified in teleost fish. Indeed, it is now generally accepted that an additional whole genome duplication event took place in the evolution of teleost fishes approximately 320-350 million years ago that was termed “fish-specific genome duplication (FSGD; reviewed in (Postlethwait, Amores et al. 2004; Meyer and Van de Peer 2005)). Most teleost gene duplicates, about 80%, encountered pseudogenization or nonfunctionalization during evolution, with elimination of one of the duplicated copy (Jaillon, Aury et al. 2004; Woods, Wilson et al. 2005). It is believed that gene duplicates have to acquire functional divergence to be maintained in the genome during evolution, encountering either neofunctionalization, an adaptive process where one copy mutates to acquire a function that was not present in the pre-duplication gene (Ohno 1970), either subfunctionalization, a neutral process where the two copies partition the ancestral function (Ohno, Wolf et al. 1968; Force, Lynch et al. 1999).

So far, no Egfr has been identified in the Protochordates, Cyclostomes and Chondrichthyes (cartilaginous fish).

4.4. EGFR structure and mode of activation

The archetypal human epidermal growth factor receptor (EGFR) was the first member of the transmembrane tyrosine kinase receptor family to be discovered (Carpenter, King et al. 1978) and sequenced (Downward, Yarden et al. 1984; Ullrich, Coussens et al. 1984). The EGFR and its pathways, that govern cell growth and differentiation during embryogenesis and adult homeostasis, is to date one of the most extensively investigated signaling systems (Yarden and Shilo 2007). The EGFR was also the first cell-surface receptor to be linked to cancer (de Larco and Todaro 1978) and its deregulation either by mutation, overexpression, or from EGFR stimulation through autocrine loops is involved in many human cancers.

EGF receptors are anchored in the cell membrane and comprise an extracellular region (~630 amino acid residues), a single transmembrane domain (~23 residues), an intracellular juxtamembrane domain (~40 residues), a tyrosine-kinase domain (~260 residues) and a C-terminal regulatory region (~232 residues) (Burgess, Cho et al. 2003; Linggi and Carpenter 2006) (Figure 1A). The extracellular region comprises four subdomains, referred to as I to IV (Lax et al. 1998b), or as L1, CR1, L2 and CR2 (Ward, Hoyne et al. 1995). They are involved in receptor activation by ligand binding and/or oligodimerization.

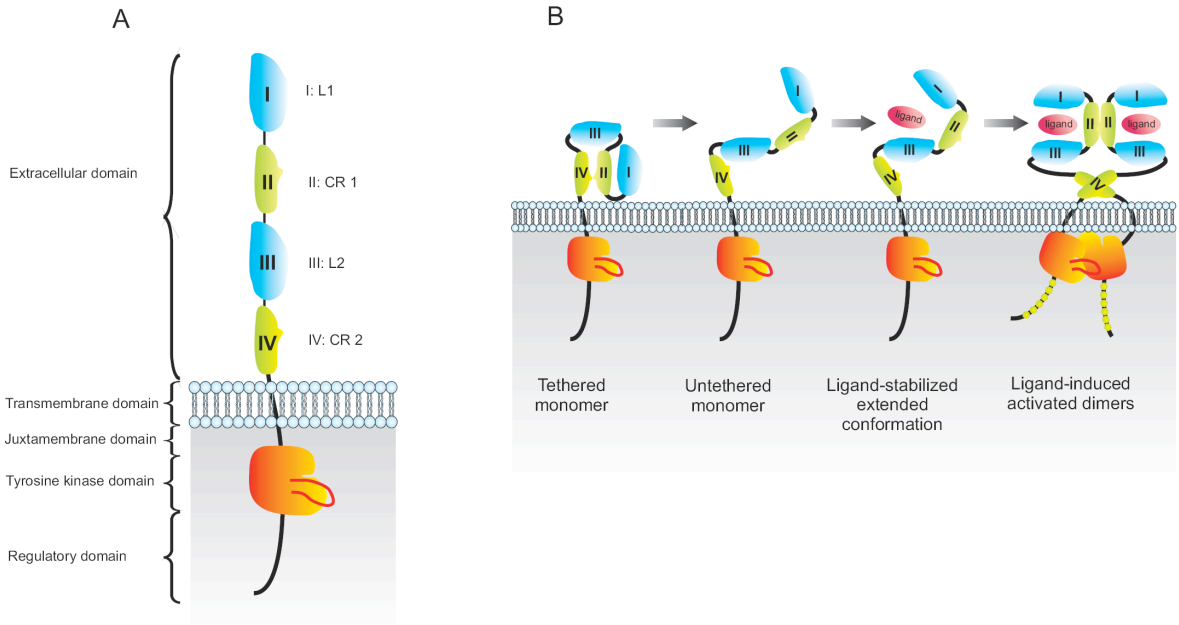

Fig. 1: EGFR structure and mode of activation. (**A**) Overall EGFR structure comprising the extracellular, transmembrane, juxtamembrane, tyrosine kinase and C-terminal regulatory domains. The extracellular region comprises four subdomains, I to IV, also referred to as large EGF binding domain 1 (L1), cysteine-rich domain 1 (CR1), L2 and CR2. (**B**) Different steps of EGFR activation: autoinhibitory tethered monomer, untethered monomer, ligand-stabilized extended, and ligand-induced activated dimer conformations.

Subdomains I and III are homologous to each other and build the ligand-binding pocket, while subdomains II and IV are cystine rich and are implicated in receptor dimerization. Crystal structure analyses of EGFR ectodomain complexed either with EGF or with TGFA (Garrett, McKern et al. 2002; Ogiso, Ishitani et al. 2002) revealed the molecular mechanisms by which the extracellular region of the EGFR (sEGFR) shifts from a dimerization-inhibited conformation, called the tethered conformation, to a ligand-binding induced dimerization-competent conformation, called the extended conformation (Figure 1B). In the tethered conformation, the dimerization arm on subdomain II is buried by intramolecular interactions with subdomain IV (Ferguson, Berger et al. 2003). Binding of ligand within the ligand-binding pocket shaped by subdomains I and III allows subdomain rearrangement, thereby exposing the subdomain II dimerization arm which is necessary for EGFR dimerization. Recently, additional crystallographic approaches and biochemical studies from other regions of native and mutated forms of the EGFR revealed that the cytoplasmic juxtamembrane domain also plays a major role in receptor dimerization, and consequently may also be involved in the allosteric control of ligand binding (Jura, Endres et al. 2009; Red Brewer, Choi et al. 2009). In any case, EGFR dimerization upon ligand binding is thought to reposition the two cytoplasmic tyrosine kinase domains in order to allow efficient trans-phosphorylation of tyrosine residues in the kinase activation loop, the juxtamembrane region and the cytoplasmic domain (see for a review (Hubbard 2004)).

4.5. EGFR signaling

Activation of the EGF receptor by binding of extra-cellular proteins from the EGF-family of growth factors induces receptor dimerization. Subsequent phosphorylation of the cytoplasmic C-terminal part of the EGFR by autophosphorylation or by transphospshorylation (by collagen domain protein Src or Jak-2 kinases) on tyrosine residues is providing docking sites for the Src homology 2 (SH2) or phospho-tyrosine binding (PTB) domains of intracellular signal transducers and adaptors proteins (Schlessinger, 2000; Yarden and Sliwkowski, 2001; Holbro and Hynes, 2004; Citri and Yarden, 2006). Due to the diversity of molecules that complex or are phosphorylated by the EGFR, multiple signaling pathways can be simultaneously activated, the four major highly conserved signaling pathways being the Ras-mitogen-activated protein kinase (MAPK) cascade (Schlessinger, 2000), the phosphoinositide 3-kinase (PI3K)/Akt pathway (Cantlay, 2002; Davis, 2000), the Signal Transducer and Activator of Transcription (STAT) transcription factors (Silva, 2004) and the Src pathway.

The Shc, Grb2 and Ras/MAPK pathway

MAPKs cascades downstream of cell surface EGFRs are involved in the regulation of cell proliferation and survival processes. Following EGFR tyrosine kinase activation kinase by auto- or by transphosphorylation, the complex formed by the adaptor proteins Grb2 and Sos binds directly or via association with the adaptor molecule Shc to specific sites on the receptor (Sasaoka, Langlois et al. 1994). Relocation of the Grb2/Sos complex to the EGFR at the plasma membrane facilitates the interaction of membrane-associated Ras with Sos, with exchange of Ras-bound GDP for GTP and hence in Ras activation. Activated Ras activates in turn serine/threonine kinase Raf-1 (Hallberg, Rayter et al. 1994). Activated Raf-1 initiates phosphorylation, activation and nuclear translocation of Erk-1 and Erk-2. Activated Erk can translocate to the nucleus where they phosphorylate and regulate various nuclear transcription factors (e.g., Elk1, Ets2) ultimately leading to change in gene expression (Johnson and Vaillancourt 1994; Schulze, Nicke et al. 2004). Activated MAPK also acts as inhibitor in a negative feedback loop for this pathway by dissociation of the Grb2-Sos complex induced by MAPK phosphorylation of Sos (Langlois, Sasaoka et al. 1995).

Another major pathway activated upon ligand binding to the EGFR is the PI3-K/Akt pathway, involved in cellular processes including proliferation, survival, adhesion and migration (for a review see (Cantley 2002)). The association occurs between the SH2 domain of the PI3-K p85 subunit and phosphorylated C-terminal part of the EGFR. It has to be mentioned that in humans ErbB3 and not EGFR is the major binding partner of the PI3-K (Kim, Sierke et al. 1994; Ram and Ethier 1996). Once phosphorylated, PI3-K generates the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3), for which best characterized target is the Ser/Thr kinase Akt (PKB). Akt binds in turn to the lipid and is translocated to the plasma membrane where it is phosphorylated and activated by phosphoinositide-dependent-kinase-1 (PDK-1) (see for a review (Nicholson and Anderson 2002)).

Signal transducers and activators of transcription (STAT) proteins are transcription factors involved in cellular proliferation, differentiation, apoptosis and oncogenesis. They interact with phosphotyrosine residues of the Egfr via their Src homology 2 domains, dimerize and translocate from the cytoplasm to the nucleus where they bind regulatory elements in the promoter of target genes to regulate their transcription (Yu and Jove 2004).

SRC kinase (short for sarcoma) is a member of the family of nonreceptor tyrosine kinase that plays a critical role in the regulation of cell proliferation, migration, adhesion, as well as in angiogenesis and immune function (Yeatman 2004). Via activation of a series of substrates,

including focal adhesion kinase, PI3K and STAT proteins, SRC can function independently in the cytosol or cooperate with receptor tyrosine kinase signaling, serving as signal transducer and enhancer of Egfr activation (Jorissen, Walker et al. 2003).

4.6. Phylogenetic distribution of EGFR ligands

In mammals such as humans, EGFR has to date seven known ligands that all are peptide growth factors: EGF, TGFA, AREG only bind EGFR, whereas BTC, EREG, HBEGF and EPGN bind both EGFR and ErbB4 (Harris, Chung et al. 2003; Schneider and Wolf 2009). It is likely that no other EGFR ligands exist since an in-depth genome-wide search with algorithms based on genomic and cDNA structures did not reveal further potential EGFR ligands (Kochupurakkal, Harari et al. 2005).

The seven Egfr ligands characterized in humans are also present in all sequenced vertebrate genomes. Reptiles, chicken, zebra finch and also the amphibian *Xenopus tropicalis* display all seven Egfr ligands sequences in their genome. In teleost fish, all seven Egfr ligands were identified among the seven species investigated but no single teleost fish was found to harbour all seven Egfr ligands at once (Stein and Staros 2006).

However, among invertebrates, only one Egfr ligand was found in the protostomian *C.elegans*, namely Lin-3 (Hill and Sternberg 1992), while four Egfr ligands were characterized in the deuterostomian arthropod *D.melanogaster*: Gurken, Spitz, Vein and Keren (Freeman 1998) (Stein and Staros 2006).

4.7. Structure of Egfr ligands

Egfr ligands are type I transmembrane proteins which, with the exception of Egf - comprise an N-terminal region, an Egf domain (or Egf motif), a short juxtamembrane extension, a hydrophobic transmembrane domain and a cytoplasmic C-terminal tail (Figure 2A). Egf features a unique structure in mammals, with eight extracellular Egf motifs in addition to the Egfr interacting Egf domain located close to the cell membrane.

The seven Egfr ligands have a relatively low overall protein sequence homology of about 25%, but the stretch of 43 amino acids building the Egf motif with its six conserved cysteines is exhibiting high protein sequence homology (Harris, Chung et al. 2003). The six cysteine residues C1 to C6 are building three disulfide bridges between C1 and C3, C2 and C4, and C5 and C6, called the A, B and C loops, respectively (Figure 2B).

Noteworthy, AREG and HB-EGF are the only ligands to possess an additional N-terminal hydrophilic heparin-binding site, thus conveying binding to heparan sulfate proteoglycans (HSPGs) on the cell surface and within the extracellular matrix. Binding of growth factors to heparin sulfate (HS) chains have been demonstrated to modulate the cellular response to growth factors (Forsten-Williams, Chu et al. 2008). HB-EGF also serves as diphtheria toxin receptor, allowing the toxin to enter the cell via binding to and endocytosis with proHB-EGF (Mitamura, Higashiyama et al. 1995).

In mammals, the mature, soluble forms of EGFR ligands have to be released from the initially synthesized membrane-bound precursors to interact with the EGFR.

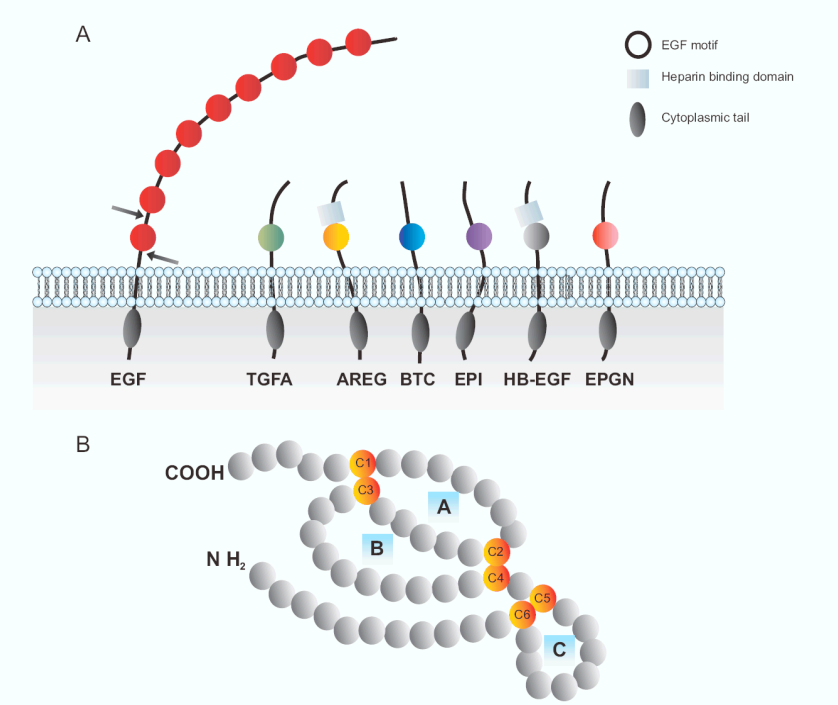

Fig. 2: EGFR ligands. (**A**) The seven EGFR ligand transmembrane precursors: epidermal growth factor (EGF), transforming growth factor alpha (TGFA), amphiregulin (AREG), betacellulin (BTC), epiregulin (EPI), heparin-binding EGF-like growth factor (HBEGF) and epigen (EPGN). (**B**) Overall structure of a mature EGFR ligand with the 6 conserved cysteines building the three disulfide bounds C1-C3, C2-C4 and C5-C6 that generate the A, B and C loops.

4.8. Activation of EGFR ligands by ADAM family members.

One of the most important post-translational regulation events rendering Egfr ligands available is the proteolytic cleavage of an extracellular fragment of the ligand precursor in order to release the EGF motif. Egfr ligands precursors are released from the cell membrane

by specific sheddases called ADAMs (A Disintegrin And Metalloproteases) (Sahin, Weskamp et al. 2004; Sahin and Blobel 2007).

ADAMs are membrane-anchored proteins of about 750 amino acids length that belong to the matrix metalloproteinase family. They are responsible for ectodomain shedding of cell surface proteins such as growth factors, cytokines, cell adhesion molecules, and receptors. Therefore they are involved in a number of physiological processes. Up to now, there are at least 40 ADAMs known in various species, from filamentous fungi, yeast (Saccharomyces pombe), invertebrates (*Caenorhabditis elegans*, *Drosophila melanogaster*) (Pan and Rubin 1997; Huang, Huang et al. 2003; Nakamura, Abe et al. 2004), to vertebrates (hamsters, mice and humans) (see (Reiss and Saftig 2009) for a review).

ADAMs are mostly type I transmembrane glycoproteins with a conserved domain structure consisting of an N-terminal signal sequence, a prodomain, a metalloprotease domain, a disintegrin domain, an EGF domain, a transmembrane domain and a cytoplasmic tail. G protein-coupled receptors (GPCRs) together protein kinase C (PKC) and second messengers such as Ca^{2+} and reactive oxygen species (ROS) have been shown to activate ADAMs and transactivate the EGFR (Ohtsu, Dempsey et al. 2006) (Figure 3).

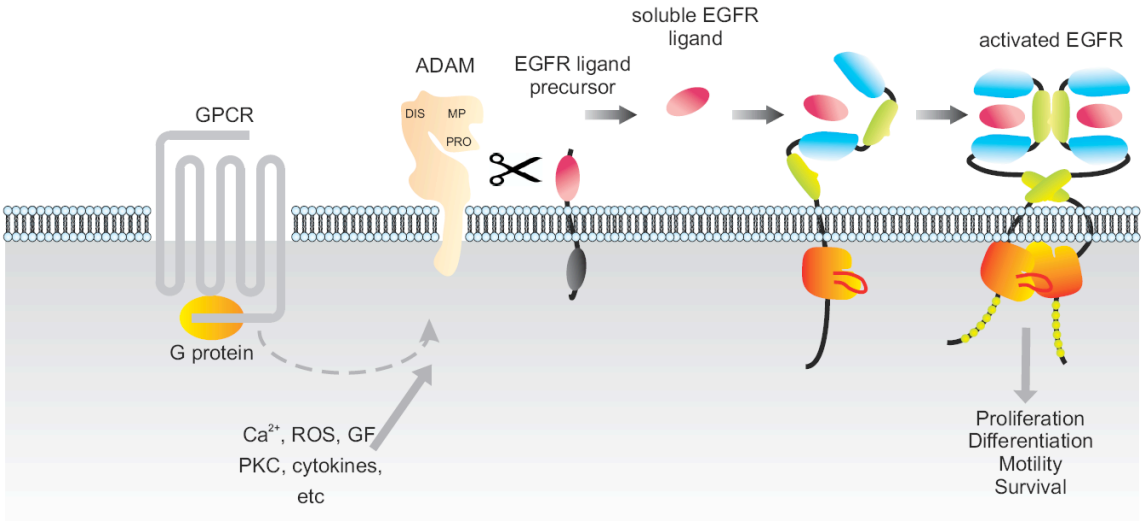

Fig. 3: EGFR transactivation through pro-EGFR ligands shedding by ADAMs. G-protein coupled receptors (GPCRs) together with second messengers (Ca^{2+}, PKC, UV-induced ROS) induce ADAM activation and thereby lead to shedding of EGFR ligand precursors that in turn activate EGFR. ADAM metalloproteases consist of a prodomain (PRO), a metalloprotease domain (MP), a disintegrin domain (DIS), a EGF-like domain (EGF), a transmembrane domain (TM) and a cytoplasmic tail (CT).

Two ADAM members are critical for shedding of most EGFR ligands: ADAM10 and ADAM17. ADAM10 has been shown to be responsible for cleavage of EGF and BTC while ADAM17 is

involved in shedding of AREG, EPGN, EREG, HBEGF and TGFA (Sahin, Weskamp et al. 2004; Sahin and Blobel 2007).

In human cancers, several ADAMs were reported to be overexpressed. Frequently, expression levels correlated with tumor progression (Rocks, Paulissen et al. 2008). This implicates a role of ADAMs in cancer progression. Consequently, ADAMs are currently tested in xenograft and preclinical models (Zhou, Peyton et al. 2006; Fridman, Caulder et al. 2007) as new targets for cancer therapy, showing encouraging results by inhibiting tumor cell growth when used in synergy with existing therapies.
Still, the mechanisms of ADAM protease regulation are only partly resolved. To date, not much is known about the transcriptional regulation of ADAMs in the normal and pathological state and further investigations that could benefit cancer therapy are needed.

4.9. EGFR oncogenic functions

As Egfr controls critical aspects of cell proliferation, motility, differentiation and survival, it is not surprising that its deregulation is implicated in many oncogenesis processes (see (Mitsudomi and Yatabe 2010) for a review).
Oncogenic Egfr deregulation can take place at multiple levels: autocrine activation, overexpression of the receptor, activating receptor mutations and defects in receptor internalization and degradation (see for a review (Normanno, De Luca et al. 2006)). Noteworthy, co-expression of different members of the EGFR family of tyrosine kinase receptors occurs in the majority of solid tumors (Singer, Hudelist et al. 2004).
EGFR is often upregulated in human breast cancers, and the co-expression of TGFa and EGFR correlates with a poor overall prognosis (Umekita, Ohi et al. 2000). EGFR is expressed in 20 to 80% of non-small cell lung cancer (NSCLC) tumors (Fontanini 1998), with deregulation mostly being the result of amplifications and mutations. Also NSCLC tumors are positive for EGFR ligands including TGFA and AREG, which correlates with a poor patient prognosis (Hodkinson, Mackinnon et al. 2008). EGFR also plays a role in colon cancer, with metastatic colon cancer cells expressing five times more EGFR than the nonmetastatic cells and the receptor-ligand pair EGFR-TGFA being a characteristic feature of more advanced tumors (Sasaki, Nakamura et al. 2008). A mutation of the EGFR, EGFRvIII, is found in more than 50% of glioblastoma multiforme (GBM), the most common and aggressive type of primary brain tumor in humans. This mutation, responsible for constitutive activation of the

ERK pathway and impairment of internalization and degradation, is implicated in uncontrolled cell proliferation (Grandal, Zandi et al. 2007).

Evidences are accumulating for a role for EGFR also in malignant melanoma. In recent publications, highly metastatic melanoma from patient biopsies and melanoma cell lines showed up-regulation of the EGFR (Fiori, Zhu et al. 2009) and human malignant melanoma cell growth was reduced in the presence of an EGFR inhibitor (Djerf, Trinks et al. 2009). EGFR was previously shown to be overexpressed in highly metastatic melanomas (Hoek, Schlegel et al. 2006) and EGFR activation has been demonstrated to play an important role in melanoma cell motility (Xie, Pallero et al. 1998; Gordon-Thomson, Jones et al. 2005).

The EGF-family of peptide growth factors is also involved in the pathogenesis of human carcinomas, and in mammalian cells overexpression of EGFR is able to induce transformation only in presence of appropriate levels of ligands. EGFR ligands are overexpressed in numerous solid neoplasms and anticancer agents targeting the EGFR ligands are currently under development (Yotsumoto, Sanui et al. 2009).

4.10. The *Xiphophorus* melanoma model

In the field of cancer research, one of the oldest animal model systems is the platyfish *Xiphophorus maculatus*. Already in the 1920's, Anders, Gordon, Kosswig and Haussler found that crossing the platyfish *X.maculatus* with the swordtail *X.helleri* gave rise to fish that spontaneously developed tumors resembling human malignant melanoma (Häussler 1928; Kosswig 1928; Gordon 1931). Pigment cells called macromelanophores that form characteristic black spots in the dorsal fin region (spotted dorsal or *Sd* phenotype) were found to be the source of these tumors.

However, development of malignant melanoma only occurred in the hybrid progeny after backcrossing the F1 offspring of (*X.maculatus* x *X.helleri*) with *X.helleri* (Figure 4A). The dominantly acting pigment-cell specific *Tu* (for tumor, as this is the oncogenic locus) allele is harboured on *X.maculatus* sex chromosomes, but the presence of a regulator locus (*R*) on another chromosome in *X.maculatus* impairs Tu oncogenic effect. The swordtail *X.hellerii* lacks both *Tu* and *R* and its crossing with *X.maculatus* gives rise to a hybrid F1 with fish presenting only one allele for *R* and for *Tu* on their respective chromosome pairs. After backcross of these *Tu*/-; *R*/- F1 fish with *X. hellerii*, about 25% of the offspring harbour *Tu* in the absence of *R* on their set of chromosomes and these fish will develop highly invasive malignant melanoma (Figure 4A). Another 25% of the offspring harbours one copy of the *R*

and one copy of the *Tu* locus. These fish are genotypically and phenotypically similar to the F1 generation and display non-malignant hyperpigmentation and overproliferation of pigment cells. Interestingly, re-introduction of the *R* locus completely impairs melanoma formation in subsequent generations. Altogether, the *Tu* locus, in absence of the *R* locus, is responsible for the onset of tumor formation in pigment cells and this due to the molecular nature of the oncogene that is present at this locus.

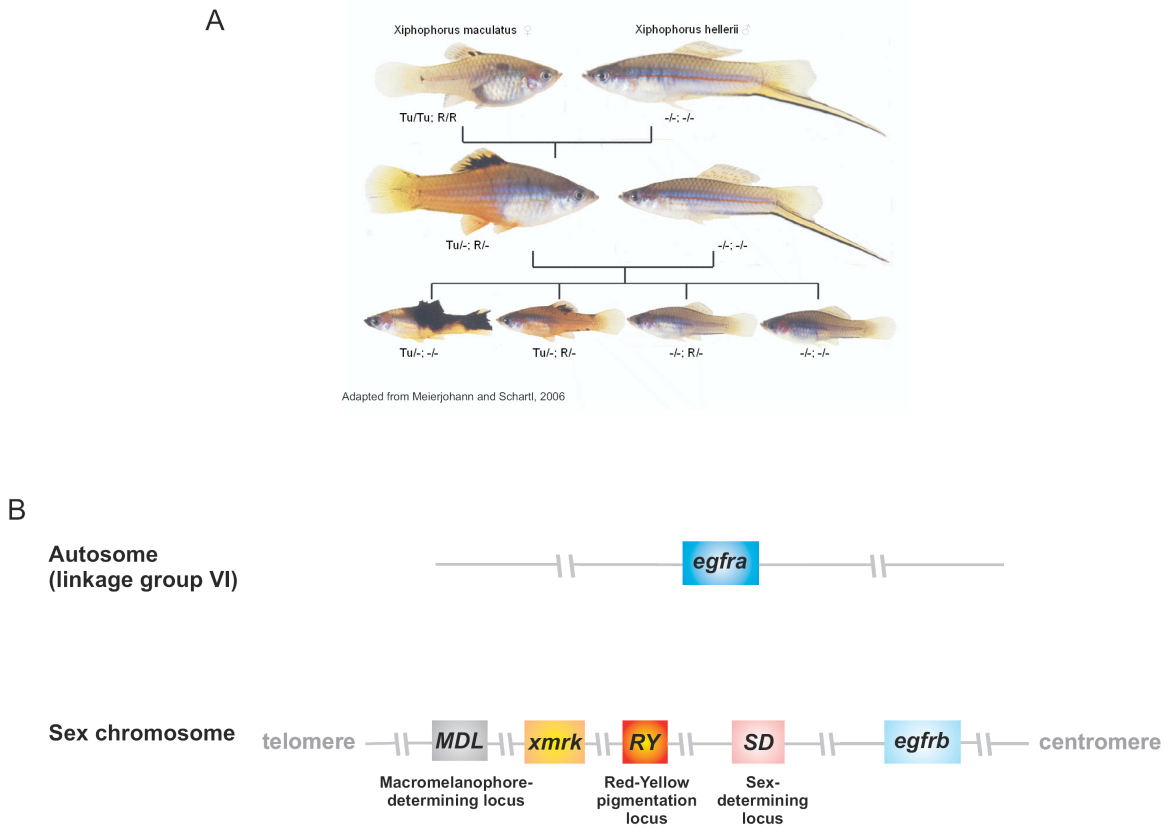

Fig. 4: Xiphophorus melanoma model. (**A**) Classical Gordon–Kosswig–Anders cross leading to generation of *Xiphophorus* fish spontaneously developing melanoma. *Tu*: tumor locus, *R*: tumor repressor locus. Fish that only harbour *Tu* in the absence of the *R* locus develop highly invasive melanoma. Adapted from (Meierjohann and Schartl 2006). (**B**) Chromosomal locations of xiphophorus *egfra*, *egfrb* (proto-oncogene), and *xmrk* (oncogene). The *Tu* locus harbouring *xmrk* is located close to important loci, like the macromelanophore-determining locus (Mdl), the red-yellow locus (RY) and the sex-determining region (SD).

Positional cloning approaches allocated a gene encoding for a novel receptor tyrosine kinase to the *Tu* locus. This gene was found to be homologous to the human EGFR and was called *xmrk* for *Xiphophorus* melanoma receptor kinase (Wittbrodt, Adam et al. 1989). Importantly, *X.maculatus* mutants that lost the property to induce tumor formation when crossed to

X.helleri showed disruption of the *xmrk* gene by a transposable element (Schartl, Hornung et al. 1999).

In *Xiphophorus*, the above mentioned fish-specific genome duplication (FSGD) generated two orthologous copies for the *egfr* gene that have been maintained during evolution and that encode two different receptor proteins: Egfra and Egfrb. While *egfra* gene is located on an autosome on linkage group VI (Harless, Svensson et al. 1990), *egfrb* gene is located in the subtelomeric part of both sex chromosomes. In addition, a more recent intrachromosomal segmental duplication of a genomic region including *egfrb* led to the generation of the oncogenic *xmrk* (Gutbrod and Schartl 1999; Nanda, Volff et al. 2000; Froschauer, Korting et al. 2001). As *xmrk* originates from *egfrb,* the latter can be consideres as proto-oncogene. *Xmrk* is located about 1 Mb away from *egfrb* on the sex chromosomes, and therefore it linked to a new 5' region that affects its transcription (Adam, Dimitrijevic et al. 1993; Volff, Korting et al. 2003) (Figure 4B).

While classical epidermal growth factor receptors require the binding of one or several ligands in order to dimerize and become activated, Xmrk can form activated dimers without any ligand binding. Two activating mutations present on Xmrk extracellular part, namely G359R and C578S, have been shown to be responsible for conformational changes that allow spontaneous dimer formation (Gomez, Wellbrock et al. 2001) (Meierjohann, Mueller et al., 2006).

In *Xiphophorus* macromelanophores, overexpression of the oncogenic receptor tyrosine kinase Xmrk together with its constitutive activation massively activates tumorigenic signaling pathways. Three major signaling pathways have been identified downstream of activated Xmrk. They are very similar to Egfr signaling pathways already characterized in invertebrates and vertebrates (Nagaraj and Banerjee 2004; Sundaram 2006) (Burgess 2008) (Figure 5). Firstly, Xmrk activation and subsequent tyrosine residue phosphorylation on the intracellular carboxy-terminus of the receptor is generating docking sites for adaptor proteins with *src* homology (SH) domains. In the *Xiphophorus* malignant melanoma derived cell line PSM, Xmrk has been shown to be associated to the adaptor protein Grb2 directly and via the SH2 and SH3 domains of Shc. This association is followed by the induction of the Ras/Raf/MAPK signaling cascade responsible for cell proliferation, differentiation and tumor cell survival at ectopic sites (Wellbrock, Fischer et al. 1999; Wellbrock and Schartl 2000). Secondly, PI3-K (phosphatidylinositol 3-kinase) can also bind to Xmrk C-terminal phosphorylated tyrosine residues and be activated (Wellbrock, Fischer et al. 1999; Wellbrock and Schartl 2000). This binding is leading to the induction of anti-apoptotic signaling via the

downstream effector Akt. Thirdly, Xmrk-dependent activation of the STAT5 transcription factor also induces cell proliferation and resistance to apoptosis by interacting with the PI3K pathway (Baudler, Schartl et al. 1999; Wellbrock and Schartl 2000; Morcinek, Weisser et al. 2002). Finally, the Src kinase Fyn has been shown to play an important role in Xmrk signaling, since this protein was found to interact into a ternary complex with Xmrk and PI3K and to activate PI3K when bound to Xmrk (Wellbrock and Schartl 2000). Fyn Src kinase when bound to the C-terminal part of Xmrk can also enhance MAPK pathway signaling by inhibition of the MAPK phosphatase 1 (MKP-1) (Wellbrock, Weisser et al. 2002). Moreover, Xmrk activation of Fyn that in turns interacts with activated focal adhesion kinase (FAK) induces migration of pigment cells from mouse and fish origins (Meierjohann, Wende et al. 2006; Teutschbein, Schartl et al. 2009).

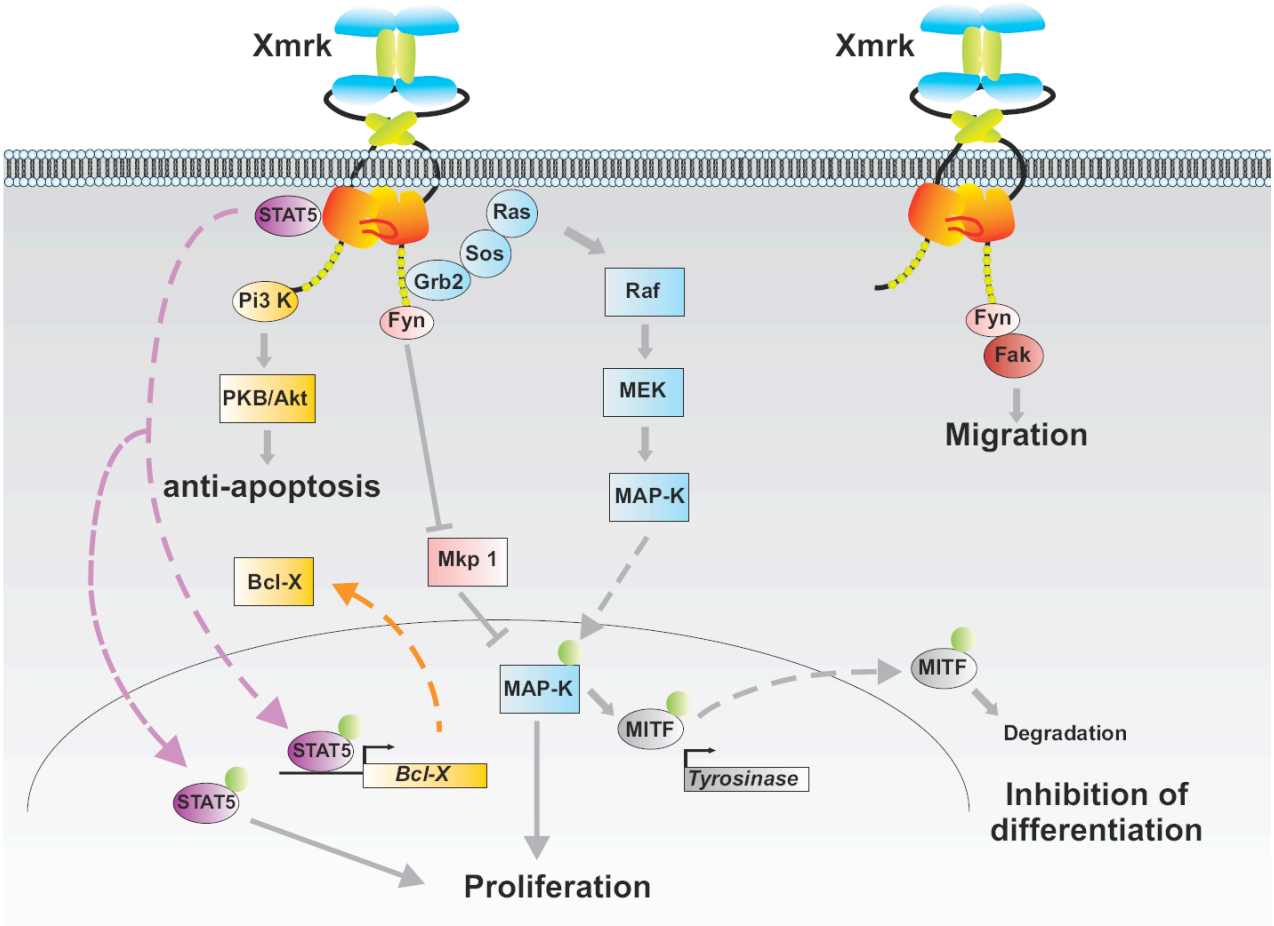

Fig. 5: Molecular signaling pathways induced by Xmrk. Activation of STAT5 promotes proliferation event while STAT5 together with the PI3K pathway lead to anti-apoptotic event. Xmrk also induces the Ras-Raf-MAPK pathway which promotes proliferation event but also differentiation

by reducing the stability of the pigment specific MITF transcription factor. The tyrosine kinase Fyn is strongly activated by Xmrk and contributes both to proliferation and cell migration events. Adapted from (Meierjohann and Schartl 2006).

4.11. Aim of the PhD thesis

The X*mrk* oncogene, responsible for the initiation and progression of highly aggressive metastatic melanoma in the *Xiphophorus* fish is encoding for a mutated version of the Egfr. The two mutations which are independently responsible for Xmrk constitutive activation and subsequent oncogenic signaling take place in the receptor extracellular domain. These mutations generate new intramolecular disulfide bridges that lead to permanent Xmrk dimerization (Meierjohann, Mueller et al. 2006). The first mutation, G336R, occurs in Xmrk subdomain 2 which contains the dimerization loop that usually responds to ligand binding. Consequently, G336R mutation-induced structure resembles the Egfr in its ligand-bound state but still does not affect the ligand-binding pocket built by subdomains 1 and 3. The second mutation, C555S, creates a new disulfide bridge between two subdomain 4 monomers, subdomain originally involved in receptor dimerization. Here again, the mutation does not affect the ligand-binding pocket. Therefore, it remains unclear if Xmrk despite its mutations is still able to bind Egfr ligands and moreover, it is not known if Xmrk is having an effect on Egfr ligands regulation. The aim of my PhD was to investigate the Egfr/Egfr ligands system with regard to Xmrk oncogenic signaling.

Firstly, I started to investigate the Egfr/Egfr ligands system from an evolutionary point of view to better understand the situation in teleosts. I performed the phylogeny, synteny and expression analyses with a special interest for medakafish since we are currently working on a newly established transgenic *mitf*::*xmrk* medaka melanoma model for which I analyzed Xmrk signaling profiles in non-tumorigenic and tumorigenic tissues.

Secondly, I analyzed the Egfr/Egfr ligands system in response to Xmrk oncogenic signal both *in vitro* and *in vivo*. I analyzed the expression of Egfr ligand and related sheddase genes in melanocytes carrying an inducible version of Xmrk and investigated the expression of Egfr/Egfr ligand system members in tumorigenic and non-tumorigenic tissues from adult transgenic *mitf*::*xmrk* medakafish. Finally I looked for *Xiphophorus* Egfrb functionality and for the possibility for Xmrk to interact with Egfr ligands.

5. Results and Discussion

5.1. Evolution of the Egfr/Egfr ligand system in vertebrates

As key regulator in normal cellular processes, the epidermal growth factor receptor (Egfr) is one of the most investigated receptor tyrosine kinases and is highly conserved from triploblastic invertebrates to vertebrates (Stein and Staros 2006; van Kesteren, Gagatek et al. 2008). While one gene is encoding for the Egfr in the tetrapod lineage, the situation appears to be different in teleosts due to the teleost-specific genome duplication (TSGD) event that occurred about 350 million years ago in the actinopterygian stem lineage (Postlethwait, Amores et al. 2004; Meyer and Van de Peer 2005; Froschauer, Braasch et al. 2006).

5.1.1. Potential subfunctionalization of teleost *egfr*

Due to the lineage-specific genome duplication, teleosts often possess two copies of various genes. Whereas about 76 to 80% of duplicated genes become secondarily inactive and get lost during evolution (Jaillon, Aury et al. 2004; Woods, Wilson et al. 2005), about 15% of gene paralogs have been retained in duplicates over time. In this context, we found that duplicated genes for Egfr are present in the six teleost species investigated. They are termed *egfra* and *egfrb* (Figure 1A). On the contrary, the Egfr-related protein Erbb2, that shares a common ancestor gene with Egfr, has not been maintained in two copies during evolution (Figure 1A).

Noteworthy, Erbb2 is the sole Egfr-related family member without a ligand-binding site (Citri, Skaria et al. 2003). Egfr-related proteins ErbB3 and ErbB4, which are known to interact with their respective ligands in human and mouse, have also been retained in duplicates in medaka and three other teleost species (Froschauer, Braasch et al. 2006).

The situation is different for Egfr ligand genes, where only one copy for each of the seven tetrapod Egfr ligands has been retained in known teleost genomes, with the only exception being zebrafish *hbegf* (Laisney, Braasch et al. 2010).

Transcription analysis of all Egf receptors and ligands in adult medaka tissues, revealed differential expression of *egfra* and *egfrb* as well as their ligands in the tissues. *Egfra* was expressed mainly in spleen, kidney and skin together with *hbegf*, whereas *egfrb* was prominently expressed in gills, ovary and liver, together with *tgfa*, *areg* and *ereg* (Table 1).

In his classic book, *Evolution by Gene Duplication* (1970) the geneticist and evolutionary biologist Susumu Ohno (February 1, 1928 – January 13, 2000) postulated that gene

duplication was the most important force in the evolution of higher organisms. In his theory, one of the duplicated genes when maintained in the genome can either loose its function and become inactive ("nonfunctionalization") or acquire novel and beneficial functions ("neofunctionalization"). More recently, the theory of subfunctionalization stating that both gene duplicates acquire mutations resulting in the partition of the ancestral gene functions among the two duplicates emerged (Force, Lynch et al. 1999). Our expression pattern analyses suggest a scenario in which the two medaka Egfr copies may have different functions which justifies their maintenance in the medaka genome in line with the subfunctionalization process that is well-documented in teleosts (Force, Lynch et al. 1999; Postlethwait, Amores et al. 2004; Rastogi and Liberles 2005). In addition, according to their dissimilar expression patterns together with the receptors, Egfr ligands may also bind Egfra and Egfrb with different affinities.

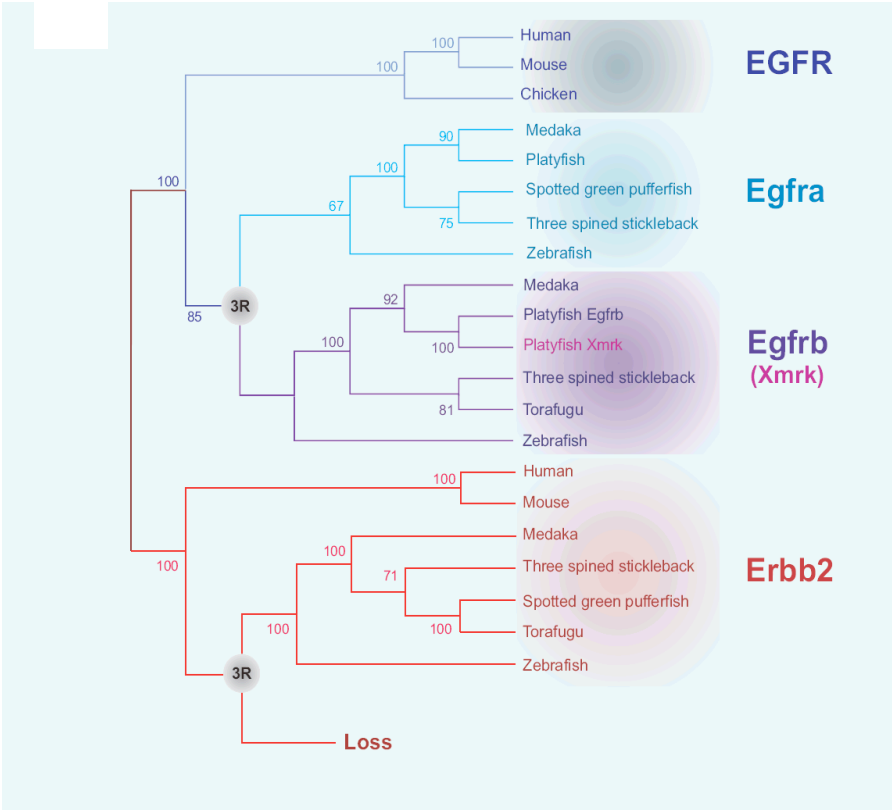

Figure 1: Phylogeny of Egfr and Egfr ligand genes.

The consensus tree is based on a 958 amino acid alignment using the maximum-likehood method (LG substitution model, 1,000 pseudosamples); bootstrap values for major nodes are given as percentages. Only bootstrap values above 50% are shown. The presumed third genome duplication event (3R) is shown and fish-specific gene duplicates are quoted as Egfra and Egfrb.

Bootstrap values for Maximum Likehood and Neighbor Joining methods are shown. The tree was rooted on the branch leading to *Egf* sequences. Only bootstrap values above 50% are shown.

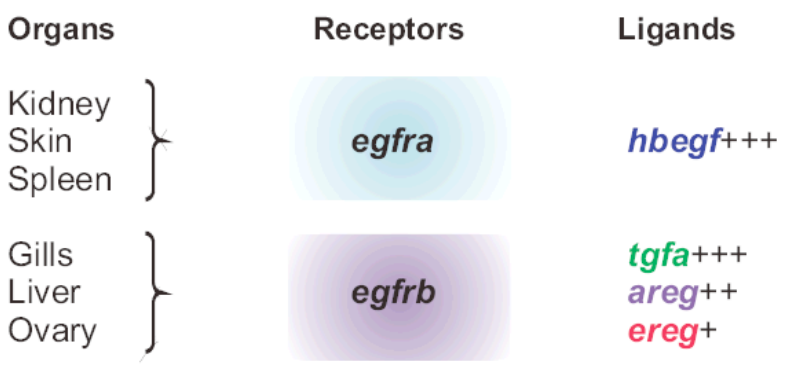

Table 1: co-expression of Egfr and Egfr ligand genes in medaka organs.

5.1.2. Evolution of the vertebrate Egfr ligand gene family

Phylogenetic analyses based on the alignment of the mature part of the Egfr ligands gave well supported orthology only for a subset of tetrapod and teleost ligand genes, namely *Egf*, *Tgfa*, *Hbegf* and *Ereg*. Therefore additional micro- and macro-synteny conservation analyses were performed to obtain further informations about the relationship between teleost Egfr ligands and their tetrapod counterparts. The genomic surroundings of the respective human, zebrafish, tetraodon and medaka Egfr ligand genes were analyzed with the Synteny Database (Catchen, Conery et al. 2009). Altogether, by combination of phylogenetic and synteny analyses, true orthologues for Egfr ligands were identified in all teleost genomes available.

Synteny Database dot plots analyses of paralogous genes within the human genome demonstrated that the *TGFA* containing region on Hsa2, the *EGF* and ligand cluster (*Epgn*, *Ereg*, *Areg* and *Btc*) containing region on Hsa4, the *HBEGF* containing region on Hsa5 were highly synthenic to each other, as well as with a region of Hsa10 devoid of EGFR ligand gene (Figure 2).

These results confirm previous studies proposing that parts of chromosomes Hsa2, Hsa4, Hsa5 and Hsa10 all derive from vertebrate protochromosome *C*. Protochromosome *C* was duplicated twice during the R1/R2 whole genome duplication giving rise to the gnathostome protochromosomes *C0*, *C1*, *C2* and *C3* (Nakatani, Takeda et al. 2007).

Chromosomal locations of Egfr ligand genes in the chicken and teleost genomes are also compatible with being derived from vertebrate protochromosome *C* and its four gnathostome derivates, leading to the hypothesis that all Egfr ligand genes in the present vertebrate genomes can be traced back to a single pre-R1/R2 Egfr ligand gene.

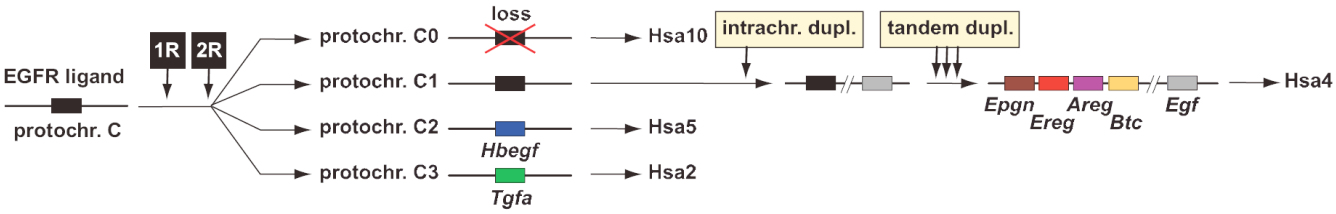

Figure 2: Model of Egfr ligand gene family evolution in vertebrates.

An ancestral gene on vertebrate protochromosome *C* was duplicated twice during the R1 and R2 whole genome duplications. The gene on gnathostome protochromosome *C0* was lost, while the gene on protochromosome *C1* was duplicated intrachromosomally. One of the two descendent genes became *Egf*, the other one was duplicated in tandem giving rise to the ligand cluster. The protochromosome nomenclature follows ref. (Nakatani, Takeda et al. 2007).

5.1.3. Evolutionary diversity of the receptor-ligand interface between tetrapods and teleosts

The observed persistence of duplicated Egfr genes in teleosts, combined with the differential tissue expression of Egfr ligands in medaka (Laisney, Braasch et al. 2010) (Table 1), suggest differences in structure and function between the two fish Egfr copies. In addition, it is not clear how far teleost and tetrapod Egfrs are functionally conserved and to which extend a structure/function relationship is represented in the conservation of the primary structure, *i.e.* on the aminoacid level. Looking at the similarity values between tetrapod and teleost Egfr subdomains, we found that the extracellular domain, implicated in receptor dimerization and ligand-binding, is the least conserved part of the receptor.

With the use of the bioinformatic ConSurf tool, we projected the amino acid alignment of tetrapod and teleost Egfr orthologues on a given 3D structure of the human EGFR/TGFA complex. The representation indicated that most of the Egfr residues building the ligand-binding pocket are highly variable, thus they are not evolutionarily conserved between tetrapods and teleosts (Figure 3).

A detailed examination of all known human Egfr amino acids that directly contact EGF or TGFA revealed that their medaka counterparts were mostly not conserved. More strikingly, some of them even showed a radical amino acid shift, i.e. a replacement by an amino acid with completely different physico-chemical properties. These data suggested a poor affinity of fish Egfr to mammalian EGFR ligands and the other way round. Therefore we tested the stimulation potential of human and medaka Egfr by human EGF, using melanocytes that expressed the respective receptor. Western blot analyses revealed that human EGF was able to

induce human EGFR phosphorylation, as well as phosphorylation of subsequent signaling pathway components. On the opposite, human EGF was not able to induce a response in cells expressing medaka Egfra. Further functional analyses demonstrated that the species-specific medaka Egfr ligands Egf, Areg and Hbegf were able to stimulate medaka Egfra (Figure 4).

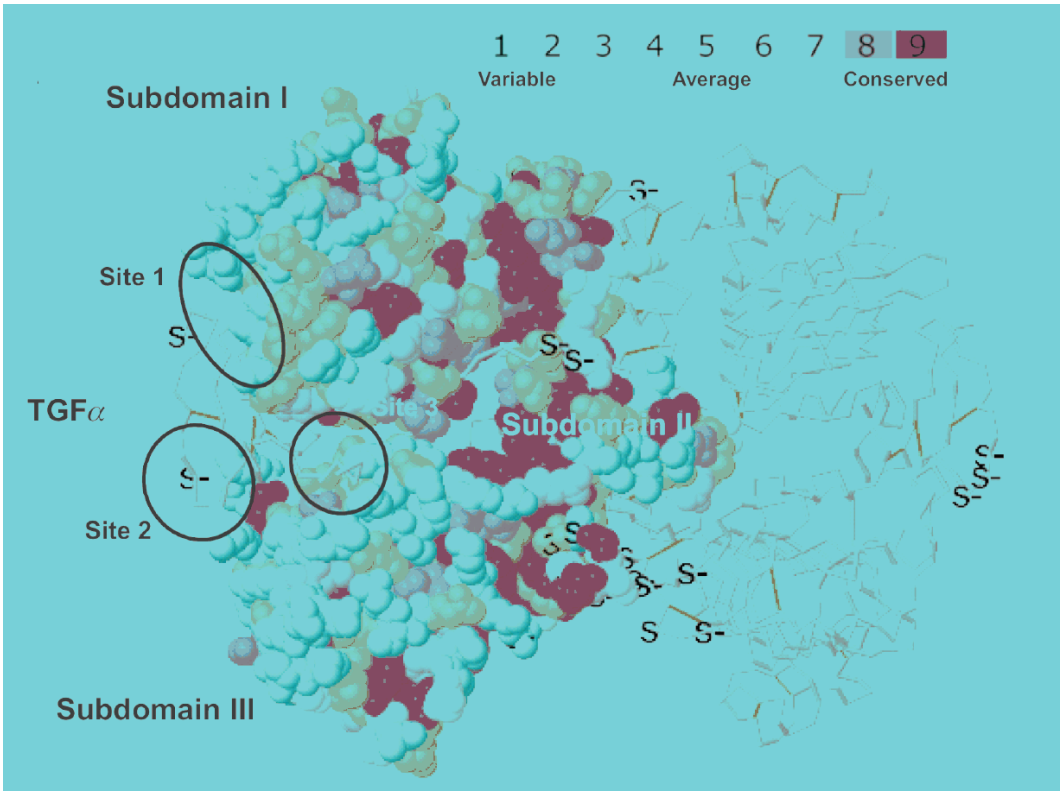

Figure 3: ConSurf evolutionary conservation analysis of the Egfr ligand binding pocket residues between tetrapods and teleosts.

A) Overall strucure of extracellular subdomains I and III of Egfr in complex with Tgfa, 3 interface sites are outlined. Non-conserved residues are colored in turquoise whereas conserved residues are coloured in pink and maroon. Yellow color indicates amino acids for which data were not sufficient to calculate reliable conservation values (http://consurf.tau.ac.il/) (Landau, Mayrose et al. 2005).

Altogether, these data speak against the use of heterologous ligands to stimulate fish Egf receptors. In most studies implying fish Egfr, heterologous growth factors from the EGF-family are commonly used *e.g.* human or mouse EGF.

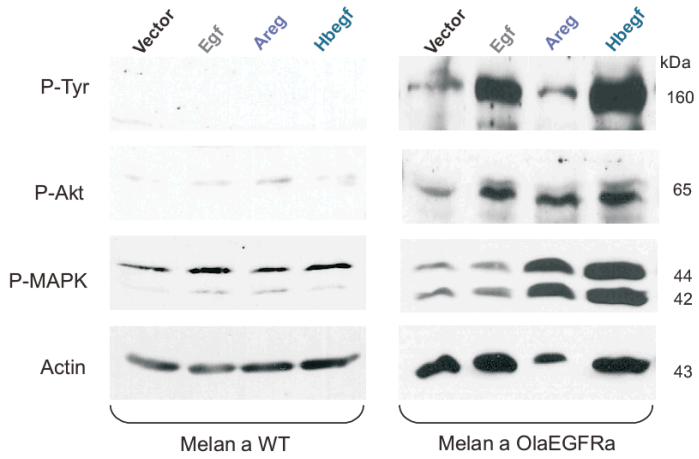

Figure 4: Western blot analysis of medaka Egfra activation by medaka Egfr ligands.
Melan-a wildtype cells or melan-a Ola-*egfra* stimulated for 30 minutes at 37°C with overnight conditioned supernatant of 293-T cells expressing vector alone, medaka *egf*, medaka *areg* or medaka *hbegf*. P-Tyr: phospho-tyrosine antibody; P-Akt: phospho-Akt antibody; P-Erk1/2: phospho Erk1/2. The anti-P-Tyr antibody recognizes a band of the size of Egfra, thus indicating activated receptor.

To explore potential paracrine regulatory mechanisms in zebrafish pituitary cells, use of heterologous human EGF was made but did not have any effect on the target genes investigated (Lin and Ge 2009). As zebrafish pituitary cells express Egfr (Lin and Ge 2009) and as EGF is having effects on pituitary cells in rat (Childs and Unabia 2001), it may be that use of heterelogous ligand in this case is inappropriate and would explain the fact that zebrafish pituitary cells do not react to human EGF.
In goldfish, examination of prostaglandin synthesis showed lack of responsiveness for one of three classes of ovarian vitellogenic follicles expressing the Egfr when stimulated with mouse EGF (MacDougall and Van Der Kraak 1998). This lack of responsiveness can either be due to a change in the actions of EGF during ovarian follicular development in goldfish, but can also be the consequence of impaired cross-reactivity between fish Egfr and murine EGF that may occur with a different responsiveness when compared to the homologous goldfish Egfr-Egfr ligands system.

5.2. The transgenic mitf::xmrk medaka in addition to Xiphophorus as animal model for melanoma

Natural occurrence of melanoma has been described only in few species, namely human, opossums, dogs, cats, minipigs, horses and the platyfish *Xiphophorus*. Mice are protected

from natural development of melanoma since their epidermis is devoid of melanocytes, present in the hair follicle instead.

With the spontaneous development of highly invasive and metastatic melanoma in certain hybrid genotypes, the *Xiphophorus* fish is one of the oldest animal models for human cancer. The overexpressed oncogene responsible for the onset of melanoma is encoding for a mutated version of the Egf receptor and was called *Xmrk* for *Xiphophorus* melanoma receptor kinase (for a review see (Meierjohann and Schartl 2006)). While melanoma is a multistep process in humans and mice, comprising oncogene activation, tumor-suppressor gene inactivation as well as epigenetic changes during tumor progression, oncogenic *Xiphophorus* Xmrk alone is able to trigger the onset of tumor formation as well as to sustain melanoma progression, at least in fish. By recruiting and activating multiple transcription factors, kinases and other adaptor proteins, Xmrk alone allows tumor initiation and progression by blocking cell differentiation, inducing cell proliferation and migration, and protecting from apoptosis events. While Xmrk downstream signaling events have been relatively well characterized, it is still unclear to what extent *Xiphophorus* the hybrid genetic background of *Xiphophorus* contributes to Xmrk induced tumorigenesis. Furthermore, *Xiphophorus* fish are live-bearing, thus impairing large-scale high-throughput analyses and transgenic approaches. The closely related fish species medaka (*Oryzias latipes*) is a widely used small-animal model system which offers several practical advantages when compared to *Xiphophorus*. Medaka fish produce externally developing transparent embryos, the full genome has been sequenced (Kasahara, Naruse et al. 2007), transgenic approaches are available (Thermes, Grabher et al. 2002; Wittbrodt, Shima et al. 2002), and a number of *in vitro* systems have been established (Hong, Winkler et al. 1998; Hong, Liu et al. 2004). Therefore, we chose to examine *xmrk*-dependent melanoma development in medaka. We generated stable transgenic medaka lines that express *xmrk* under the control of the pigment-cell specific *mitf* promoter.

5.2.1. Melanoma phenotypes in the transgenic medaka

The *mitfa* promoter from medaka was fused to the full-length cDNA of *Xiphophorus xmrk* oncogene and the resulting construct was injected into medaka embryos from the *Carbio* and albino (*i-3*) strains. Injected fish developed various pigmentation abnormalities due to the activity of the transgene. Embryos developing three-dimensional growth of darkly pigmented cells, also histologically diagnosed melanotic tumors, did not hatch and did not survive, while embryos developing large areas of hyperpigmentation or progressively growing pigment cell

tumors as juvenile or adult animal survived. Two types of tumors developed in the stable transgenic lines, namely pigment cell tumors of the red and yellow pigment-containing xanthophores and erythrophores (xanthoerythrophoromas or XE tumors) and melanoma, sometimes even on the same individual (Figure 5).

Melanoma were detected at sites harbouring extracutaneous pigment cells (body musculature, internal organs, chorioidea of the eye, spinal cord, intestine), but also secondarily at sites devoid of extracutaneous pigment cells (liver). The latter had no connection to the main tumor mass and were therefore considered as metastases. A notable dosage effect was observed when comparing heterozygous and homozygous *mitf*::*xmrk* transgenic fish. Heterozygous fish developed f-nevi or cutaneous hyperpigmentation but also melanoma in the fins and the trunk at about 6-10 weeks of age and most of them developed hyperpigmentation of the xanthophore/erythrophore lineage, with presence of melanoma cells in between XE tumors (mixed tumors). Homozygous fish exhibited pigmentation disorders much earlier, and within 2-6 weeks hyperpigmentated areas transformed into malignant tumors.

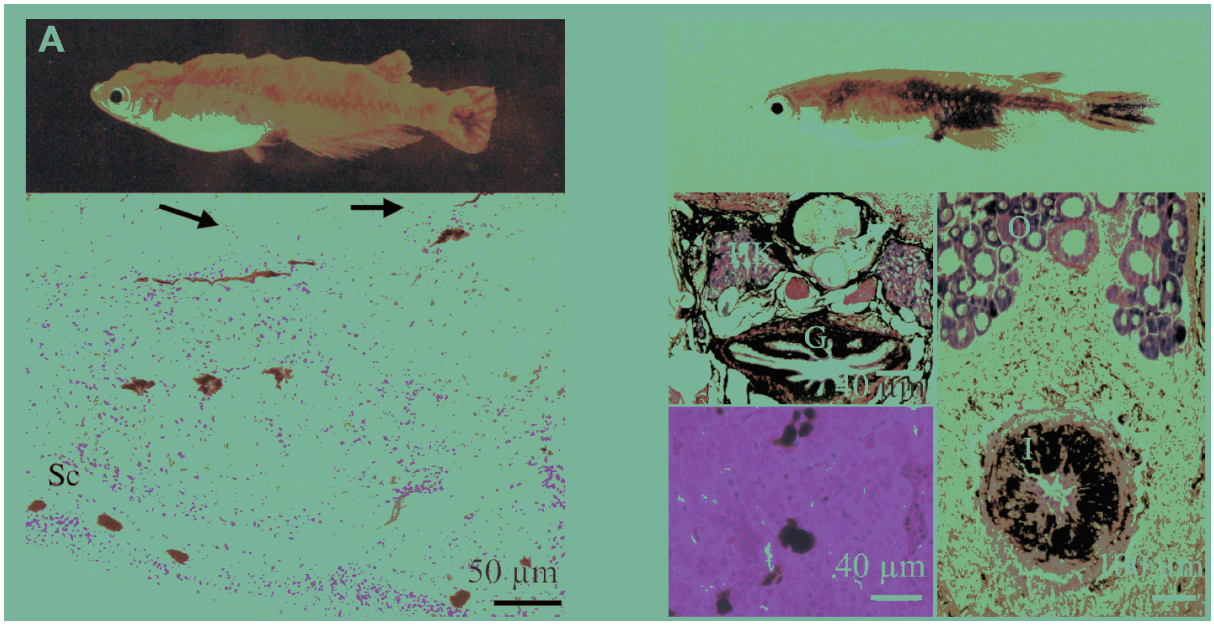

Figure 5: Pigment cell tumors in *mitf*::*xmrk* transgenic medaka.

A) Macroscopic and microscopic patterns of exophytic xanthoerythrophoroma. Upper image: 10-weeks old female medaka (*Carbio*) with exophytically growing xanthoerythrophoroma. Lower image: Xanthoerythrophoroma growing in the dermal compartment and locally invading the underlying trunk musculature (arrows). Bar = 40µm. B) Macroscopic and microscopic patterns of invasive extracutaneous melanotic melanoma. Upper image: 4-weeks old juvenile medaka (*Carbio*) with melanotic tumor filling the abdomen and growing invasively into the musculature. Lower image: melanoma metastasis in the liver. Bar = 40µm.

5.2.2. Xmrk-induced signal transduction and gene expression in tumors

Expression of the *xmrk* transgene in medaka tumors was high on transcriptional level and low to very high on protein level. Activation of Xmrk signal transducers that have been already characterized, like PI3K/AKT (Wellbrock, Fischer et al. 1999), MAPK/Erk (MEK) kinase and mitogen-activated protein kinase (MAPK) (Wellbrock, Weisser et al. 2002) was detected in transgenic medaka XE tumors and melanoma, but also, albeit at lower levels, in healthy fish skin. Interestingly, strong activation of signal transducer and activator of transcription 5 (Stat5) correlated best with the level of xmrk expression in the tumors and reached the highest levels in the most malignant pigment cell type, the melanoma. Stat5 was already demonstrated to be of great importance for Xmrk-driven tumorigenesis events, inducing proliferation and antiapoptotic signaling of cultured cells (Wellbrock, Geissinger et al. 1998) (Baudler, Schartl et al. 1999) (Morcinek, Weisser et al. 2002). STAT5 is also implicated in cell survival and interferon resistance in human melanoma (Wellbrock, Weisser et al. 2005; Mirmohammadsadegh, Hassan et al. 2006) (Figure 6).

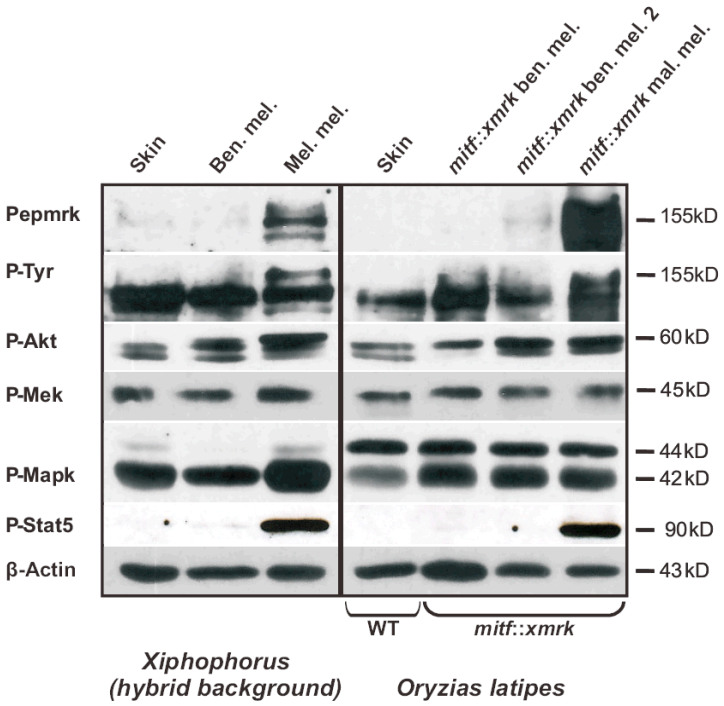

Figure 6: Western blot analysis of *Xiphophorus* hybrid and transgenic medaka tumors.
Protein lysate of Carbio skin, xanthoerythrophoroma (XE tumors) and melanoma was analyzed using Western blot. Antibodies were directed against Xmrk ("Pepmrk), phosphotyrosine (P-Tyr), phosphorylated Mek, Mapk, Akt and Stat5. The upper Akt band is the specific one and is indicated by an arrow. Beta-actin is used as loading control.

The myc-family basic helix-loop-helix/leucine zipper microphtalmia-associated transcription factor (*mitf*) was found to be strongly upregulated along with *xmrk* expression in the more malignant medaka melanoma. The effect of MITF levels on human melanoma development is still under debate. It was reported that high MITF levels have an anti-proliferative effect on melanoma cells (Wellbrock and Marais 2005) whereas low levels of MITF were found in invasive melanoma (Hoek, Eichhoff et al. 2008) and are associated with bad prognosis and clinical disease progression (Zhuang, Lee et al. 2007). Still, the *MITF* gene was found to be amplified in 15-20% of metastatic melanomas (Garraway, Widlund et al. 2005) and expression of *MITF* in intermediate-thickness cutaneous melanoma is inversely correlated with overall survival (Salti, Manougian et al. 2000). Retention of *MITF* expression in most primary melanomas and non-pigmented tumors led to the common use of MITF as a prognostic marker in these malignancies (Chang and Folpe 2001). It is most likely that MITF plays both pro- and antiproliferative roles, depending in its level of expression and activity (Goding and Meyskens 2006). Noteworthy, at the protein level, MITF is the target of a range of modifications, *e.g.* phosphorylation and sumoylation, which control its expression or activity. The controversy of the MITF role in melanoma, anti-proliferative versus pro-survival, is still not fully understood, and the strong expression of *mitf* in transgenic medaka malignant melanomas would rather support the hypothesis of a pro-tumorigenic and pro-survival role in the transgenic *mitf::xmrk* medaka.

Another factor that discriminated between the most malignant medaka tumors and the other tissue was the antiapoptotic protein Bcl-2, which was strongly upregulated only in melanoma. Interestingly, on transcriptional level *bcl-2* was upregulated both in XE tumors and melanoma, suggesting that Bcl-2, like Mitf, are more strongly regulated by posttranscriptional than by transcriptional mechanisms in medaka tumors. In human, *BCL-2* is a direct target gene of MITF (McGill, Horstmann et al. 2002). BCL-2 is one of the most important regulators of melanoma cell apoptosis (Soengas and Lowe 2003) and anti-sense suppression of *BCL-2* or mutations in *MITF* lead to decreased melanoma cell survival and increased sensitivity to chemotherapy (Jansen, Schlagbauer-Wadl et al. 1998; Gautschi, Tschopp et al. 2001; McGill, Horstmann et al. 2002). Consequently, *BCL-2* is extensively investigated as therapeutic target in cancer and new BCL-2-targeting drugs currently undergo clinical trials (for a review see (Patel, Masood et al. 2009)).

Another antiapoptotic gene, namely *survivin1* was found to be transcriptionally upregulated in melanoma but not in uveal melanoma in transgenic *mitf::xmrk* medaka. Survivin1 is a member of the inhibitor of apoptosis (IAP) family and a human melanoma progression

marker (Chen, Gong et al. 2009). Its expression in human melanomas correlates with disease recurrence and poor survival (Piras, Murtas et al. 2007). Survivin expression was also found in human uveal melanoma, where it correlates with resistance to the pro-apoptotic tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (Li, Niederkorn et al. 2005).
Altogether, activation of signaling pathways and expression of genes known to be important in melanomagenesis or melanoma progression in human and/or Xiphophorus confirm that transgenic *mitf*::*xmrk* medaka is suitable as new fish melanoma model. This model could be especially useful to contribute to a better understanding of prosurvival signaling in pigment cell tumors, as upregulation of antiapoptotic Stat5, *bcl-2* and *survivin 1* both in XE tumors and melanoma was monitored.

5.2.3. Genetic melanoma modifiers

Both xanthoerythrophoromas and melanomas were observed in the transgenic medaka Carbio strain, which is a non-inbred line with mixed genetic background. To investigate Xmrk-dependent oncogenicity according to genetic modifiers, the transgene was crossed into different medaka lines of defined genetic backgrounds. Strong differences were already observed after two generations. Fish from the *CabR'* background developed almost exclusively xanthoerythrophoromas that invaded the underlying trunk musculature only at terminal stages. About 2-5% developed additionally nodular or invasive melanoma. On the contrary, fish from the *HB32C* genetic background developed mostly invasive extracutaneous melanoma, while xanthoerythrophoroma were extremely rare. The third group, fish from *i-3* albino background, showed hyperpigmentation of xanthophores in the skin that rarely evolved into tumors, and about 30% developed extracutaneous poorly pigmented melanoma from the intestine. Interestingly, about 40% of these fish also exhibited unilateral or bilateral eye melanoma.

5.3. Xmrk melanoma model and Egfr ligands.

Being a mutated version of the Egfr, it is conceivable that oncogenic Xmrk may interact with members of the Egfr ligand family. Moreover, receptor activation by autocrine loops is a common feature in receptor tyrosine kinase driven tumors. It is not known if Xmrk can bind Egfr ligand but previous modeling studies of Xmrk extracellular domain structure could show that subdomains I and III, which build the interacting interface for the ligand, are not

affected by the two mutations that are responsible for constitutive Xmrk activation (Meierjohann, Mueller et al. 2006). This means that ligand binding to Xmrk, even in a pre-dimerized state, may occur. It was already demonstrated that cell-surface EGFR dimers, either formed due to mutational activation (Moriki, Maruyama et al. 2001), or spontaneously formed by wildtype EGFR (Tao and Maruyama 2008), can bind EGF.

5.3.1. Induction of EGFR ligands and related sheddases by human EGFR and Xmrk

To investigate the role of human EGFR and Xmrk in the transcriptional regulation of EGFR ligands, we used immortalized mouse melanocytes cell line (melan-a) transgenic for human EGFR or an inducible version of Xmrk (called HERmrk; a chimera of the extracellular part of human EGFR and the intracellular part of Xmrk, thus activatable by human EGF). As melan-a cells do not express endogenous EGFR (Wellbrock, Weisser et al. 2002), EGF-dependent activation could only occur due to the respective transgene. Melan-a HER cells or melan-a HERmrk were stimulated with EGF and activation of the receptors and downstream signaling pathways was validated by Western blot analysis. Then, RNA expression of the seven Egfr ligands was monitored by quantitative realtime-PCR. *Areg*, *Btc* and *Hbegf* were strongly transcriptionally upregulated in melanocytes upon human EGFR activation (Figure 7). Simulation of HERmrk was even more potent and also led to induction of *Ereg* and *Epgn* in addition. However, *Hbegf* and *Areg* were the strongest upregulated ligand genes 2 hours post-stimulation.

The role of EGF family genes in melanoma onset and progression is attracting recently more and more attention. It is well-known that human melanoma cell lines frequently express high levels of *TGFA* (Marquardt and Todaro 1982; Richmond, Lawson et al. 1985), suggesting a possible autocrine EGFR/TGFA pathway. In the melan-a cells, we could not detect any transcriptional upregulation of *Tgfa* by either EGFR or HERmrk, but an autocrine receptor induction occurs through other ligands (see below).

Of relevance in the context of the results exposed here, AREG and HB-EGF were found to be the two major EGFR ligands expressed in most human cancers cells. They are considered as suitable targets for cancer therapy (Yotsumoto, Yagi et al. 2008). In addition, Singh et al. reported UV induced activation of the EGFR by AREG in human cancer cells and observed overexpression of HB-EGF, AREG and BTC (and also TGFA) in different melanoma cell lines (Singh, Schneider et al. 2009).

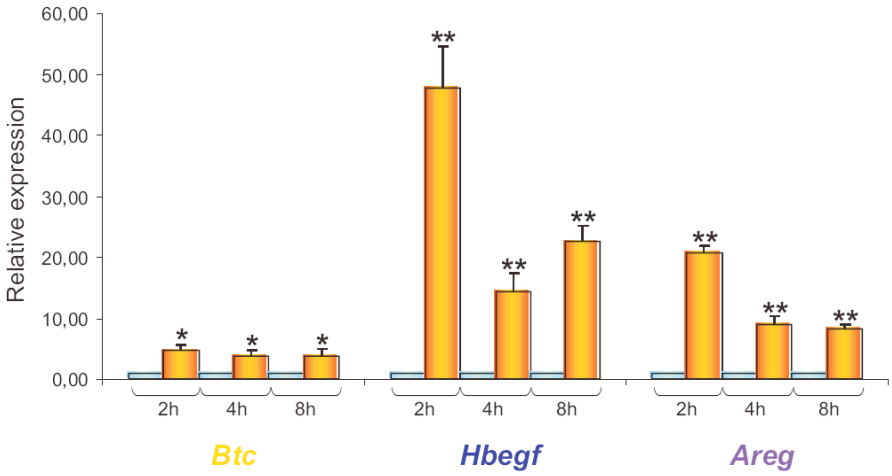

Figure 7: Expression of mouse EGFR ligands in melan-a HERmrk cells upon addition of human EGF.

Expression of *Btc*, *Hbegf* and *Areg* in melan-a HERmrk cells stimulated or not with human EGF (100 ng/ml) for 2, 4 and 8 hours.

In the mouse melanocyte system, the strong upregulation of *Hb-egf* and Areg both in activated HER and HERmrk melan-a cells was particularly striking. Both genes encode for the only EGFR ligands that possess an additional amino-terminal heparin-binding site in their precursor form. Heparin-binding sites enable binding of heparin sulphate proteoglycans (HSPGs) which are essential components of the cell surface and the extracellular matrix, allowing structural integrity and storage of growth factors and chemokines. HSPGs are overexpressed in many forms of cancer including melanoma (Ikuta, Nakatsura et al. 2005) and overexpression correlates with melanoma metastatic potential (Timar, Ladanyi et al. 1992; O'Connell, Fiori et al. 2009). Still, their functions are largely unclear and still need to be investigated.

To further elucidate the signaling pathways necessary for the strong upregulation of *Hbegf* and *Areg* in activated HERmrk melan-a cells, I investigated their transcriptional regulation upon addition of diverse inhibitors. We demonstrated that inhibition of HERmrk, MEK and SRC kinases could abolish *Hbegf* and *Areg* upregulation, whereas inhibition of the PI3K pathway had no effect (Laisney et al., in preparation).

All Egfr ligand precursors are transmembrane proteins that are shedded by members of the “a disintegrin and metalloprotease” (ADAM) family to release the mature part of the ligand that interacts with its receptor. ADAM sheddases are therefore a major factor for posttranslational regulation of EGFR ligands and a rate limiting step in autocrine EGFR activation. They are

currently investigated as targets for the therapy of cancer and inflammatory diseases (see for a review (Tortorella, Malfait et al. 2009)). In mice, the two major ADAM proteins involved in EGFR ligand shedding are ADAM10 and ADAM17 (Sahin, Weskamp et al. 2004). In the HERmrk melanocyte model, we could observe that both ADAM10 and ADAM17 genes were slightly upregulated upon receptor activation, suggesting a role for ADAM metalloproteases in the EGFR ligand autocrine loop.

5.3.2. Xmrk induces production of functional Egfr growth factors

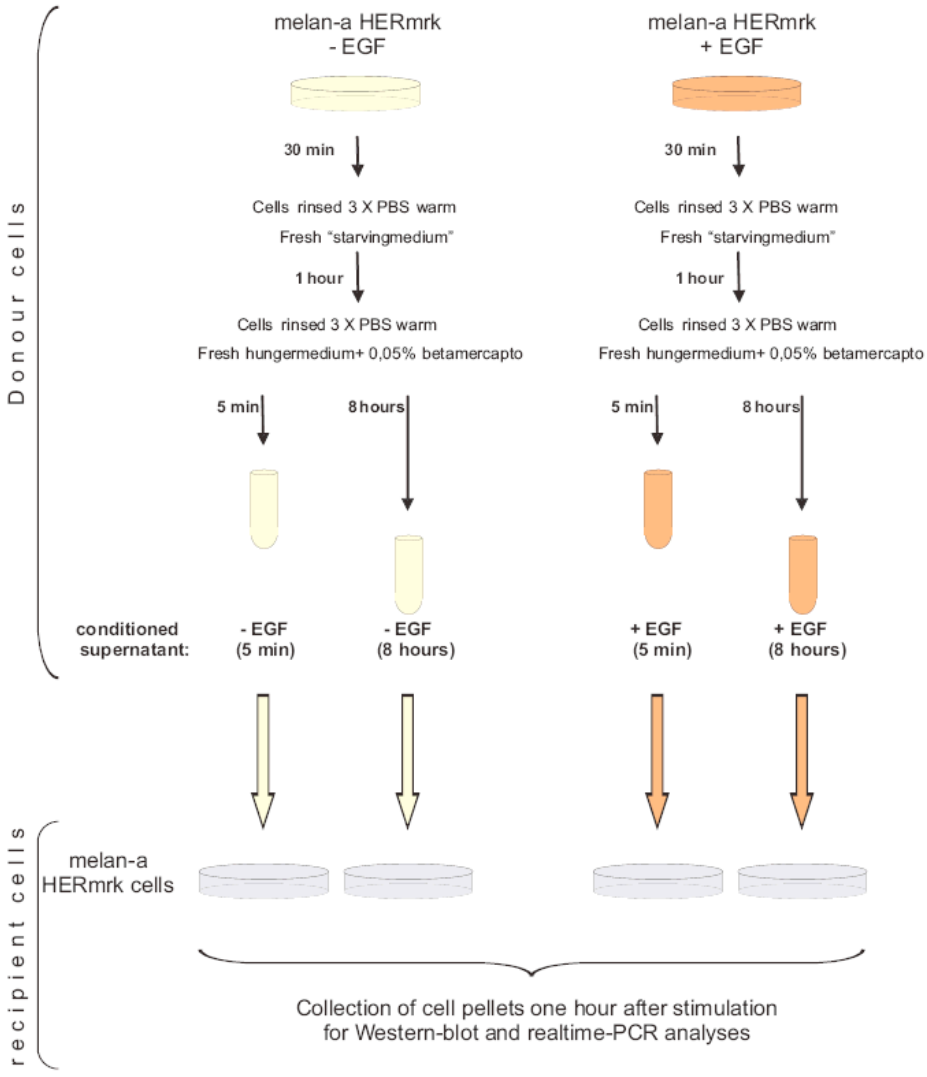

Figure 8: schematic representation of the conditioned HERmrk supernatants experiment.

After demonstrating regulation of Egfr/Egfr ligands on the transcriptional level in Hermrk transgenic cells, we wanted to elucidate whether Xmrk was able to induce secretion of active EGF family growth factors. We designed a "conditioned supernatant" experiment where supernatants from non-activated and activated HERmrk "donor" cells were collected and incubated together with unactivated HERmrk "receptor" cells (Figure 8). Only conditioned supernatant from stimulated Hermrk cells was able to trigger a signaling response in recipient HERmrk cells (Laisney et al., in preparation). Activation of HERmrk construct was monitored on protein level with Western blot analysis showing receptor, Akt and MAPK phosphorylations, but also on transcriptional level with realtime-PCR analyses displaying upregulation of known Hermrk target genes *Osteopontin* (*Opn*) and *Egr1*.

5.3.3. Hyperactivation of Xmrk and related downstream signaling by autocrine stimulation.

The Xmrk melanoma oncogene is a constitutively active mutated version of the Egfr, with at least two intermolecular disulfide bridges that allow disruption of the autoinhibitory tether and subsequent ligand-independent dimerization of the receptor (Gomez, Wellbrock et al. 2001). Analysis of the structure of Xmrk extracellular domain (Meierjohann, Mueller et al. 2006) revealed that these mutations do not affect the ligand binding pocket and do not obliterate the possibility for ligand binding to the pre-dimerized Xmrk. As we were using melan-a cells expressing whole *Xiphophorus* Xmrk protein, we decided to produce fish Egfr ligands in order to gain efficient receptor stimulation (Laisney, Braasch et al. 2010) (see chapter 5.1.3). Human Embryonic Kidney 293 cells (293-T) were transiently transfected with expression vectors for medaka Egf, Hbegf or Areg in order to gain conditioned supernatant containing the respective ligand. Then, wildtype melan-a cells or melan-a cells expressing *Xiphophorus* Egfrb (X-Egfrb) or *Xiphophorus* Xmrk were stimulated with these conditioned supernatants. Supernatants containing either medaka Egf or Hbegf were able to activate X-Egfrb, as demonstrated by enhanced phosphorylation of Akt and MAPK on the Western blot analysis (Figure 8) and upregulation of known Egfr target genes *p21*, *Mmp3* and *Opn* on realtime-PCR analyses. Interestingly, the already detectable Xmrk activation was further enhanced upon addition of conditioned supernatant with medaka Hbegf, demonstrated both on protein and transcriptional levels (Figure 9).

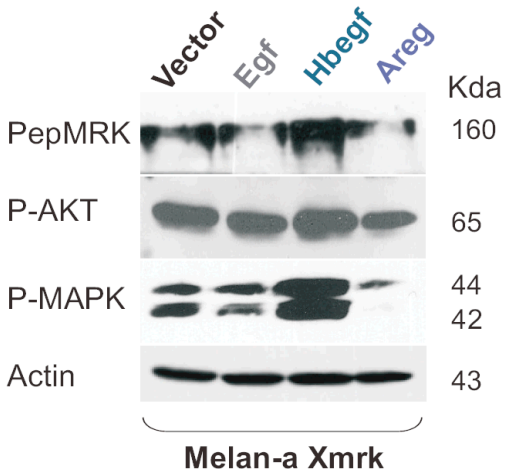

Figure 9: activation of *Xiphophorus* Xmrk by medaka Egfr ligands.

Melan-a Xmrk was stimulated for one hour with supernatants from 293-T cells transiently transfected with vector alone, or with expression constructs for medaka Egf, Hb-egf or Areg. Antibodies were directed against Xmrk (Pepmrk), P-AKT and P-MAPK. β-actin was used as loading control. Protein size is indicated in kDa.

Activation mechanism of EGFR family proteins is complex. The extracellular part of the receptor is divided into four subdomains, I to IV. Subdomains I and III build the ligand binding interface, and in the closed conformation subdomains II and IV form a contact that keeps the receptor in an inactive tethered, auto-inhibited, conformation. Binding of ligand leads to a rotation between domains I and II, disrupting the interaction between domains II and IV, thus allowing an open conformation. In this state, the dimerization arm of domain II is exposed, enabling dimerization of two neighboring molecules (reviewed in (Riese, Gallo et al. 2007) (Ward, Lawrence et al. 2007)). Xmrk mutations in the extracellular domain create independently new disulfide bridges that disrupt the tethered conformation and additionally stabilize the close proximity of both intracellular kinase domains, thus allowing receptor activation without ligand binding. It is likely that Xmrk, in this pre-dimerized open conformation, is well accessible to ligand binding. In this case, ligand binding may lead to flexible rotation of the juxtamembrane region that would place intracellular tyrosine kinase domains in a position that will increase crossactivation and therefore enhance Xmrk signalling when compared to unbound receptor. This phenomenon has already been described for mutation-induced cell-surface localized EGFR dimers which were able to bind EGF (Moriki, Maruyama et al. 2001).

5.3.4. Regulation of the members of the autocrine Egfr loop in transgenic mitf::xmrk medaka

To find out whether enhanced activation of oncogenic Xmrk may also take place *in vivo*, expression of Egfr ligands in *xmrk*-expressing medaka tumors was investigated.

The expression of *xmrk*, *egfr*a, *egfrb*, all *egfr* ligands, *adam10* and *adam17* was monitored in the two major medaka pigment cell tumors, the XE tumors and the melanoma. Gene expression levels in medaka *Carbio* healthy skin was taken as reference. A positive correlation was observed between expression of *egfrb*, *areg* and *epgn* in advanced stages of melanoma (Laisney et al., in preparation). An increased gene expression for *egf*, *tgfa* and *btc* was additionally monitored in the pigment cell tumors that occurred in fish also displaying metastatic melanoma. *Adam10* and *adam17* genes were also found to be slightly upregulated in the latter tumors. Altogether, we could provide many clues for the presence of an autocrine Egfr loop in the Xmrk-driven fish tumors. However, a discrepancy between the subset of upregulated Egfr ligand genes found in the Xmrk cell culture model (*Areg*, *Btc* and *Hb-egf*) and in the Xmrk transgenic fish tumors (*Egf*, *Areg* and *Epgn*) was observed. It is possible that this divergence may result from tumor niche effects (*in vivo*) or may reflect different states of oncogenic transformation.

In regard to these data, Egfr ligands may possibly play a role in Xmrk-driven melanoma since overexpression of Egfr ligands in tumors from fish with melanoma metastases was observed. Still, it has to be elucidated how this autocrine Xmrk loop contributes to melanoma progression. It is also very likely that in Xmrk-driven tumors, overexpressed Egfr ligands can interact in the tumor microenvironment with native wildtype Egfrs present either on melanocyte cell surface, either with Egfr present on adjacent cells cell surface, e.g. epithelial cells of blood vessels, contributing in both cases to melanoma progression, tumor angiogenesis or vasculogenesis. In mice it has been demonstrated that not only melanoma but melanoma-derived endothelial cells show gain in EGFR expression when compared to normal endothelial cells. Moreover, melanoma-derived endothelial cells are responsive to EGF that induces endothelial cell proliferation (Amin, Hida et al. 2006). Additionally, as demonstrated in chapter 5.3.3., Egfr ligands may also interact with Xmrk to enhance its oncogenic signaling (Figure 10).

The prominent role of EGFR ligands in cancer is already well established. For example, aggressive breast cancers co-over express EGFR and AR (LeJeune, Leek et al. 1993) and an EGFR-AR autocrine loop was identified in human breast cancer cells (Willmarth and Ethier 2006). HBEGF has been showed to be overexpressed in ovarian cancer and proposed as

suitable target for cancer therapy (Miyamoto, Hirata et al. 2004). In lung cancer cells, the constitutive activation of EGFR is achieved by several mechanisms, including increased production of ligands (Fujimoto, Wislez et al. 2005). Anti-EGFR most clinically advanced targeted therapies are based on inhibition strategies including small-molecule inhibition of EGFR intracellular tyrosine kinase domain (gefitinib and erlotinib) and monoclonal antibody-mediated blockade of EGFR extracellular ligand-binding domain (cetuximab) (Kataoka 2009). However, solid tumors are often resistant to conventional treatment modalities, raising the need in developing novel therapeutic approaches. Lately, targeting of EGFR ligands has been proposed as promising for cancer therapy (Yotsumoto, Sanui et al. 2009) but is still in its early stages, with a phase I study of the use of a specific inhibitor of HBEGF for patients with recurrent ovarian cancer (Miyamoto, Fukami et al. 2009) and development of EGFR ligand-sequestering drug (Cardo-Vila, Giordano et al. 2010).

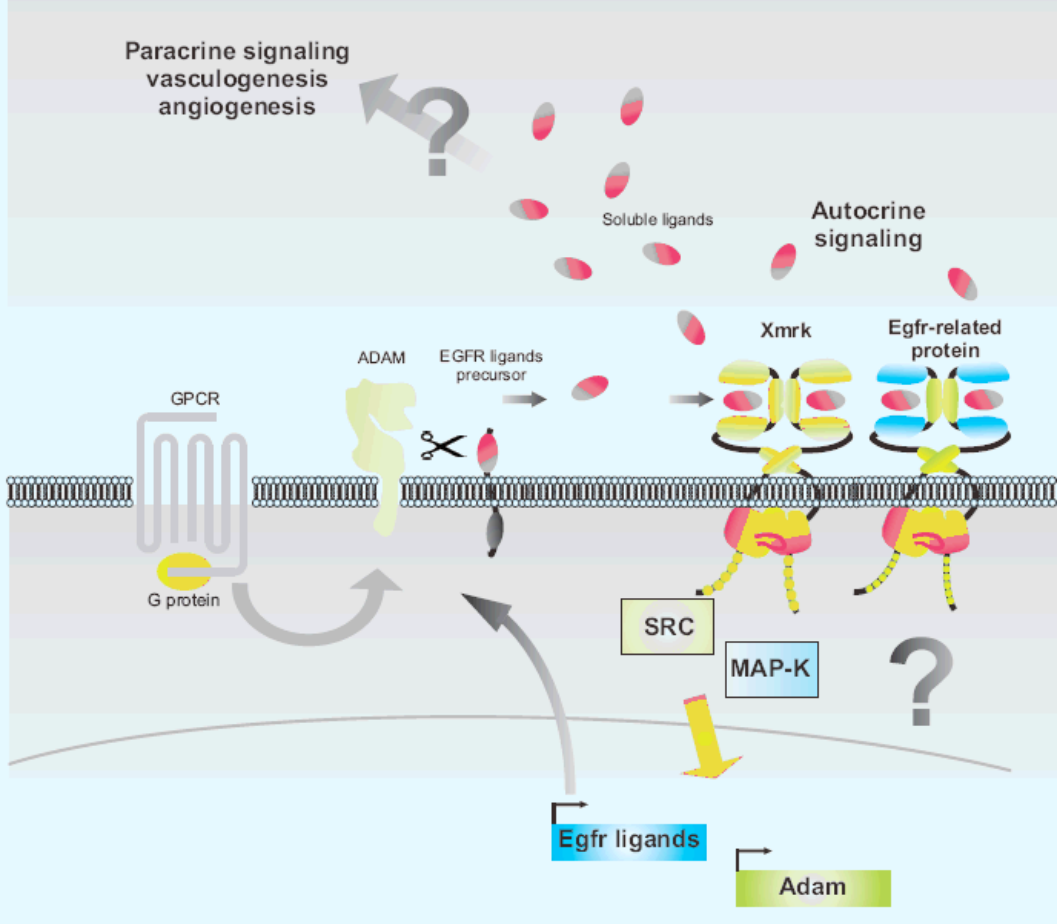

Figure 10: Model for an autocrine activation of Egfr family members in Xmrk-driven melanoma. In tumor cells microenvironment, members from the Egfr family are activated by various mechanisms including mutation (Xmrk), overexpression and autocrine or paracrine production of Egf. Xmrk signaling induces Egfr ligands and respective sheddases expression through MAP-K and Src

pathways. Egfr ligands are secreted by the tumor cells either to stimulate Egfr family members located on the cell surface of tumor cells (autocrine mode of action) but may also stimulate members from the Egfr family located on adjacent cells (paracrine mode of action), like for exemple on melanoma-derived endothelial cells from blood vessels, allowing vasculogenesis and angiogenesis events (Amin, Hida et al. 2006).

6. Conclusions

Naturally occurring melanomagenesis in *Xiphophorus* fish is due to expression of the *xmrk* oncogene that encodes for a mutated version of the Egfr. Previous to this work, mechanisms of the constitutive activation of Xmrk and its cellular effects were thoroughly elucidated. Still it was unclear if Xmrk is able to bind Egfr ligands despite its permanent dimerization and also uncertain if Egfr ligands may play a role in Xmrk-driven melanoma.

To better understand the situation of the Egfr/Egfr ligands system in teleost fish, I firstly investigated this system from an evolutionary point of view. Astonishingly, in all teleost species investigated, whereas Egfr gene has been kept in duplicates after the teleost-specific genome duplication, only one copy remains for each of all seven Egfr ligand. As I could show differential expression profiles for *egfra* and *egfrb* together with different subsets of ligands in adult medaka organs, we suggest a possible subfunctionalization of the Egfr in this fish, with partition of the ancestral gene functions between Egfra and Egfrb, which would explain the retention of the two gene copies during evolution. Also unexpected was the lack of conservation between tetrapod and teleost Egfr extracellular subdomains involved in ligand-binding, implicating use of homologous, or closely-related species Egfr ligands as a prerequisite in experiment involving teleost fish Egfr, which is not the case actually.

The next question was whether Xmrk can regulate the Egfr/Egfr ligands system. In melanocytes, I could demonstrate that Xmrk transcriptionally regulates Egfr ligand expression through MAPK and SRC pathways, suggesting an autocrine mode of activation. Taking benefit of our recently established transgenic *mitf*::*xmrk* medaka melanoma model, where I demonstrated Xmrk-induced signalling profiles are similar in *Xiphophorus* fish and in the newly established *mitf*::*xmrk* transgenic medaka fish were similar in non-tumorigenic and in tumorigenic tissues. These data together with histological and expression analyses confirm *mitf*::*xmrk* transgenic medaka as a suitable melanoma model in addition to *Xiphophorus*.

I could show that a specific subset of Egfr ligands was higher upregulated in malignant tumors when compared to benign tumors. In addition, genes encoding for ADAM10 and ADAM17, which are sheddases critical for the release of soluble Egfr ligands, and one of the two medaka constitutional Egfr copies, Egfrb, were found to be upregulated in tumors from fish carrying metastases. These data strengthen the hypothesis of an autocrine loop in Xmrk-driven melanoma, which may also involve a second Egf receptor, namely Egfrb.

Importantly, medaka Hbegf was also able to enhance the already activated state of Xmrk. These results confirm that the *Xiphophorus* Egfrb/Xmrk/Egfr ligand system is functional and

that Xmrk can still binds its ligands, which enhance its activation and might contribute to its oncogenic potential.

Self-suffiency in growth signals by autocrine growth factor production is one of the six hallmarks of cancer. In melanoma, oncogenic activation of mutated receptor tyrosine kinases (RTKs), like the fibroblast growth factor receptor (FGFR), the cytokine receptor KIT, or the mesenchymal-epithelial transition factor (MET) are frequently associated to autocrine production of their specific ligands. Melanoma is usually resistant to chemotherapy and most of primary and metastatic melanomas express EGFR but respond badly to EGFR tyrosine kinase inhibitor alone. While most research has concentrated on the intracellular signal transduction, it would be of great interest to analyze into details melanoma cells secretome, a biological fluid that may be enriched with secreted and/or shed proteins. This approach should give better insight into the processes that lead to self-sustainability of the tumor and would help to define new targets for melanoma treatment.

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8. Original publications

8.1. Lineage-specific co-evolution of the Egf receptor/ligand signaling system.

Laisney, J. A., I. Braasch, et al. (2010). "Lineage-specific co-evolution of the Egf receptor/ligand signaling system." BMC Evol Biol **10**: 27.

8.2. A mutated EGFR is sufficient to induce malignant melanoma with genetic background-dependent histopathologies.

Schartl, M., B. Wilde, et al. (2009). "A Mutated EGFR Is Sufficient to Induce Malignant Melanoma with Genetic Background-Dependent Histopathologies." J Invest Dermatol.

8.3. Dimerized and oncogenic EGFR variants can induce an autocrine loop to enhance EGFR activation.

Submitted

Dimerized and oncogenic EGFR variants can induce an autocrine loop to enhance EGFR activation

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Running Title:

Autocrine stimulation of oncogenic receptor tyrosine kinase

Keywords: Xmrk, EGFR, melanoma, oncogene, autocrine signaling.

Abstract

Autocrine production of growth factors is a well-known phenomenon in receptor tyrosine kinase-related tumors. As a consequence, the tumor cells become largely independent of the stimulating effecs of neighboring cells, often leading to unrestricted proliferation. An aberrantly activated MAPK pathway, e.g. caused by BRAF- or RAS mutations, can lead to the production of several EGFR ligands which contributes to the tumorigenic potential of the oncogenically modified cell. Similarly, receptor tyrosine kinases can induce their own ligands, as described for EGFR, FGFR and PDGFR.

In the *Xiphophorus* melanoma model, an oncogenic orthologue of the human EGF receptor drives the formation of aggressive melanoma. However, the fish harbours two further EGFR orthologues which can be expressed in the tumor or the tumor niche. To investigate whether autocrine activation of Egfr orthologues may contribute to tumor formation in this melanoma model, we investigated the transcriptional regulation of Egfr ligands by Xmrk.

Using a cell culture model in which a chimeric and inducible form of Xmrk is expressed, we found a strong upregulation of secreted and active *Hbegf* and *Areg* after Xmrk stimulation. This was dependent on MAPK and SRC family kinase pathways. Accordingly, Xmrk-positive melanoma from *mitf::xmrk* transgenic medaka also displayed high levels of several Egfr ligands and, in addition, the two Egfr isoforms that are present in this species. We also investigated whether these ligands are capable of activating the fish receptors, and surprisingly found that not only the proto-oncogenic Egfr, but also the already dimerized Xmrk was further stimulated by fish-derived Egf and Hbegf.

Altogether, we provide evidence for the presence of a functional Xmrk-dependent autocrine loop *in vitro* and *in vivo*. This autocrine hyperactivation might be a general process which contributes to the oncogenic potential of those receptor tyrosine kinases that still maintain a functional ligand binding domain.

Introduction

Receptor tyrosine kinases of the epidermal growth factor receptor (EGFR) family are implicated in the development of many cancer types, e.g. breast cancer, non-small-cell lung cancer, colorectal cancer and glioblastoma (Salomon, Brandt et al. 1995; Blume-Jensen and Hunter 2001; Yarden and Sliwkowski 2001). Together with the breast cancer oncogene ERBB2, EGFR poses a promising target for medical intervention, and specific antibodies or small molecule inhibitors against EGFR are either already successfully used in the clinic

(NSCLC, colorectal cancer (Govindan 2010; Ortega, Vigil et al. 2010)) or are in experimental stages, undergoing clinical trials (see for a review (Modjtahedi and Essapen 2009; Mitsudomi and Yatabe 2010)). Increased EGFR signalling in cancer cells can occur through several mechanisms. It can be due to increased receptor expression (often caused by gene amplification) activating EGFR mutations or autocrine loops mediated by enhanced ligand production (Miyamoto, Fukami et al. 2009).

Activating EGFR mutations can be categorized into two main groups, characterized either by kinase domain mutations or by the deletion or mutation of extracellular parts. Non-small-cell lung carcinomas often display mutations of the first kind. In many glioblastomas, alterations of the second group are present, where the main part of the extracellular domain, including the ligand binding parts, is missing due to a deletion of exons 2-7 (Huang, Cavenee et al. 2007; Laurent-Puig, Lievre et al. 2009). In addition, some cases of point mutations affecting extracellular Cys residues were also reported (Gomez, Wellbrock et al. 2001; Sihto, Puputti et al. 2005). These mutations lead to the disruption of intramolecular disulfide bridges and may facilitate the formation of intermolecular disulfide bridges.

A role for the EGFR/EGFR ligand system is also emerging in initiation and progression of malignant melanoma, the most dangerous form of skin cancer. Here it was reported to be involved in many key melanomagenic processes including proliferation, cell migration, differentiation, survival and tissue homeostasis (Yarden and Sliwkowski 2001; Bardeesy, Kim et al. 2005; Djerf, Trinks et al. 2009; Singh, Schneider et al. 2009).

Tumor development and progression can occur if cells escape from regulatory mechanisms. To be able to proliferate uncontrollably, cells must acquire growth autonomy. Usually, keratinocytes regulate melanocyte growth and behaviour by cell-cell adhesion molecules and growth factor production. Soluble growth factors can bind and activate receptors by paracrine, juxtacrine or autocrine modes of activation. Melanoma cells can escape control by keratinocytes using several mechanisms, including production of autocrine growth factors and enhanced expression of the respective receptors. It was previously described that melanoma cells are producing growth factors which activate the EGFR (Todaro, Fryling et al. 1980), such as transforming growth factor alpha (TGFA) and epidermal growth factor (EGF) (Krasagakis, Garbe et al. 1995). Correspondingly, EGFR expression or overexpression occurs in melanoma cells, thus contributing to their proliferation (de Wit, Moretti et al. 1992; Mattei, Colombo et al. 1994; Sparrow and Heenan 1999; Bardeesy, Kim et al. 2005; Akslen, Puntervoll et al. 2008; Djerf, Trinks et al. 2009). In addition, not only the melanoma tissue,

but also the adjacent endothelial cells need EGFR signalling to allow tumor vasculature and growth (Amin, Hida et al. 2006).

The *Xiphophorus* melanoma system provides an animal model where melanoma development is naturally induced by overexpression of a constitutively active version of the EGFR, called *Xiphophorus* melanoma receptor kinase (Xmrk) (Meierjohann, Schartl et al. 2004). The receptor is permanently dimerized due to the formation of intermolecular disulfide bridges (Gomez, Wellbrock et al. 2001; Winnemoeller, Wellbrock et al. 2005); however, structure predictions suggest that the ligand binding domain might still be accessible (Meierjohann, Mueller et al. 2006). Recently, a transgenic medaka melanoma model was produced by expressing the oncogene *xmrk* under the control of the pigment cell specific *mitf* promoter (Schartl, Wilde et al. 2009). Medaka is closely related to *Xiphophorus* but offers many additional advantages, like available transgenic technologies (Wittbrodt, Shima et al. 2002), *in vitro* systems (Hong, Winkler et al. 1998; Hong, Liu et al. 2004), and, importantly, a fully sequenced and well-annotated genome (Kasahara, Naruse et al. 2007). Moreover, all seven EGFR ligands have been characterized in medaka and their true orthology has been confirmed (Laisney, Braasch et al. 2010).

Here, we investigate the role of the known EGFR ligands in an oncogenic EGFR-driven tumor, using the Xmrk medaka melanoma model as well as melanocytes transgenic for an inducible version of Xmrk. We demonstrate the production of biologically active secreted ligands by Xmrk and human EGFR. Importantly, we observed an enhanced activation of already predimerized receptor by the produced autocrine factors. These results imply the involvement of autocrine factors in cancer progression, even if the receptor is already active due to oncogenic mutations that lead to a constitutively dimerized form.

Results

Activated human EGFR and Xmrk induce expression of EGFR ligands

An oncogenic form of human EGFR, namely the truncated EGFRvIII, was previously reported to induce EGFR ligands such as HBEGF and TGFA in glioblastoma (Ramnarain, Park et al. 2006). Although these ligands cannot bind the truncated receptor, they can stimulate intact EGFR which is also present on glioblastoma cells.

In order to check whether the oncogenic EGF receptor Xmrk also regulates transcription of EGFR ligands, we made use of a murine melanocyte model expressing the HERmrk chimera (melan-a HERmrk). This chimera consists of the extracellular part of human EGFR, fused to the intracellular part of *Xiphophorus* Xmrk. While Xmrk is constitutively active due to the existence of extracellular and intermolecular disulfide bridges, HERmrk signalling can be induced by adding EGF. Wildtype melan-a cells do not respond to EGF due to a lack of endogenous EGFR (Meierjohann, Wende et al. 2006; Leikam, Hufnagel et al. 2008).

Expectedly, EGF treatment of melan-a HERmrk induced a strong time-dependent phosphorylation of the chimeric receptor and of its downstream targets AKT and MAPK (Figure S1A). Also, transcription of the known target gene *Osteopontin*, which is mediated by the MAPK pathway, was enhanced at all time points upon Xmrk activation (Figure S1B). The EGFR inhibitor AG1478 completely abolished all HERmrk signalling (Figure S1A and S1B).

After this successful examination of HERmrk stimulation, we investigated the expression of all seven EGFR ligands by quantitative realtime-PCR analysis. All ligands except *Tgfa* and *Egf* showed transcriptional upregulation with maximal values 2 hours post stimulation (Figure 1A). In particular, *Hbegf* and *Areg* displayed the highest upregulation (47.8 and 20.6 fold induction, respectively). We also investigated the effect of activating human full-length EGFR on transcriptional regulation of EGFR ligands in melanocytes. We made use of the melan-a cells transgenic for human EGFR (melan-a HER), stimulated them for one hour with human EGF and monitored expression of the seven EGFR ligands in both unstimulated and hEGF stimulated cells. A strong upregulation was observed for three of the seven ligands genes: *Areg*, *Btc* and *Hbegf* (Figure 1B). Importantly, the parental melan-a cell line, which does not express detectable amounts of endogenous EGFR, did not respond to EGF stimulation (data not shown and (Laisney, Braasch et al. 2010)).

Activated Xmrk induces expression of *Adam10* and *Adam17*

Since “a disintegrin and a metalloprotease (ADAM) 10 and ADAM17 are the two major sheddases involved in membrane-anchored EGFR proligand release in mouse (Sahin, Weskamp et al. 2004), we investigated their regulation in melan-a HERmrk cells. Stimulation of melan-a HERmrk cells with human EGF resulted in a 2.6 fold increase of metalloproteinase *Adam10* transcript and a 1.8 fold increase of *Adam17* compared to the unstimulated control (Figure 1C).

MEK and SRC kinases mediate the upregulation of autocrine factors

As *Hbegf* and *Amphiregulin* displayed the highest upregulation upon HERmrk activation in melanocytes, we were interested in their upstream regulation. Thus, we applied EGFR inhibitor AG1478, MEK inhibitor U0126, SRC kinase inhibitor PP2 and PI3K inhibitor LY294002 in combination with hEGF. Inhibition of EGFR, MEK and SRC kinases, but not PI3K completely abolished the hEGF-mediated *Hbegf* and *Areg* upregulation (Figure 2).

Metastatic melanomas express high levels of *egfrb*, *egf*, *areg*, *epgn*, *adam10* and *adam17*

Recently we described the generation of a new melanoma model, where stable expression of *xmrk* under the control of the *mitf* promoter drives pigment cell tumors in medaka (Schartl, Wilde et al. 2009). Here, two major types of pigment cell tumors are commonly observed. The first type is the exophytically growing xanthoerythrophoroma (derived from orange/yellow-coloured pigment cells that naturally occur in these fish), which has a low metastatic potential. The second tumor type is the melanoma which mostly derives from extracutaneous sites and is characterized by a high metastatic and invasive behaviour. Exophytic cutaneous tumors can either occur independently, or in combination with malignant and metastatic melanoma. In the latter situation, they are more advanced and contain nests of melanoma cells intermingled with the xanthoerythrophoroma (XE) cells (Schartl, Wilde et al. 2009). We compared exophytic tumour tissues from fish without melanoma with those from fish that additionaly displayed metastatic melanoma for their capacity to induce an autocrine EGFR loop (Figure 3A). The expression of the respective gene in skin tissue was used as reference. Expression of *xmrk*, the two *egfr* genes copy that occur in fish (*egfra* and *egfrb*), all Egfr ligands, *adam10* as well as *adam17* was monitored by quantitative realtime-PCR. *Egfra* and *egfrb* were expressed in all tumors, with a significant overexpression of *egfrb* in tumors that co-occured with metastatic melanoma (Figure 3B)

Similarly, the EGFR ligands *egf*, *tgfa*, *areg, btc* and *epgn* were in most cases more strongly expressed in the tumors connected to metastatic melanoma when compared to those without melanocytic tumors (Figure 3C). However, in both tumor types the overall expression of *tgfa* was much lower than in skin tissue. The *btc* levels of the normal exophytic tumors were also lower than those observed in skin, but exophytic tumors linked to melanoma displayed levels comparable to levels in skin. No significant changes were noticed for *ereg* and *hbegf*

Furthermore, *adam10* and *adam17* displayed a slight trend towards stronger expression in tumors linked to metastatic melanoma (Figure 3D), though this was not significant.

Altogether, the data indicate a positive correlation between the expression of *egfrb* and the EGFR ligands *areg* and *epgn* and advanced stages of melanoma in the fish model.

HERmrk stimulation leads to the production of functional EGFR growth factors

So far, the transcriptional expression of EGFR ligands was demonstrated in HERmrk-transgenic cells and in *xmrk*-transgenic medaka melanoma. To demonstrate the secretion of functional EGF family growth factors, we designed a "conditioned supernatant" experiment where the secreted factors from stimulated "donor" HERmrk melanocytes were used to stimulate a "receptor" cell population. The receptor cell population consisted of unstimulated HERmrk melanocytes. These cells only display activation of their HERmrk receptor when the conditioned supernatant of the hEGF-treated donor cells, which is transferred to the receptor cells, contains secreted EGFR ligands. Importantly, after stimulation with hEGF the donor cells were washed repeatedly to prevent the presence of hEGF in the conditioned supernatant. In addition, a short-time stimulation control was applied.

The Western blot clearly demonstrated that only supernatant from activated HERmrk cells was able to trigger a signalling response (Figure 4A). Only here, phosphorylation of activation-associated sites in HERmrk, MAPK and AKT was visible, indicating that secreted and active growth factors were produced in the 8 h-conditioned supernatant. Furthermore, induction of the HERmrk target genes *Osteopontin* and *Egr-1* was also exclusively observed in the receptor cells treated with supernatant from stimulated cells (Figure 4B and 4C).

The oncogenic Xmrk induces an autocrine loop that enhances oncogenic signaling

After having observed that HERmrk was able to induce an autocrine feedback loop, we wanted to find out whether the melanoma oncogene Xmrk itself was also responsive to its potential ligands. As the receptor is permanently dimerized due to the existence of at least two intermolecular disulfide bridges, an effect of ligand binding on receptor signaling was not previously expected. In order to monitor stimulation of Xmrk that – as opposed to HERmrk – is no human-fish protein chimera but the entire *Xiphophorus* protein, we had to use fish EGFR ligands to gain specificity. It was shown previously that Xmrk and its proto-oncogene Egfrb do not bind mammalian EGF (Gomez, Volff et al. 2004) (own unpublished data). To produce fish EGFR ligands, we transiently transfected 293T cells with expression plasmids containing medaka *egf*, *hbegf* or *areg* (Laisney, Braasch et al. 2010), or with an empty expression plasmid as a control. The supernatant of these cells was then collected to stimulate

wild-type melan-a cells (melan-a WT), or melan-a cells stably expressing either *Xiphophorus* Egfrb (melan-a X-Egfrb) as control, or Xmrk (melan-a Xmrk). As expected, melan-a WT cells treated with control or conditioned supernatant were not stimulated. (Figure 5A).

However, medaka Egf and Hbegf containing supernatants clearly activated the proto-oncogenic X-Egfrb, indicated by a strong phosphorylation of AKT and MAPK (Figure 5A). Additionally, the downstream genes *p21* and *Mmp3* displayed a robust induction when compared to controls (Figure 5C). Medaka Areg, however had no effect on Egfrb signaling, neither on protein nor on RNA level (Figure 5A and 5C). When we looked at the signaling capacity of constitutively activated Xmrk, we expectedly observed that melan-a Xmrk cells already showed receptor, AKT and MAPK activation with the control supernatant (Figure 5A). Surprisingly, medaka Hbegf was able to further activate Xmrk, with considerable increase in AKT and MAPK phosphorylation. On RNA level, a slight but significant upregulation was observed for *p21* and *Mmp3* after stimulation of melan-a Xmrk cells with medaka Hbegf and Egf (Figure 5D). Altogether, these data indicate that although Xmrk is a predimerized oncogenic receptor, its activation is further enhanced by ligands which are produced in an autocrine manner by the receptor itself.

Discussion

Autocrine stimulation of receptor tyrosine kinases is a common feature of many tumors and contributes to their development. An oncogenic form of human EGFR, namely the truncated EGFRvIII, was previously reported to induce EGFR ligands such as HBEGF and TGFA in glioblastoma (Ramnarain, Park et al. 2006). Although these ligands cannot bind the truncated receptor, they can stimulate intact EGFR which is also present on glioblastoma cells.

We report here that induction of EGFR ligands downstream of HERmrk is dependent on MAPK and SRC kinases. These observations are in concordance with published data, where SRC family kinases inhibitors hinder EGFR autocrine production of AREG (Kansra, Stoll et al. 2005) and HBEGF (Zhuang, Kinsey et al. 2008). Furthermore, the EGFR-RAS-RAF-MEK-ERK axis is also known to upregulate EGFR ligands (Roberts and Der 2007), as shown in fibroblasts and human epithelial breast cells (McCarthy, Samuels et al. 1995) (Schulze, Lehmann et al. 2001). MAPK are responsible for phosphorylation and activation of JUN and FOS proteins that constitute the activator protein-1 (AP-1) transcription factor complex (Li, Ma et al. 2003). The transcriptional induction of *Hbegf* is directly mediated by AP-1 binding to their binding sites in the *Hbegf* promoter (Park, Adam et al. 1999; Kitamura, Miyazaki et

al. 2001; Sakai, Tsukada et al. 2001; Li, Cheung et al. 2009). As SRC kinases such as FYN are involved in maintaining high levels of MAPK activation (Wellbrock, Weisser et al. 2002), it is possible that the observed blocking effect of the SRC kinase inhibitor is due the decrease of MAPK- and consequently AP-1 activity.

Both human activated EGFR and HERmrk expressing melanocytes displayed highest expression levels for *Hbegf* and *Areg* among all seven EGFR ligands. Recently both ligands were demonstrated to be the most aberrantly expressed EGFR ligands in numerous human cancer cells, and even discussed as targets for cancer therapy (Yotsumoto, Yagi et al. 2008). Areg and Hbegf are the only EGFR ligands with an amino-terminal heparin-binding site, thus enabling binding of heparan sulphate proteoglycans (HSPGs) in addition to EGFR. HSPGs are essential components of both the cell surface, thus helping to provide structural integrity, and the extracellular matrix (ECM), being involved in storage of growth factors and chemokines. The mitogenic action of HBEGF has been shown to be increased when the ligand was associated with HSPG, through the formation of high-affinity ternary complexes stabilizing the receptor-ligand interaction (Higashiyama, Abraham et al. 1993). HSPGs are overexpressed in many forms of cancer, including melanoma (Ikuta, Nakatsura et al. 2005), but their role in melanoma in particular is scarcely documented. Former studies showed absolute or relative dominance of HSPGs over chondroitin sulphate proteoglycans (CSPGs) at the cell surface of metastatic melanoma tumor cells (Timar, Ladanyi et al. 1992). Recently, HSPGs increase in expression was demonstrated to be correlated with melanoma progression (O'Connell, Fiori et al. 2009), indicating a possible involvement/implication for HSPGs regarding melanoma metastatic potential.

Interestingly, we observed that medaka Egf and Hbegf could further enhance the signalling downstream of predimerized Xmrk, as indicated by increased phosphorylation of AKT and ERK1/2 and the induction of downstream target genes. How can this be achieved? An explanation is probably found in the complex activation mechanism of EGFR family proteins. The extracellular domain (ECD) of EGFR is arranged into four domains. Domains I and III constitute the binding interface for the ligand. In the closed conformation, domains II and IV form a contact that keeps the receptor in a tethered conformation and therefore in an auto-inhibited state. Binding of ligand leads to a rotation between domains I and II, thus disrupting the interaction between II and IV and allowing an open conformation. In this state, the dimerization arm of domain II is exposed, which enables the dimerization of two neighboring molecules that are both present in the open conformation (reviewed in (Riese, Gallo et al. 2007; Ward, Lawrence et al. 2007)).

According to the structural model for Xmrk, the mutations in the ECD lead to a free accessibility of cysteines 305 and 564. Both form a disulfide bridge with the corresponding residue of a neighboring receptor monomer, albeit not all molecules are predicted to contain both disulfide bridges (Meierjohann, Mueller et al. 2006). Importantly, Cys305 and Cys564 are located in domains II and IV, respectively, with Cys564 being directly adjacent to those amino acid residues that are essential for the inhibitory interaction between domains II and IV (Mattoon, Klein et al. 2004; Dawson, Bu et al. 2007). This means that disulfide bridges at either Cys305 or at Cys564 disrupt the tethered conformation of the receptor and, in addition, stabilize the close proximity of both intracellular kinase domains. This explains the basal activity of Xmrk in absence of ligand. This model is supported by analogous observations by Moriki et al., who observed spontaneous dimerization of EGFR after introduction of cysteine residues into the extracellular juxtamembrane region of the receptor (Moriki, Maruyama et al. 2001). Even in absence of ligand, the expression of some of these constructs was sufficient to cause cellular transformation of NIH3T3 cells, as indicated e.g. by soft agar growth (Moriki, Maruyama et al. 2001). A similar effect is observed with cells expressing a dimerizing EGFR mutant with insertions on both sides of the transmembrane region (Sorokin 1995). Still, even in the pre-dimerized state ligand binding and further receptor activation are possible. Several groups have observed that cell-surface localized EGFR dimers – either formed due to mutational activation (Moriki, Maruyama et al. 2001), or, to a smaller extent, spontaneously formed by wildtype EGFR (Tao and Maruyama 2008), can bind EGF. This induces a flexible rotation of the juxtamembrane region which is translated into the cytoplasmic domain and eventually leads to enhanced kinase activation. We propose a similar mechanism for Xmrk, which is arranged in a conformation where the autoinhibitory tether is disrupted by at least one of the two disulfide bridges. The open conformation that is stabilized by the dimer is well accessible to ligand, and ligand binding may lead to the described flexible rotation that places the intracellular kinase domains in a position that is optimal and allows a higher degree of crossactivation compared to the unbound receptor.

In summary, we describe the induction of EGFR ligands by HERmrk/Xmrk in transformed pigment cells in-vitro as well as in a whole melanoma animal model. The ligands are able to enhance the activation state of the oncogene, and thereby contribute to the tumor phenotype. It is very likely that the same concept is valid in human tumors that contain activated oncogenes, such as *c-KIT*, *EGFR* or *FGFR* (Allerstorfer, Sonvilla et al. 2008; Hofmann, Kauczok-Vetter et al. 2009).

Methods

Cell culture

Melan-a, melan-a HERmrk (Wittbrodt, Lammers et al. 1992), melan-a HER and melan-a X-Egfrb cells (Gomez, Volff et al. 2004) were cultured as described earlier (Meierjohann, Wende et al. 2006).

HEK 293T cells (human embryonic kidney fibroblasts with SV40 T-antigen) were grown in DMEM supplemented with 10% FCS, 1% glutamine and antibiotics.

For the stimulation experiments, melan-a HERmrk and melan-a HER were cultivated in starving medium (Dulbecco's Modified Eagle's Medium with 1, 5 % dialysed foetal calf serum (Gibco/Invitrogen, Karlsruhe, Germany) for three days before EGF treatment. Where indicated, human EGF (100 ng/ml) (Tebu-bio, Offenbach, Germany) was added and melan-a HERmrk cells were collected after 2, 4 and 6 hours post-stimulation for melan-a Hermrk and 2 hours post-stimulation for melan-a HER cells. Where indicated, EGFR inhibitor AG1478 (10 μM), PI3K inhibitor LY294002 (10 μM), Fyn inhibitor PP2 (20 μM) or MEK inhibitor U0126 (10 μM) (all purchased from Calbiochem, Merck Chemicals Ltd., Darmstadt, Germany) were added to the starving medium one hour prior to the EGF stimulation.

DNA transfection

Melan-a cells were transfected with a *Xiphophorus* Egfrb expression plasmid (melan-a X-*egfrb*) (Gomez, Volff et al. 2004) using Fugene transfection reagent (Roche) according to manufacturer's recommendations. Expression constructs pCS2+-Ola-*egf*, pCS2+-Ola-*areg* and pCS2+-Ola-*hbegf* were transiently transfected into HEK 293T cells using the calcium-phosphate method (Chen and Okayama 1987).

Cell lysis and Western blotting

Cells were trypsinized, rinsed twice with PBS and lyzed in 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 200 μmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 100 mmol/L NaF. 50 μg of protein lysate was separated by SDS-PAGE and analyzed by Western blotting onto nitrocellulose. Membranes were blocked for 60 minutes with TBS [10 mmol/L Tris-HCl (pH 7.9) and 150 mmol/L NaCl], 0.1% Tween-20, and 5% bovine serum albumin (BSA) and were incubated overnight at 4°C with the first

antibody. Monoclonal anti-phosphotyrosine antibody PTyr (PY20) was from BD Biosciences (San Jose, CA). Phospho-p44/42 MAPK (Thr202/Tyr204) antibody was purchased from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal anti-phospho AKT was obtained from New England Biolabs (Ipswich, MA). Polyclonal anti-mrk recognising the C-terminal part of Xmrk was generated by Biogenes (Berlin, Germany).Anti-b-actin was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The secondary antibodies were conjugated with horseradish peroxidase and were directed against mouse (Pierce, Rockford, IL) or rabbit (Bio-Rad Laboratories, Redmond, WA).

Fishes

Fishes were kept under standard conditions in the aquarium facility of the Biozentrum at the University of Würzburg. The medaka (*Oryzias latipes*) strains *tg*(*mitf::xmrk*), genetic background Carbio (Schartl, Wilde et al. 2009) and wild-type Carbio were used in this study.

RNA extraction, cDNA synthesis and realtime−PCR analysis

RNA extraction from melan-a HERmrk cells and medaka tissues was done using Total RNA Isolation Reagent (ABgene, Epsom, UK) as recommended by the manufacturer.
For all melanocyte cell lines and healthy medaka skin as well as *mitf::xmrk* medaka tumors, cDNA was prepared from total RNA cDNA was prepared using the RevertAid kit with random hexamer primers (Fermentas, Burlington, Canada). For analysis of gene expression, real-time PCR was done with primers designed using Primer3 software. primer sequences are available upon request. Each PCR was carried out in duplicate and was repeated 3 times independently in a 25 µl volume using a home-made SYBR green containing master mix for 4 minutes at 95°C followed by 40 cycles of 95°C for 30 seconds and 60°C for 60 seconds in the iCycler IQ (Bio-Rad, Hercules, CA). All PCR products were checked by sequencing using the CEQ DTCS dye terminator cycle sequencing kit and run on a CEQ 2000XL DNA sequencing system (Beckmann Coulter, Krefeld, Germany).Values for each gene were normalized to expression levels of beta-actin (*actin*) using the 2-DDCT method (Livak and Schmittgen 2001). Data are presented as mean ± standard deviation.

Cloning of the expression constructs

Full length cDNA encoding medaka *egfra* and medaka *hbegf* were PCR-amplified from medaka kidney cDNA, and medaka *egf* and *areg* were amplified from medaka brain cDNA. The respective primers were designed from the medaka genome database available on

Ensembl Genome Browser (www.ensembl.org). To generate the different pCS2+ expression constructs, EcoRI and XbaI restriction sites were added to the 5' and 3' termini of medaka *egf*, EcoRI and XhoI were added to the 5' and 3' termini of medaka *areg* and ClaI and XbaI were added at the 5' and 3' termini of medaka *hbegf*, using PCR. The PCR products were digested with the respective enzymes and were ligated into the correspondingly digested pCS2+ vector. This gave rise to the vectors pCS2+-Ola-*egf*, pCS2+-Ola-*areg* and pCS2+-Ola-*hbegf*. For the cloning of medaka *egfra*, Xba I and SnaBI restriction sites were added at the 5' and 3' termini, respectively. The PCR product was ligated into the XbaI and SnaBI digested pCS2+ expression vector to create the pCS2+-Ola-*egfra* construct. Gene accession numbers is given in supplementary table S1.

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Figure legends

Figure 1: Transcriptional regulation of EGFR ligands and their sheddases in response to HER and HERmrk stimulation (A-C). A: Expression of mouse EGFR ligands in melan-a HERmrk cells unstimulated or stimulated with human EGF (100 ng/ml) for 2, 4 and 8 hours. B: Expression of mouse EGFR ligands in melan-a HER cells unstimulated or stimulated with human EGF (100 ng/ml) for 2 hours. C: Expression of murine *Adam10* and *Adam17* in melan-a HERmrk cells under the same conditions as in 1A. Results are average values of three independent quantitative realtime-PCR analyses normalized to *β-actin* expression level.

Figure 2: *Areg* and *Hbegf* expression is regulated by SRC kinases and MAPK.

Areg and *Hbegf* expression in melan-a HERmrk cells stimulated for 4 hours with human EGF in absence or presence of PI3K inhibitor (LY294002) (10 μM), SRC kinase inhibitor (PP2) (20 μM) or MEK inhibitor (U0126) (10 μM). Results are average values of three independent quantitative realtime-PCR analyses normalized to *β-actin* expression level.

Figure 3: Expression of medaka EGF receptors, their ligands and sheddases in Xmrk-driven tumors of different malignancy (exophytic tumors of fish without metastases and with (*) melanoma metastases) (A-D). A: *mitf*::*xmrk* transgenic medaka showing differences in tumor development. Left image: cutaneous exophytic xanthoerythrophoroma. Right image: extracutaneous invasive melanotic melanoma. B: Expression of medaka *egfr-a* and *egfr-b*. C: Expression of all Egfr ligands. D: Expression of *adam10* and *adam17*. The expression was normalized to expression of the respective gene in medaka wild-type normal skin. 6 individual fish were used for each group displayed by the box and whiskers representation, and each individual value is the average value of three independent quantitative realtime-PCR analyses normalized to *ef1a1* expression.

Figure 4: Recipient melan-a HERmrk cells show HERmrk activation only upon addition of conditioned supernatant from stimulated donor melan-a HERmrk cells (A-C).
Recipient melan-a HERmrk cells were stimulated for 1 h with four different conditioned supernatants from donor melan-a HERmrk cells that were previously treated either for 5 minutes or for 8 hours with or without recombinant human EGF. A: Western blot analysis. Antibodies were directed against phosphorylated tyrosine kinase (PTK), AKT (P-AKT) and MAPK (P-MAPK). β-actin was used as loading control. Protein size is indicated in kDa. B and C: Realtime analysis displaying the expression of murine *Opn* (B) and *Egr1* (C) in recipient melan-a HERmrk cells incubated for 1 h with the conditioned supernatants from melan-a HERmrk donor cells. Results are average values of three independent quantitative realtime-PCR analyses normalized to *β-actin*.

Figure 5: Egfrb and Xmrk are both stimulated by Egfr ligands (A-D). Melan-a wild type (WT), X-Egfrb or Xmrk were stimulated for one hour with supernatants from 293-T cells transiently transfected with vector alone or with expression constructs for medaka Egf, Hbegf or Areg. A: Western blot analysis. Antibodies were directed against Xmrk (Pepmrk), P-AKT and P-MAPK. β-actin was used as loading control. Protein size is indicated in kDa. B-D: Realtime PCR of Egfrb and Xmrk target genes. Expression of *p21* (B), *Mmp3* (C) and *Opn*

(D) in melan-a wild type, melan-a X-Egfrb or melan-a Xmrk. Results are average value of three independent quantitative realtime-PCR analyses normalized to *β-actin* expression level.

Supplementary figure 1:

Activated melan-a Hermrk cells show activation of downstream pathways (A-B). A: phosphorylation of the chimeric Hermrk receptor, AKT and MAPK, as shown by Western blot analysis. Where indicated, melan-a HERmrk cells were stimulated with human EGF (100 ng/ml) for 2, 4 or 8 hours in absence or presence of EGFR inhibitor AG1478 (10 μM). Antibodies were directed against Xmrk (Pepmrk), phosphorylated tyrosine kinase (PTK), AKT (P-AKT) and MAPK (P-MAPK). β-actin was used as loading control. Protein size is indicated in kDa. B: Expression of the target gene *Opn* in melan-a HERmrk cells stimulated with human EGF (100 ng/ml) for 2 hours, 4 hours or 8 hours in absence or presence of EGFR inhibitor AG1478 (10 μM). Results are average values of three independent quantitative realtime-PCR analyses normalized to *β-actin* expression level.

Figure 1

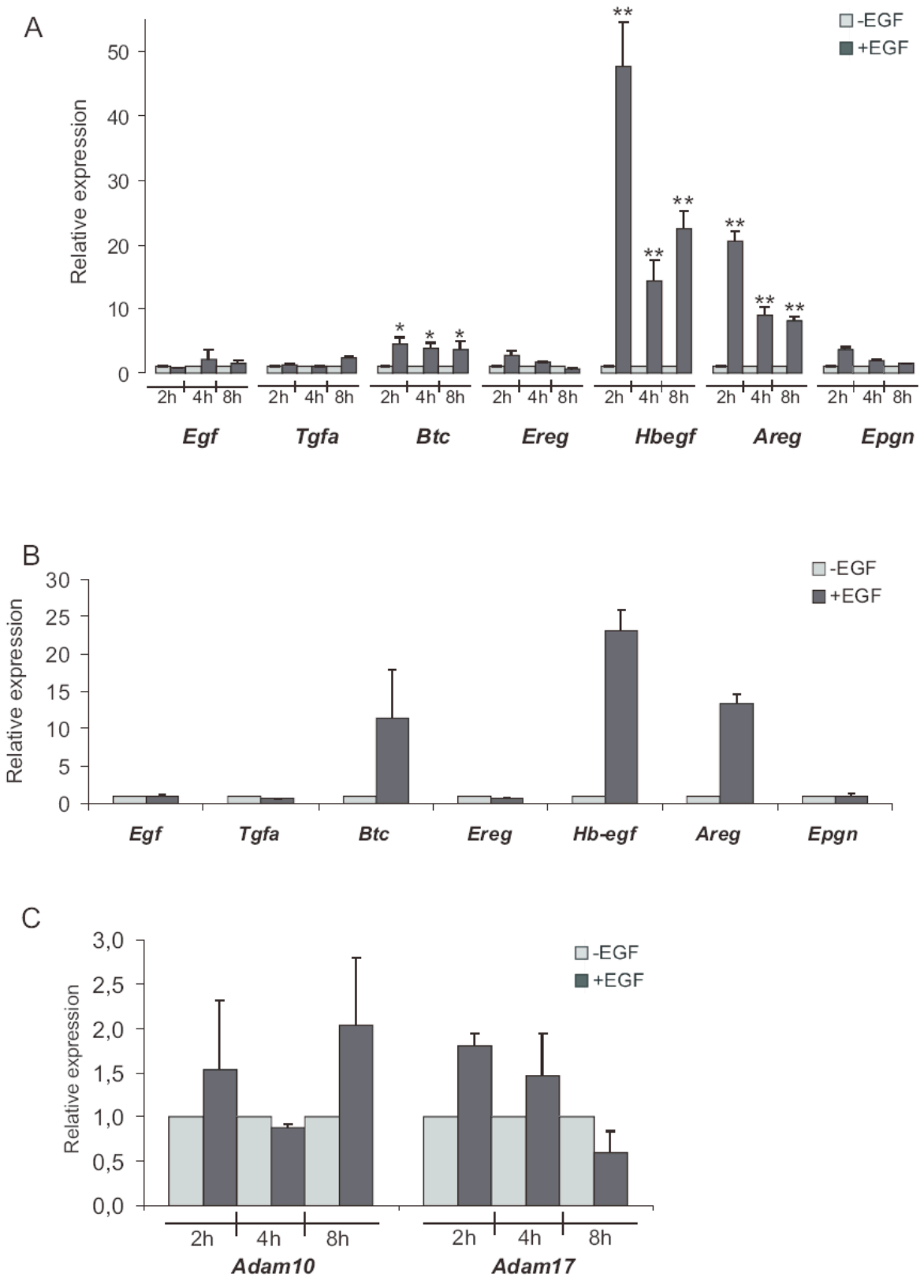

A
-EGF
+EGF
Relative expression
0
10
20
30
40
50
2h 4h 8h
Egf
Tgfa
Btc
Ereg
Hbegf
Areg
Epgn
B
-EGF
+EGF
Relative expression
0
5
10
15
20
25
30
Egf
Tgfa
Btc
Ereg
Hb-egf
Areg
Epgn
C
-EGF
+EGF
Relative expression
0,0
0,5
1,0
1,5
2,0
2,5
3,0
2h 4h 8h
Adam10
Adam17

Figure 2

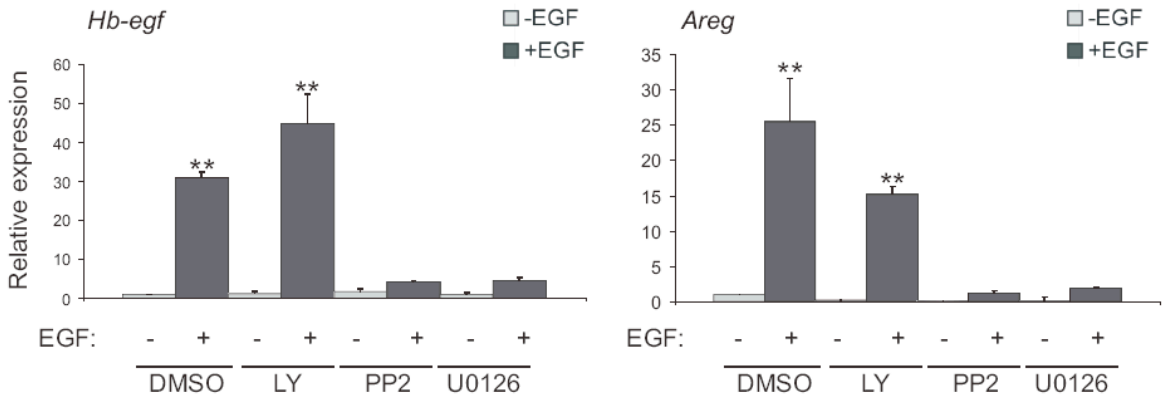

Hb-egf
-EGF
+EGF
Relative expression
60
50
40
30
20
10
0
**
**
EGF: - + - + - + - +
DMSO
LY
PP2
U0126
Areg
-EGF
+EGF
35
30
25
20
15
10
5
0
**
**
EGF: - + - + - + - +
DMSO
LY
PP2
U0126

Figure 3

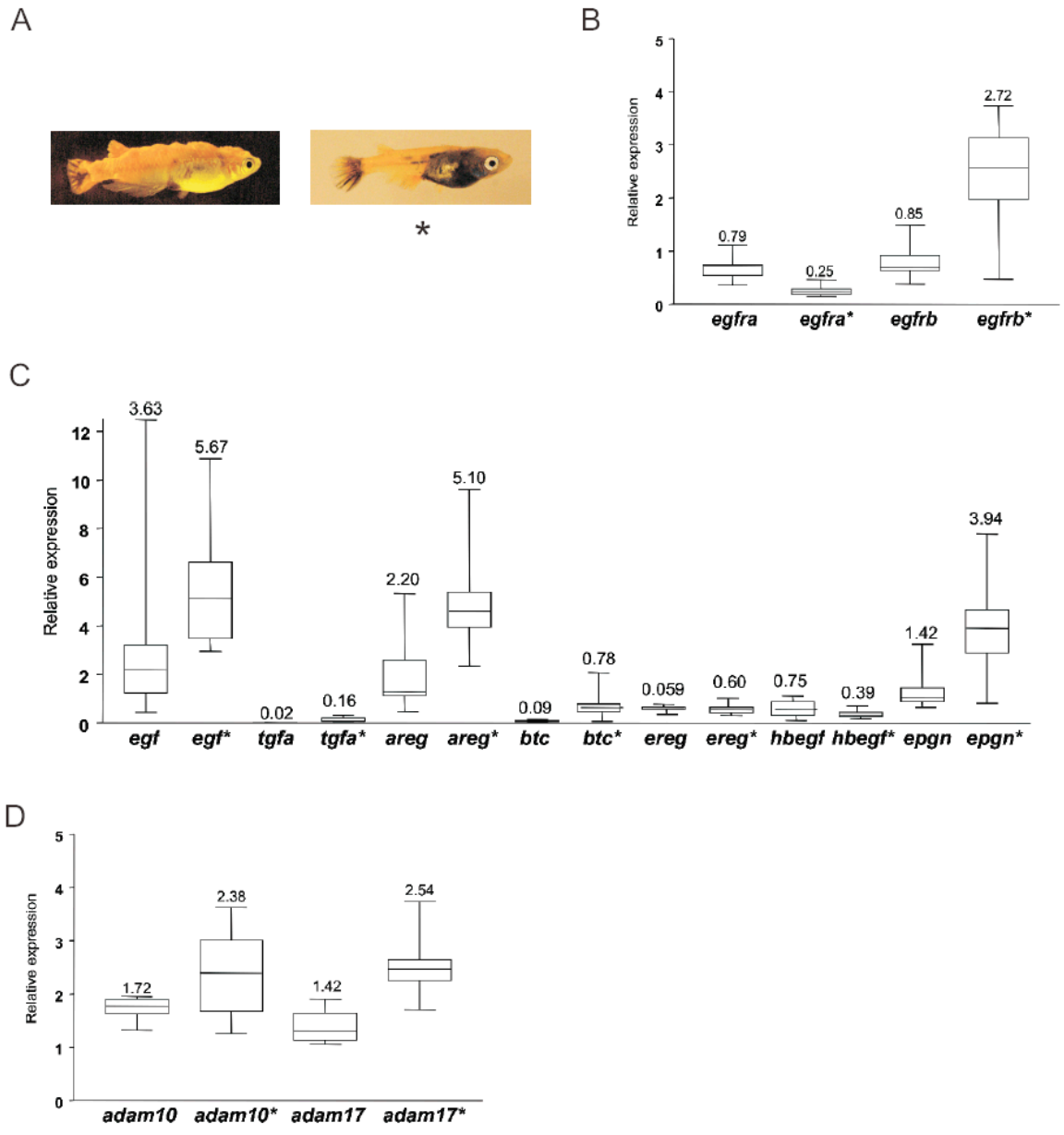
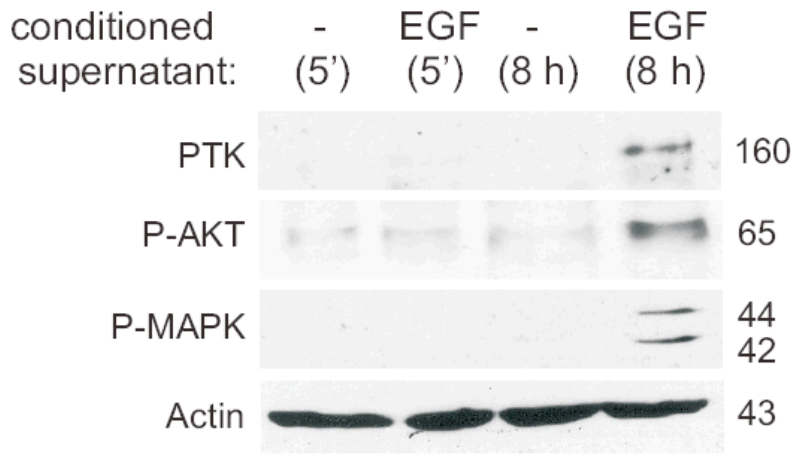
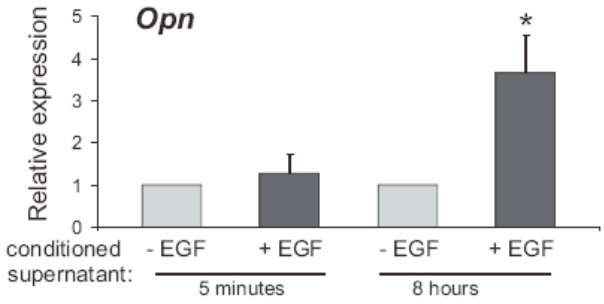

A
*
B
Relative expression
0.79
0.25
0.85
2.72
egfra
egfra*
egfrb
egfrb*
C
Relative expression
3.63
5.67
0.02
0.16
2.20
5.10
0.09
0.78
0.059
0.60
0.75
0.39
1.42
3.94
egf
egf*
tgfa
tgfa*
areg
areg*
btc
btc*
ereg
ereg*
hbegf
hbegf*
epgn
epgn*
D
Relative expression
1.72
2.38
1.42
2.54
adam10
adam10*
adam17
adam17*

Figure 4

A


conditioned supernatant:
-
(5')
EGF
(5')
-
(8 h)
EGF
(8 h)
PTK
160
P-AKT
65
P-MAPK
44
42
Actin
43

B


Opn
Relative expression
0
1
2
3
4
5
*
conditioned supernatant:
- EGF
+ EGF
- EGF
+ EGF
5 minutes
8 hours

C

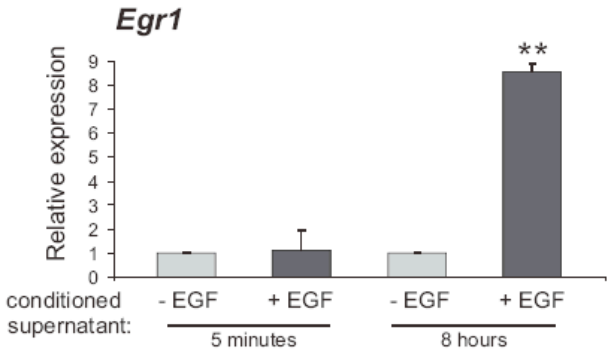
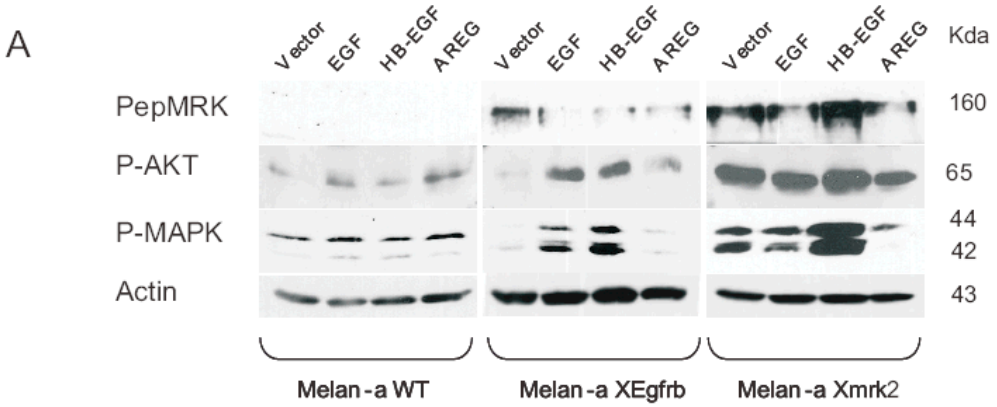
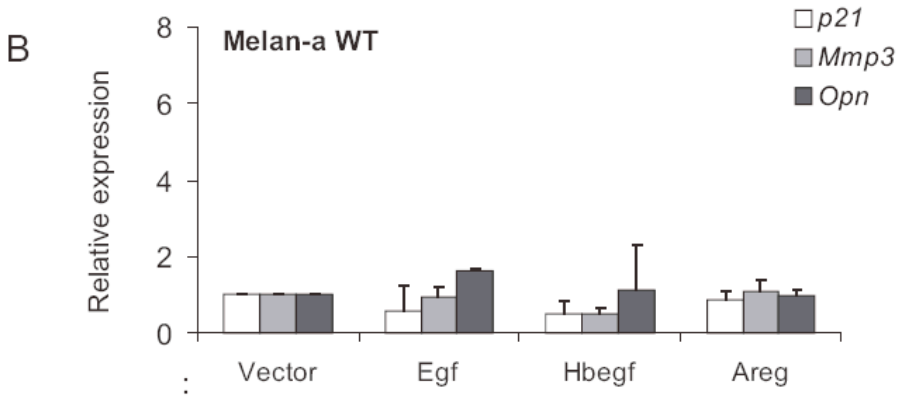
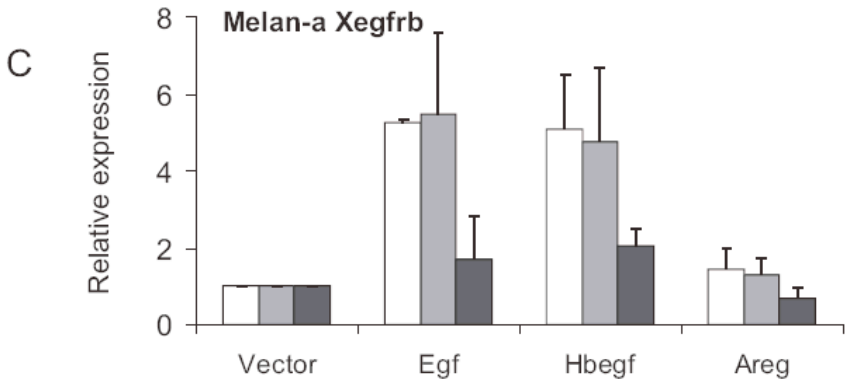

Egr1
Relative expression
0
1
2
3
4
5
6
7
8
9
**
conditioned supernatant:
- EGF
+ EGF
- EGF
+ EGF
5 minutes
8 hours

Figure 5


A
Vector
EGF
HB-EGF
AREG
Vector
EGF
HB-EGF
AREG
Vector
EGF
HB-EGF
AREG
Kda
PepMRK
P-AKT
P-MAPK
Actin
160
65
44
42
43
Melan-a WT
Melan-a XEgfrb
Melan-a Xmrk2


B
Melan-a WT
p21
Mmp3
Opn
Relative expression
8
6
4
2
0
Vector
Egf
Hbegf
Areg


C
Melan-a Xegfrb
Relative expression
8
6
4
2
0
Vector
Egf
Hbegf
Areg

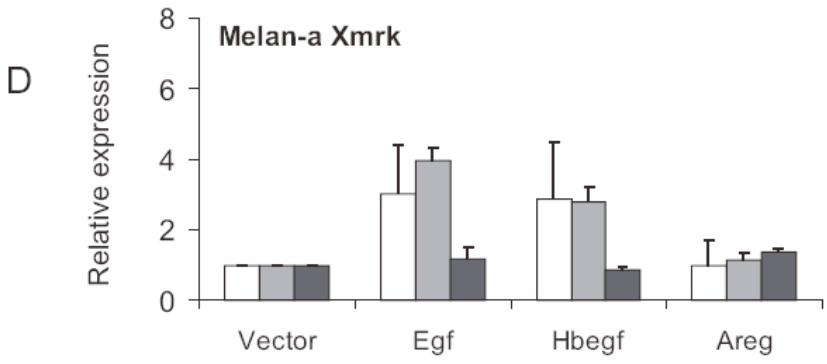
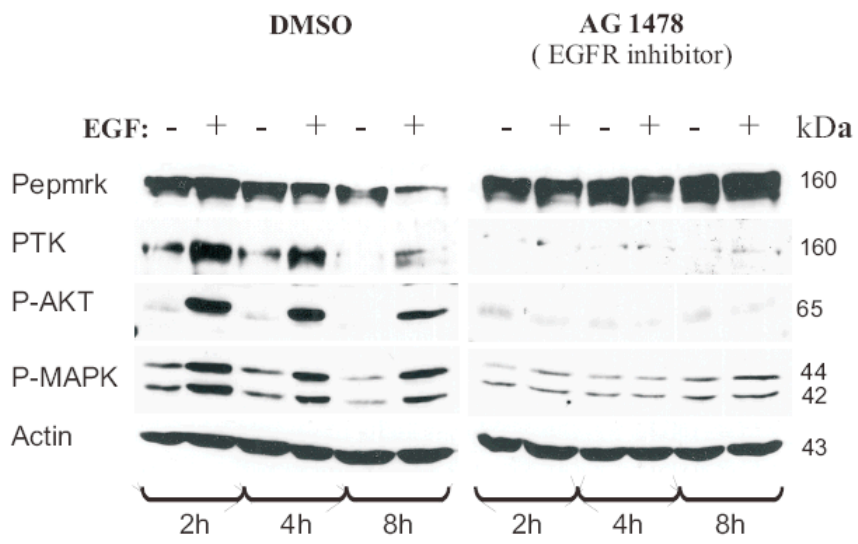
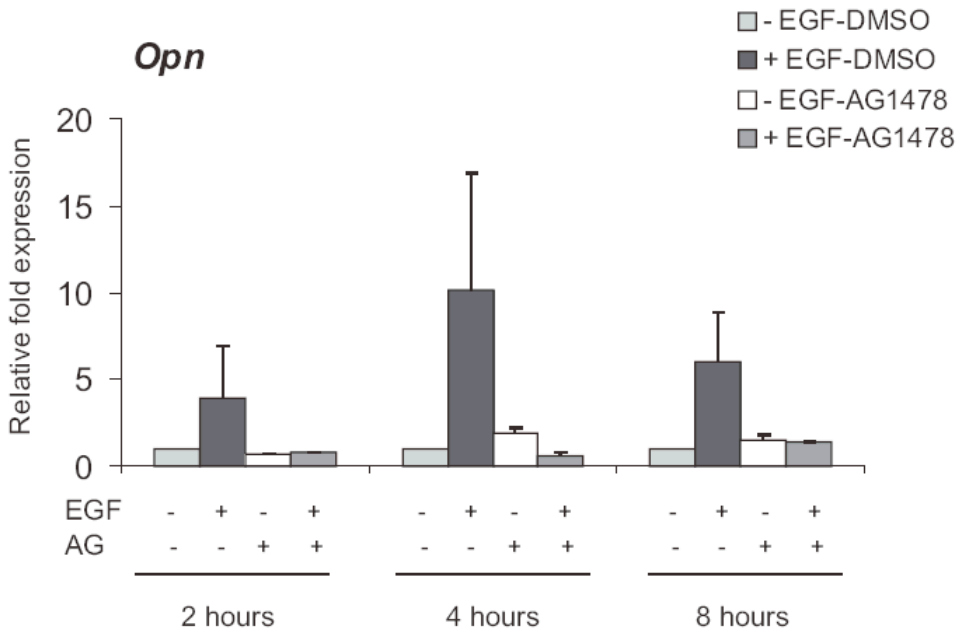

D
Melan-a Xmrk
Relative expression
8
6
4
2
0
Vector
Egf
Hbegf
Areg

Figure S1

A


DMSO
AG 1478
(EGFR inhibitor)
EGF: - + - + - + - + - + - +
kDa
Pepmrk
PTK
P-AKT
P-MAPK
Actin
160
160
65
44
42
43
2h
4h
8h
2h
4h
8h

B


Opn
- EGF-DMSO
+ EGF-DMSO
- EGF-AG1478
+ EGF-AG1478
Relative fold expression
20
15
10
5
0
EGF - + - + - + - + - + - +
AG - - + + - - + + - - + +
2 hours
4 hours
8 hours