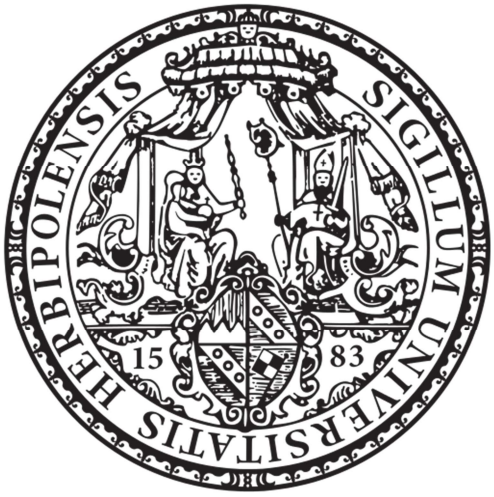

Transcriptional regulation of cancer genes in the *Xiphophorus* melanoma system

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Janine Regneri

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Würzburg, Juni 2013

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Janine Regneri

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Abstract

The *Xiphophorus* melanoma system is a useful animal model for the study of the genetic basis of tumor formation. The development of hereditary melanomas in interspecific hybrids of *Xiphophorus* is connected to pigment cell specific overexpression of the mutationally activated receptor tyrosine kinase Xmrk. In purebred fish the oncogenic function of *xmrk* is suppressed by the molecularly still unidentified locus *R*. The *xmrk* oncogene was generated by a gene duplication event from the *Xiphophorus egfrb* gene and thereby has acquired a new 5' regulatory sequence, which has probably altered the transcriptional control of the oncogene. So far, the *xmrk* promoter region was still poorly characterized and the molecular mechanism by which *R* controls *xmrk*-induced melanoma formation in *Xiphophorus* still remained to be elucidated.

To test the hypothesis that *R* controls melanoma development in *Xiphophorus* on the transcriptional level, the first aim of the thesis was to gain a deeper insight into the transcriptional regulation of the *xmrk* oncogene. To this end, a quantitative analysis of *xmrk* transcript levels in different *Xiphophorus* genotypes carrying either the highly tumorigenic $xmrk^B$ or the non-tumorigenic $xmrk^A$ allele was performed. I was able to demonstrate that expression of the tumorigenic $xmrk^B$ allele is strongly increased in malignant melanomas of *R*-free backcross hybrids compared to benign lesions, macromelanophore spots, and healthy skin. The expression level of the non-tumorigenic $xmrk^A$ allele, in contrast, is not influenced by the presence or absence of *R*. These findings strongly indicate that differential transcriptional regulation of the *xmrk* promoter triggers the tumorigenic potential of these *xmrk* alleles.

To functionally characterize the *xmrk* promoter region, I established a luciferase assay using BAC clones containing the genomic regions where *xmrk* and *egfrb* are located for generation of reporter constructs. This approach showed for the first time a melanoma cell specific transcriptional activation of $xmrk^B$ by its flanking regions, thereby providing the first functional evidence that the *xmrk* oncogene is controlled by a pigment cell specific promoter region. Subsequent analysis of different deletion constructs of the $xmrk^B$ BAC reporter construct strongly indicated that the regulatory elements responsible for the tumor-inducing overexpression of $xmrk^B$ in melanoma cells are located within 67 kb upstream of the *xmrk* oncogene. Taken together, these data indicate that melanoma formation in *Xiphophorus* is

regulated by a tight transcriptional control of the *xmrk* oncogene and that the *R* locus acts through this mechanism.

As the identification of the *R*-encoded gene(s) is necessary to fully understand how melanoma formation in *Xiphophorus* is regulated, I furthermore searched for alternative *R* candidate genes in this study. To this end, three genes, which are located in the genomic region where *R* has been mapped, were evaluated for their potential to be a crucial constituent of the regulator locus *R*. Among these genes, I identified *pdcd4a*, the ortholog of the human tumor suppressor gene *PDCD4*, as promising new candidate, because this gene showed the expression pattern expected from the crucial tumor suppressor gene encoded at the *R* locus.

Zusammenfassung

Fische der Gattung *Xiphophorus* sind ein gut etabliertes Modellsystem zur Analyse der genetischen Grundlagen der Tumorentwicklung. Die Entwicklung hereditärer Melanome in bestimmten interspezifischen *Xiphophorus*-Hybriden wird durch die pigmentzellspezifische Überexpression des Onkogens *xmrk* ausgelöst. Dieses Gen codiert für eine durch Mutationen aktivierte Rezeptortyrosinkinase. In den reinerbigen Elterntieren wird die onkogene Funktion von *xmrk* durch den Regulator-Locus *R* unterdrückt, welcher jedoch auf molekularer Ebene noch nicht identifiziert wurde. Das Onkogen *xmrk* ist durch eine Genduplikation aus dem Protoonkogen *egfrb* entstanden und hat dabei eine neue regulatorische 5‘ Region erhalten, welche mit hoher Wahrscheinlichkeit die transkriptionelle Regulation des Onkogens verändert hat. Die Promotorregion von *xmr*k war allerdings bisher nur unzureichend charakterisiert und der molekulare Mechanismus, durch den der *R*-Locus die *xmrk*-induzierte Melanomentwicklung kontrolliert, war noch weitgehend unbekannt.

Um zu analysieren, ob der *R*-Locus die Melanomentwicklung in *Xiphophorus* auf transkriptioneller Ebene kontrolliert, war das erste Ziel dieser Arbeit die transkriptionelle Regulation des *xmrk* Onkogens genauer zu untersuchen. Zu diesem Zweck habe ich eine quantitative Analyse der *xmrk* Expressionslevel in Geweben verschiedener *Xiphophorus*-Genotypen durchgeführt, welche entweder das stark tumorigene $xmrk^B$ oder das nicht-tumorigene $xmrk^A$ Allel besitzen. Ich konnte zeigen, dass im Vergleich zu benignen Läsionen, Macromelanophoren und gesunder Haut, die Expression des tumorigenen $xmrk^B$ Allels in den malignen Melanomen der *R*-defizienten Rückkreuzungshybride stark erhöht ist. Das Expressionslevel des $xmrk^A$ Allels wird hingegen nicht durch den *R*-Locus beeinflusst. Dieses Ergebnis deutet darauf hin, dass eine differenzielle transkriptionelle Regulierung des *xmrk* Promotors für die Unterschiede im onkogenen Potential dieser Allele verantwortlich ist.

Um die *xmrk* Promotorregion funktional zu charakterisieren, habe ich in der hier vorliegenden Studie einen Luciferase-Assay etabliert, für den BAC-Klone, welche die *xmrk*- oder *egfrb*-Region enthalten, zur Herstellung von Reporterkonstrukten verwendet wurden. Mit Hilfe dieses Ansatzes konnte ich zum ersten Mal eine melanomzellspezifische Aktivierung des $xmrk^B$ Gens durch seine regulatorischen Regionen zeigen. Dies liefert den ersten funktionalen Beweis, dass das *xmrk* Onkogen tatsächlich durch einen pigmentzellspezifischen Promotor kontrolliert wird. Durch die nachfolgende Analyse einer Deletionsserie des $xmrk^B$ Reporterkonstrukts konnte gezeigt werden, dass die regulatorischen Elemente, welche die

starke Überexpression von *xmrk* in Melanomzellen steuern, in den proximalen 67 kb der *xmrk* 5‘ Region lokalisiert sind. Zusammengefasst deuten diese Ergebnisse darauf hin, dass die Melanomentwicklung in *Xiphophorus* durch eine strikte transkriptionelle Kontrolle des *xmrk* Onkogens reguliert wird und dass der Regulator-Locus *R* seine tumorsuppressive Funktion über diesen Mechanismus ausübt.

Da die Identifizierung des *R*-Locus-Gens entscheidend ist, um die Melanomentwicklung in *Xiphophorus* vollständig zu verstehen, habe ich im zweiten Teil dieser Arbeit drei Gene, welche in derselben genomischen Region liegen in der *R* lokalisiert wurde, genauer untersucht, um zu testen, ob es sich bei einem dieser Gene um eine entscheidende tumorsuppressive Komponente des *R*-Locus handelt. Von diesen Genen wurde *pdcd4a*, welches das Ortholog zum humanen Tumorsuppressorgen *PDCD4* ist, als vielversprechendes neues Kandidatengen identifiziert, da das Expressionsmuster von *pdcd4a* mit dem zu erwartenden Expressionsmuster des am *R*-Locus codierten Tumorsuppressorgens übereinstimmt.

Introduction

Cutaneous malignant melanoma (CMM) is a highly aggressive tumor that arises from the malignant transformation of melanocytes in the skin. Malignant melanoma is the deadliest form of skin cancer with rapidly rising incidence rates worldwide (Houghton and Polsky, 2002; Linos et al., 2009; MacKie et al., 2009). Once metastasized, CMM becomes highly resistant to current therapies (Garbe et al., 2011; Lee et al., 2012). A better understanding of the genetic and environmental factors involved in the complex process of melanomagenesis is therefore essential to optimize melanoma diagnosis and treatment in the future. Animal models, where melanoma development is initiated by clearly defined genetic events, are invaluable tools to study the molecular processes that make a normal pigment cell become a fully malignant cancer cell. One of the oldest animal models for studying the genetic basis of melanoma is the *Xiphophorus* fish model, in which spontaneous melanoma formation can be initiated by standardized crossing procedures that guarantee the development of an extremely uniform tumor with respect to molecular and pathological features.

The *Xiphophorus* melanoma model

In the late 1920s it was discovered that hybrids between certain strains of platyfish (*X. maculatus*) and swordtails (*X. hellerii*) spontaneously develop a highly malignant pigment cell tumor classified as melanoma (Gordon-Kosswig-Anders cross) (Fig. 1) (Gordon, 1927; Häussler, 1928; Kosswig, 1928). These hybrid melanomas originate from giant melanophores, so-called macromelanophores, which form distinct pigment patterns in parental *X. maculatus* fish. Macromelanophores are huge nevus-like cells (300-500 µm in diameter) that often have multiple nuclei and tend to overgrow each other. In contrast to micromelanophores, which produce the grayish wildtype pigmentation of the fish, macromelanophores are present only in some *Xiphophorus* species, where they give rise to heritable spot patterns (Borowsky, 1984; Kallman, 1975).

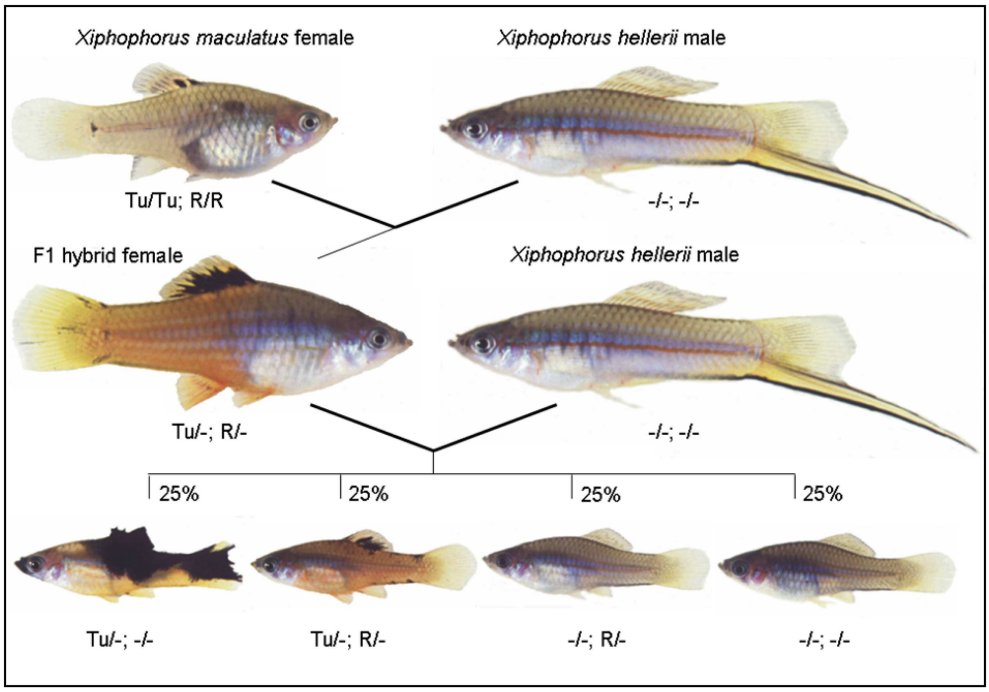


Figure 1: Classical crossing experiment (Gordon-Kosswig-Anders cross) leading to spontaneous formation of benign hyperpigmentation and malignant melanoma in interspecific backcross hybrids. Female platyfish (*X. maculatus*) harboring the sex-chromosomal *Tu* and the autosomal *R* locus are crossed with swordtail males (*X. hellerii*) that are devoid of both loci. The F1 offspring, which is heterozygous for *Tu* and *R*, is backcrossed to *X. hellerii* males resulting in segregation of the *Tu* and the *R* locus. 25% of the backcross segregants develop malignant melanoma caused by the presence of *Tu* in the absence of *R*, 25% develop a benign hyperpigmentation like the F1 animals and 50% are healthy fish without changes in pigmentation. The female platyfish (strain Jp163A) exhibits distinct macromelanophore spots in the dorsal fin (spotted dorsal (Sd) pigment pattern). (modified from (Meierjohann and Schartl, 2006))

Based on many elegant classical crossing experiments, a genetic model for spontaneous melanoma development in *Xiphophorus* hybrids has been proposed (Ahuja and Anders, 1976; Anders, 1967, 1991; Schartl, 1995). According to this model, melanoma formation in *Xiphophorus* is induced by generating a regulatory imbalance between a dominantly acting, pigment cell specific oncogene locus called *Tu* (for "tumor") and a *Tu*-suppressing regulatory locus called *R* (for "regulator") (Fig. 1). Because *Tu* and *R* are located on different chromosomes, they can be separated by selective breeding using a crossing partner that is devoid of *Tu* and furthermore lacks the *R* locus in general or has a "nonfunctional" *R* (meaning not properly interacting with *Tu*). In fish of parental *Xiphophorus* species that harbor the *Tu* locus (e.g. *X. maculatus*), the oncogenic function of *Tu* is repressed by the *R* locus, which acts as a tumor suppressor. In this genetic constitution, the restricted activity of *Tu* is visible as limited local overproduction of macromelanophores that form distinct pigment spots, for instance in the dorsal fin (Fig. 1). Crossing of *X. maculatus* with fish of a *Xiphophorus* species containing neither of the two loci (e.g. *X. hellerii*) produces offspring that are heterozygous for both *Tu* and *R*. These F1 hybrids develop a locally restricted and benign hyperproliferation of melanophores, which can be explained by the partly relieved

activity of *Tu* due to loss of one *R* allele. Using *X. hellerii* as the recurrent parent for backcrossing of the F1 animals produces 25% offspring heterozygous for *Tu*, but lacking the *R* locus. In these fish, the *R*-mediated suppression of *Tu* is totally abolished, which results in the formation of a fast growing and highly invasive melanoma arising from the macromelanophores. Backcross hybrids that have inherited *Tu* and only one copy of *R* are phenotypically like the F1 animals (for review see (Meierjohann and Schartl, 2006; Patton et al., 2010)).

The *xmrk* oncogene

Positional cloning has revealed that the *Tu* locus contains an oncogenic receptor tyrosine kinase belonging to the epidermal growth factor receptor family (Wittbrodt et al., 1989). This gene, termed *xmrk* (for *Xiphophorus* melanoma receptor kinase), was generated by a local event of gene duplication from the preexisting proto-oncogene *egfrb*, which is one of the two fish co-orthologs of the human EGFR (Adam et al., 1993; Volff et al., 2003). The evidence that *xmrk* is the tumor-determining gene of the *Tu* locus comes from the observation that disruption of this gene, either by deletion or insertion of a transposable element, leads to loss of function with respect to melanoma formation (Schartl et al., 1999; Wittbrodt et al., 1989).

Activating mutations in the Xmrk receptor

The *xmrk* and *egfrb* coding sequences are still highly similar and differ only by a number of nucleotide exchanges that have accumulated since the duplication event (Fig. S1). Depending on the *xmrk* and *egfrb* allele, these exchanges lead to approximately 19 amino acid substitutions between the mature Xmrk and Egfrb proteins (Fig. S2) (Dimitrijevic et al., 1998). The high tumorigenic potential of Xmrk compared to Egfrb results from two amino acid exchanges (G336R and C555S) in the extracellular domain of the growth factor receptor. Both mutations independently interfere with the formation of stabilizing intramolecular disulfide bridges, thereby leading to free cysteine residues in the extracellular part of the Xmrk receptor monomer. If two of these receptor monomers with unbound cysteines come closely together, intermolecular disulfide bridges are formed (Gomez et al., 2001). The resulting dimer mimics the structure of a ligand-bound wildtype receptor and is sufficient for cross-transactivation of the intracellular kinase domains, resulting in constitutive autophosphorylation of Xmrk (Meierjohann et al., 2006a). The ligand-independent activity of Xmrk causes a permanent activation of the intracellular signal transduction network, which

triggers a variety of cellular responses that determine the full neoplastic phenotype of the cell. That the mere presence of Xmrk alone is sufficient to induce tumor development has been demonstrated by gene transfer experiments. Ectopic expression of *xmrk* in transgenic medakas (*Oryzias latipes*), which are normally not cancer-prone, induces tumor formation with high frequency and after short latency periods (Schartl et al., 2009; Winkler et al., 1994).

Oncogenic signaling of Xmrk

The main signal transduction pathways involved in mediating Xmrk-induced proliferation, dedifferentiation, anti-apoptosis, cell mobility, and tumor angiogenesis have already been identified. Activation of the transcription factor Stat5 by Xmrk promotes proliferation and helps to suppress apoptosis by increasing the amount of the anti-apoptotic protein Bcl-x (Baudler et al., 1999; Morcinek et al., 2002). Binding of the adapter protein Grb2 to Xmrk initiates activation of the Ras/Raf/Erk1/2 pathway. Activated Erk1/2 inhibits pigment cell differentiation by reducing the stability of the pigment cell specific transcription factor Mitf (Delfgaauw et al., 2003; Wellbrock et al., 2002). Furthermore, activated Erk1/2 induces transcription of many Xmrk-induced target genes, among them *osteopontin (opn)* and *matrix metalloproteases* (*mmp*s). The secreted protein Opn binds to integrins on the cell surface, thereby protecting the cell from apoptosis, which enables survival of transformed melanocytes at ectopic sites (Geissinger et al., 2002). Activation of Mmps in Xmrk-expressing melanocytes is required for an efficient passage through the cell cycle and thus has a pro-proliferative effect (Meierjohann et al., 2010; Meierjohann et al., 2006b). The Src kinase family member Fyn becomes directly activated by Xmrk (Wellbrock and Schartl, 1999). By inhibiting Mkp-1, Fyn is involved in maintaining activated Erk1/2 levels and thus enhances the above mentioned Erk1/2-mediated effects (Wellbrock et al., 2002). Moreover, Fyn interacts with Fak, which results in an increased turnover of focal adhesions and finally leads to Xmrk-induced migration (Meierjohann et al., 2006b). In addition, Fyn forms a complex with Xmrk and phosphoinositide-3 kinase (Pi3k) to activate the Pi3k/Akt pathway, which also promotes proliferation (Wellbrock et al., 1999; Wellbrock and Schartl, 2000). Moreover, Xmrk induces tumor angiogenesis by ROS-driven activation of NF-κB, which leads to secretion of pro-angiogenic factors, most prominently angiogenin (Schaafhausen et al., 2013, Journal of Cell Science, in press). In summary, Xmrk is able to induce all essential steps in tumor development.

Control of *xmrk* expression

Shortly after identification of *xmrk* as the oncogenic determinant encoded at the *Tu* locus, numerous studies have demonstrated that besides activating mutations in the Xmrk receptor, the second precondition for Xmrk-induced melanoma development is transcriptional activation of *xmrk* in pigment cells. Several Northern blot or semi-quantitative RT-PCR based expression studies revealed that the *xmrk* oncogene is overexpressed exclusively in malignant melanomas of backcross hybrids, whereas lower transcript levels were found in benign precursor lesions (Adam et al., 1991; Mäueler et al., 1993; Schartl et al., 1999). In normal tissues and organs of tumor-bearing backcross hybrids and parental *X. maculatus*, low *xmrk* mRNA levels were detected in several, but not all tissues (Schartl et al., 1999; Woolcock et al., 1994). The proto-oncogene *egfrb*, in contrast, was transcribed at low levels in most organs (Schartl et al., 1999; Woolcock et al., 1994). These findings indicated that transcriptional activation of *xmrk* is restricted to the black pigment cell lineage of *R*-free backcross hybrids and furthermore, linked melanoma development to overexpression of the *xmrk* oncogene. Based on these data, it has been postulated that a release of the transcriptional control of *xmrk* in the melanocyte lineage in the absence of *R* is the initiating event in melanoma formation and consequently, that the *R* locus exerts its tumor suppressor function by directly or indirectly controlling *xmrk* expression on the transcriptional level.

The altered transcriptional regulation of *xmrk* compared to *egfrb* is proposed to be due to a new 5' upstream flanking region, which the oncogene has acquired after the duplication event. Apart from a 460 bp fragment with more than 97% nucleotide identity (Volff et al., 2003), the putative promoter regions of *xmrk* and *egfrb* are completely different from each other. After the duplication a single copy of a repetitive DNA element called *D* locus (*D* for "donor") was integrated directly upstream of the transcriptional start site of *xmrk* (Adam et al., 1993; Förnzler et al., 1996; Nanda et al., 1996). Interestingly, the new *D* locus-derived upstream sequence contains TATA- and CAAT-like sequences at the expected distance from the transcriptional start site of *xmrk* (Adam et al., 1993). Moreover, the *xmrk* oncogene is located in an extremely unstable genomic region, where it is surrounded by numerous repetitive sequences, particularly by a multitude of different retroelements. In addition, several pseudogenes were found in this region (Froschauer et al., 2001; Nanda et al., 2000; Volff et al., 2003). Thus, it is possible that these newly acquired DNA elements upstream of *xmrk* contain transcriptional control elements contributing to the altered transcriptional regulation of the *xmrk* oncogene.

The massive overexpression of *xmrk* exclusively in melanoma tissues of backcross hybrids strongly indicates the presence of pigment cell specific regulatory elements in the *xmrk* promoter region. Further evidence for the melanocyte specificity of the *xmrk* promoter comes from transgenic experiments showing that *xmrk,* when expressed under control of a ubiquitously active promoter in embryos of the closely related medakafish, is able to transform a variety of cell types other than melanocytes (Winkler et al., 1994; Winnemoeller et al., 2005). Hence, the restriction to melanoma in *Xiphophorus* backcross hybrids is apparently due to the fact that the *xmrk* promoter region drives high level expression of *xmrk* exclusively in pigment cells. The functional analysis of a 0.7 kb *xmrk* promoter fragment revealed the presence of an activating GC box element identical to the binding site described for the human Sp1 close to the transcriptional start site of the oncogene. This element mediated high level transcriptional activation in *Xiphophorus* cell lines, but the 0.7 kb fragment showed no melanoma cell specificity in reporter gene assays (Baudler et al., 1997). Subsequent attempts to identify pigment cell specific promoter elements by comparing reporter gene expression in melanoma and non-melanoma cell lines using up to 4.5 kb *xmrk* 5' flanking sequence also showed no melanoma cell specific regulation (unpublished data, Lisa Osterloh), strongly suggesting the existence of additional regulatory elements outside the region so far analyzed. In addition to specific transcription factor binding sites, epigenetic mechanisms may regulate transcriptional activation of *xmrk.* Interestingly, it has been shown that the *xmrk* promoter is highly methylated at CpG dinucleotides in non-transformed tissues, but hypomethylated in melanoma cell lines and tissues, whereas the situation for the proto-oncogene *egfrb* is the other way around (Altschmied et al., 1997). This DNA methylation pattern is consistent with overexpression of *xmrk* exclusively in melanocytes of backcross hybrids, thereby suggesting an essential role of aberrant DNA methylation in transcriptional deregulation of the *xmrk* oncogene.

Genomic organization of the *Tu* locus

The *xmrk* oncogene and its proto-oncogene *egfrb* are located in the same subtelomeric region of the X and Y sex chromosomes. The physical distance between the two genes is at least one megabase, with *xmrk* being located closer to the telomere (Nanda et al., 2000; Schultheis et al., 2006). Other known sex-chromosomal loci located in this region are the sex-determining locus *SD*, the red/yellow pigment pattern locus *RY*, which determines the yellow, orange, and red xanto-/erythrophore pigment pattern of the fish, and the puberty locus *P*, which influences the onset of sexual maturation (Kallman, 1975; Kallman, 1984; Kallman, 1989). These loci, as well as the proto-oncogene *egfrb*, map 3' to *xmrk* (Gutbrod and Schartl, 1999). An important pigment pattern locus, which is closely linked to *xmrk*, is the so-called macromelanophore-determining locus (*mdl*). The *mdl* locus maps 5' to *xmrk*, but is not molecularly characterized so far (Gutbrod and Schartl, 1999; Weis and Schartl, 1998). As mentioned earlier, macromelanophores, which are considered to be the sites of *xmrk* overexpression and the origin of the melanoma (Meierjohann et al., 2004; Woolcock et al., 1994), produce typical spot patterns in many, but not all *Xiphophorus* species. These macromelanophore patterns are encoded by a series of codominant alleles of the *mdl* locus (Borowski, 1984; Kallman, 1975). Hence, the classical genetically defined *Tu* locus would consist of *mdl*, which determines macromelanophore development in different body compartments and the pattern phenotype, plus *xmrk*, which contributes the capacity of neoplastic transformation to the macromelanophore lineage (Weis and Schartl, 1998). As a consequence, a specific melanomagenic locus is defined by the combination of the *mdl* and the *xmrk* allele (*mdl-xmrk*). Many of these *mdl-xmrk* allele combinations exist and each gives rise to a particular pigment pattern. Interestingly, apart from the location of melanoma development, these allele combinations also differ strikingly in the temporal onset of melanoma formation and in the malignancy of the tumor. There even exist *mdl-xmrk* allele combinations that only lead to a mild melanosis or hyperpigmentation in hybrid crosses and are therefore considered as non-tumorigenic (Gutbrod and Schartl, 1999; Weis and Schartl, 1998). However, thus far it has not been determined whether these differences in the tumorigenic potential of the *mdl-xmrk* alleles are attributed to *mdl*, to differences in the region controlling *xmrk* transcription, or to structural differences in the Xmrk receptor due to differences in the coding sequence. Since *mdl* may determine, at least to a certain extent, the severity of the melanoma, it can be considered as cis-acting tumor modifier locus.

Different *mdl-xmrk* allele combinations

To date, two *xmrk* allele classes have been identified in *X. maculatus*. Class A *xmrk* alleles ($xmrk^A$) encode a full-length Egf-receptor, while class B alleles ($xmrk^B$) have a deletion that includes exon 25 and the 5' part of exon 26 and results in a protein with 35 amino acids missing from the cytoplasmic domain (Fig. S1, S2) (Adam et al., 1991; Schartl and Meierjohann, 2010). Apart from this deletion, the two Xmrk protein classes differ only by single amino acid exchanges. Besides differences in the coding sequence, sequence variations in the 5' and 3' flanking regions of the different *mdl-xmrk* allele combinations were identified (Volff et al., 2003). The *X. maculatus* strain Jp163A (origin Rio Jamapa) carries two different *mdl-xmrk* alleles, one on the X and one on the Y chromosome. The X-chromosomal mdl^{Sd}-$xmrk^B$ allele (Fig. 2a), which is equivalent to the well-known spotted dorsal locus (spotted dorsal, Sd) from the classical Gordon-Kosswig-Anders cross, is highly tumorigenic and leads to development of malignant and invasive melanomas in backcross hybrids. The Y-chromosomal mdl^{Sr}-$xmrk^A$ allele (striped, Sr) (Fig. 2b), in contrast, is not melanomagenic and leads only to a very mild hyperpigmentation in hybrid crosses. Interestingly, the non-tumorigenic allele carries an insertion (*piggyBac-like* DNA transposon) in the *D* locus sequence upstream of the transcriptional start site of *xmrk*, which is not present in the putative promoter region of the tumorigenic mdl^{Sd}-$xmrk^B$ allele (Volff et al., 2003). It is therefore reasonable to assume that this insertion might have altered the transcriptional regulation of the mdl^{Sr}-$xmrk^A$ allele. However, until now supporting mdl^{Sr}-$xmrk^A$ expression data are missing. As yet, it is not known which of these sequence variations determines the tumorigenic potential of the *mdl-xmrk* allele combinations.

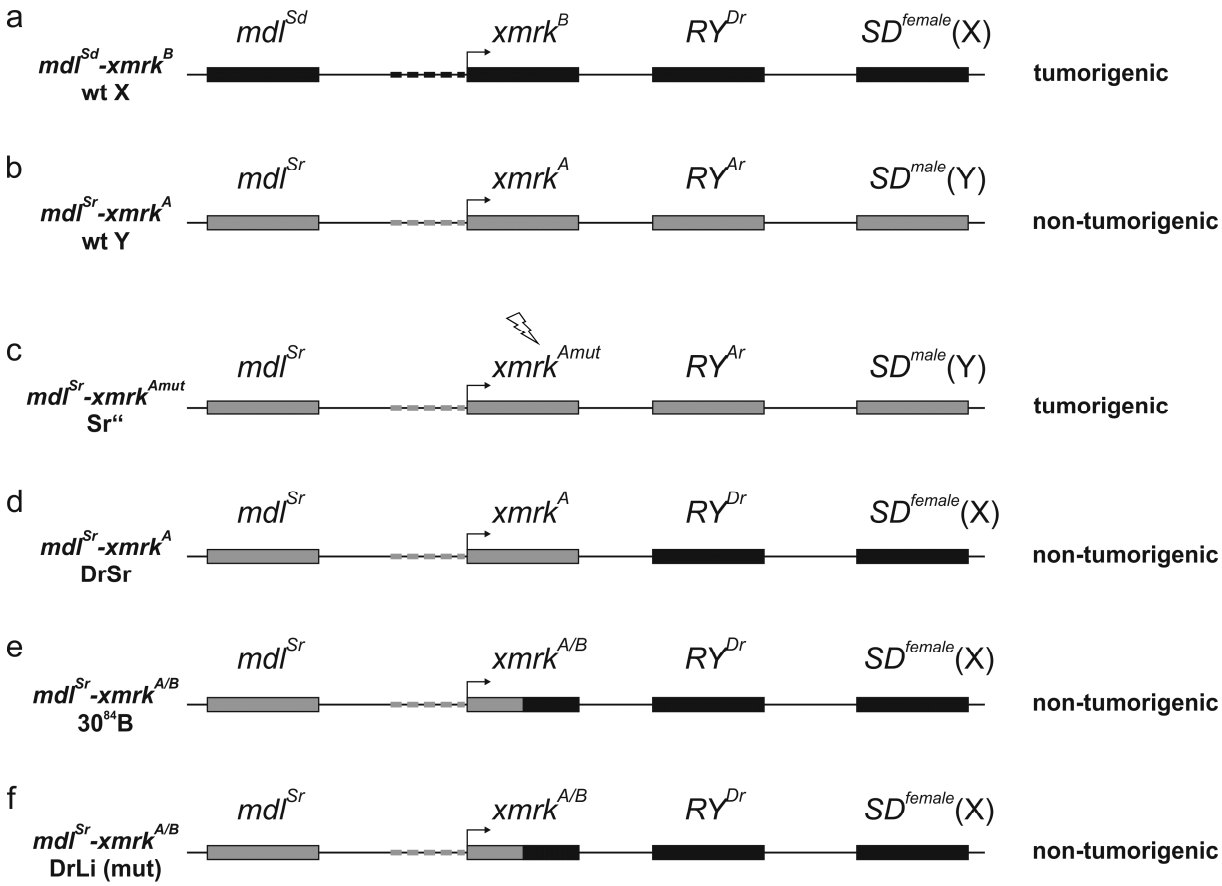


Figure 2: Genomic organization and allele structure of the wildtype X- and Y-chromosomal *mdl* and *xmrk* alleles of *X. maculatus* (Rio Jamapa, Jp163A) and of different mutant alleles. The closely linked red/yellow pigment pattern (*RY*) locus and the sex-determining (*SD*) locus are also shown. The X-chromosomal mdl^{Sd}-$xmrk^{B}$ wt allele (a) is highly tumorigenic, while the Y-chromosomal mdl^{Sr}-$xmrk^{A}$ wt allele (b) is not melanomagenic. The mutant Sr'' locus (c) is highly tumorigenic. This allele carries a so far unidentified X-ray induced mutation in the *xmrk* gene (indicated by flash). The DrSr (d) and 30^{84}B (e) mutant chromosomes were generated by X/Y sex-chromosomal crossovers. For DrSr the crossover site is located downstream of *xmrk*, whereas the crossover site of the 30^{84}B mutant chromosome is located within the *xmrk* gene. The resulting *mdl-xmrk* alleles are not melanomagenic. (f) DrLi (mut) originates from a crossover of the DrLi chromosome with the Sr'' chromosome. This allele is also not tumorigenic. (modified from (Schartl and Meierjohann, 2010))

The *R* locus

The autosomal locus *R*, which maps to linkage group V (Ahuja et al., 1980; Förnzler et al., 1991; Morizot and Siciliano, 1983), is responsible for suppressing *xmrk*-driven melanoma formation in purebred *Xiphophorus* fish. However, contrary to the *Tu* locus, where it was established that its critical constituent is the *xmrk* oncogene, the molecular identity of the *R* locus-encoded gene(s) has not been defined so far. The genetic data from the classical crossing experiments did not reveal if – on the molecular level – *R* acts up- or downstream of *xmrk*. Thus, *R* could either be involved in transcriptional regulation of *xmrk* or have an influence on Xmrk downstream signaling.

Until now, only one candidate gene for *R* has been postulated and analyzed further, namely the potential tumor suppressor gene *cdkn2ab*. *cdkn2ab* is a very attractive candidate for *R* because this gene showed the best association with the *R*–dependent melanoma phenotype in genetic linkage studies (Kazianis et al., 1998). Furthermore, the human orthologs of this gene, *CDKN2A* and *CDKN2B*, are well characterized tumor suppressors in human melanoma. As the *CDKN2A* and *B* gene products mediate their tumor-suppressing action by negatively regulating the cell cycle, this would place the tumor-suppressing activity of *R* downstream of *xmrk*. *CDKN2A* encodes two distinct proteins, $p16^{INK4a}$ and $p14^{ARF}$. Up to 40% of familial melanoma cases harbor an inactivating mutation in *CDKN2A* and furthermore, *CDKN2A* is inactivated by somatic mutations, deletions, or promoter methylations in the majority of sporadic melanomas as well. $p15^{INK4b}$, which is transcribed from *CDKN2B*, is deleted in up to 70% of melanoma cases. So far, no germline mutations of *CDKN2B* have been reported in melanoma, but somatic point mutations were described in patients with sporadic melanoma (reviewed in (Ibrahim and Haluska, 2009; van den Hurk et al., 2012)). However, contrary to the situation found in human melanoma, the *cdkn2ab* promoter region is basically unmethylated in *Xiphophorus* melanomas and *cdkn2ab* mRNA expression levels are increased in melanoma tissues compared to skin (Kazianis et al., 2000). Butler and colleagues even demonstrated that the abundance of *cdkn2ab* transcripts is positively correlated with the level of *xmrk* expression and the malignancy of the melanoma (Butler et al., 2007b). Such an expression pattern was totally unexpected for a putative tumor suppressor gene. The Cdkn2ab proteins from *X. hellerii* and *X. maculatus* differ only at two positions, a conservative amino acid exchange at position 3 (Leu/Val) and a non-conservative exchange at position 90 (Glu/Lys) (Kazianis et al., 1999). However, neither of the two mutations has been predicted to affect binding of the protein to Cdk4 and Cdk6 (Byeon et al., 1998; Russo et al., 1998) or has

been implicated in melanoma development in human (Becker et al., 2001; Harland et al., 1997; Monzon et al., 1998), suggesting that these exchanges do not represent inactivating mutations. Moreover, several sequence differences in the putative *cdkn2ab* promoter region were found between the *X. hellerii* and the *X. maculatus* allele (Kazianis et al., 1999). However, as yet it has not been determined whether these sequence differences play a role for melanoma development in *Xiphophorus*. The absence of inactivating mutations, in combination with the overexpression of *cdkn2ab* in melanomas, speaks against a potential tumor-suppressing activity of this gene in the *Xiphophorus* melanoma model, thereby indicating that *cdkn2ab* might not be the crucial *R* locus-encoded gene. Thus, there is still some reason to search for other candidate genes for *R*.

Aim of the thesis

Based on the current knowledge of the *Xiphophorus* melanoma system in general and the *xmrk* oncogene in particular, our working group postulated that melanoma formation in *Xiphophorus* is controlled on the transcriptional level. We hypothesized that the tumor-suppressive effect of the *R* locus on *xmrk* is exerted by directly or indirectly downregulating *xmrk* expression on the transcriptional level. Crossing-dependent loss of *R* would result in a release of the transcriptional control of *xmrk* in melanocytes and the resulting overexpression of the *xmrk* oncogene would consequently be the primary step that initiates melanoma formation. However, as yet the molecular identity of the *R* locus-encoded gene(s) has not been defined and there is no experimental prove that *R* has an influence on transcription of *xmrk*. Furthermore, the *xmrk* promoter region is still poorly characterized and the exact molecular mechanism underlying the melanocyte-restricted overexpression of *xmrk* in the absence of *R* still remains to be elucidated.

To test the hypothesis that *R* controls melanoma development in *Xiphophorus* on the transcriptional level, the first aim of the thesis was to gain a deeper insight into the transcriptional regulation of the *xmrk* oncogene and to identify the transcription factors and regulatory elements involved by performing a quantitative *xmrk* expression pattern analysis and a structural and functional characterization of the *xmrk* promoter region.

The second aim of the thesis was to search for and analyze new candidates for the *R* locus-encoded gene(s).

Materials and methods

Cell culture

The *Xiphophorus* melanoma cell line PSM (Wakamatsu, 1981) and the embryonic epithelial cell line A2 were cultured in F12 medium (Gibco) supplemented with 15% fetal calf serum (FCS) and penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 28°C. The PSM cell line was established from ornamental fish obtained from the aquarium trade, which carried a *Sp-type mdl* allele linked to a class A *xmrk* allele, but the sex-chromosomal origin is unknown (Schartl and Meierjohann, 2010). The A2 cell line is probably derived from the southern swordtail species *X. hellerii* according to new analyses performed during this thesis (Fig. S4, S5). Embryonic epithelial cell lines derived from *X. maculatus* (Sd/Sr24) and *X. hellerii* (XhIII, FOI8) ((Altschmied et al., 2000) and PhD thesis, Maria Pagany) were maintained in DMEM medium (Gibco), 15% FCS in the presence of penicillin/streptomycin at 28°C in a humidified atmosphere with 5% CO_2.

Experimental animals

All fish used in this study were bred under standard conditions (Kallman, 1975) in the aquarium facility of the Biocenter at the University of Würzburg. The following strains were used:

Purebred parental lines:

- WLC 1274: *X. maculatus* (origin Rio Jamapa). This strain is derived from the Jp163A strain (for reference see: The *Xiphophorus* Stock Center Manual, URL http://www.xiphophorus.txstate.edu/stockcenter.html). It is derived from few highly inbred founders but propagated by closed colony breeding rather than brother-sister mating as it is done for Jp163A. Females are homozygous for the X-chromosomal mdl^{Sd}-$xmrk^{B}$ locus (Fig. 2a) and develop macromelanophore spots in the dorsal fin (spotted dorsal pattern, Sd). Males have in addition the Y-chromosomal mdl^{Sr}-$xmrk^{A}$ locus (Fig. 2b) and have a reticulate macromelanophore pattern on the flanks of the body (striped pattern, Sr) (Fig. 3a).

- WLC 1375: *X. maculatus* laboratory strain that was established from a sex-chromosomal crossover, where mdl^{Sr}-$xmrk^{A}$ from the Y chromosome of the Jp163A strain was transferred to the X chromosome (Gutbrod and Schartl, 1999). The crossover occurred within the *xmrk* gene between the 3' end of intron 1 and exon 15. Breeding with the wildtype Y chromosome established a strain where females are homozygous for mdl^{Sr}-$xmrk^{A/B}$ (Fig. 2e) (Fig. 3b) and males are heterozygous for mdl^{Sr}-$xmrk^{A/B}$ and mdl^{Sr}-$xmrk^{A}$. These fish exhibit the wildtype Sr pattern. The strain is also known as the 30^{84}B strain.

- WLC 1750: *X. maculatus* laboratory strain established from a similar sex-chromosomal crossover event as for WLC 1375 that occurred independently in the WLC 1274 strain (Gutbrod and Schartl, 1999). The chromosomal location of the crossover site is downstream of *xmrk* (Fig. 2d). These fish are homozygous for mdl^{Sr}-$xmrk^{A}$ and are phenotypically indistinguishable from WLC 1375. This strain is also known as the DrSr strain.

- WLC 2065: *X. maculatus* (origin Rio Jamapa). These fish have neither *xmrk* nor *mdl* and do not exhibit macromelanophore patterns (Fig. 3c).

- WLC 1337: *X. hellerii* (origin Rio Lancetilla) *Db-2*$^{-}$. These fish have neither *xmrk* nor *mdl* (Weis and Schartl, 1998) and no *R*. The stock is also known as the hIII strain.

- WLC 591: *X. maculatus* laboratory strain established from an X-ray induced mutation of the wildtype mdl^{Sr}-$xmrk^{A}$ allele from the Y chromosome of the Jp163A strain (Anders et al., 1973). The mutant locus, designated Sr" (Fig. 2c), leads to a strongly enhanced Sr pattern in *X. maculatus* (Fig. 3d) and to severe melanosis and melanoma development already in F1 hybrids with *X. hellerii*. The mutation was mapped to a region within intron 1 of *xmrk* or downstream thereof (Gutbrod and Schartl, 1999), but its molecular nature is unknown so far. This *X. maculatus* strain has a W/Y sex determination system.

Hybrids

- WLC 407: Backcross hybrids of *X. maculatus* (WLC 1274) and *X. hellerii* (WLC 1337). Continuous backcrossing of mdl^{Sd}-$xmrk^{B}$ carrying fish using *X. hellerii* as the recurrent parent has been performed for more than 20 generations. This resulted in a *X. hellerii* (WLC 1337) isogenic strain that contains one copy of the mdl^{Sd}-$xmrk^{B}$

region from the *X. maculatus* X chromosome. Half of each backcross generation develops spontaneously melanoma. The severity of melanoma ranges from more benign superficially spreading melanotic hyperpigmentations that are not invasive and show only two-dimensional growth to highly malignant invasive and nodular tumors (Fig. 3e), which are fatal to the fish. Less than 10% of adult fish exhibit the more benign phenotype.

- WLC 534: Backcross hybrids, phenotypically and genotypically like WLC 407 except that these fish are heterozygous at the albino locus (*a*/+). Here, the more benign lesions make up less than 20% of adult fish.

- WLC 1748: Backcross hybrids between *X. maculatus* and *X. hellerii* (WLC 1337) using *X. hellerii* as the recurrent parent for more than 20 backcross generations. These fish are homozygous for the mdl^{Sr}-$xmrk^{A}$ locus. They develop only a slightly enhanced hyperpigmentation of the Sr pattern like wildtype *X. maculatus* (Fig. 3f). Approximately 1% of the fish develop spontaneously at older age malignant melanocytic melanoma (Fig. 3g).

- WLC 2044: Backcross hybrids of *X. maculatus* and *X. hellerii* that are homozygous for the DrLi (mut) locus (Fig. 3h). This mutant locus originates from a crossover between the DrLi (mdl^{Li}-$xmrk^{C/B}$, X-chromosomal) and the Sr'' locus. The resulting DrLi (mut) allele (mdl^{Sr}-$xmrk^{A/B}$, X-chromosomal) (Fig. 2f) produces the wildtype Sr pattern. The location of the crossover site is between the 3' end of intron 1 and exon 15 of *xmrk* (Gutbrod and Schartl, 1999).

RNA isolation, reverse transcription and expression analysis

Total RNA was extracted from cell cultures and *Xiphophorus* tissues using Total RNA Isolation Reagent (TRIR) (ABgene) according to the supplier's recommendations. Total RNA was isolated either from tissues or organs of single fish (WLC 407, 534, and 2044) or from pooled tissues (WLC 1274, 1375, 1750, 1748, and 591, 2-3 fish each). For RNA isolation from Sd pigment spots, spots from dorsal fins of 15-20 fish (WLC 1274) were accurately cut out and pooled. Benign lesions were carefully excised from dorsal fins of single backcross hybrids (WLC 407/534) and used for RNA isolation. After DNase treatment, reverse transcription was performed from 1-2 µg of total RNA using RevertAid^{TM} First Strand cDNA

Synthesis Kit (Fermentas) and random hexamer primers, according to the manufacturer's instructions. Semi-quantitative RT-PCR and quantitative real-time PCR analyses were performed on cDNA from 50 ng of total RNA. Amplification was monitored with i-Cycler (Bio-Rad) or Mastercycler ep realplex (Eppendorf) using SYBR Green reagent. For quantification, data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and normalized to expression levels of the housekeeping gene *ef1a* (*elongation factor 1 alpha*) or to the melanocyte marker gene *dct* (*dopachrome tautomerase*). PCR values for each cDNA were determined from triplicates. Unless noted otherwise, data are presented as mean ± standard deviation of at least three independent reverse-transcribed RNA samples. Significance was determined using Welch's t-test (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). PCR primers were designed using Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/primer3/). The primers had the following sequences:

gene	primer name	sequence
ef1a	ef1a_Xmac_f01	AGTGAAATCCGTGGAGATGC
	ef1a_Xmac_r01	ATCTGACCTGGGTGGTTCAG
***xmrk* (both allele classes)**	qPCR_3UTR_xmrk_f01	ACGCATCTGGAAAATGAACA
	qPCR_3UTR_xmrk_r01	AGCGCCCAGGATTAAAACAT
***xmrk*^{A}**	RT_Xmrk_Y_f03	GCTGCCATACAAACGGATAA
	RT_Xmrk_Y_r03	GGAGATGTTCCGGAGAGTG
***xmrk*^{B}**	Xmrk_X_copy_f07	GCTGGTGGGCGAGTTCTCC
	Xmrk_X_copy_r07	GTTTCACTCCCAGGCGGC
egfrb	qPCR_3UTR_egfrb_f0	TCTGGAAAATGGAGCCAAAC
	qPCR_3UTR_egfrb_r01	CCACATGTGAGAGCAGGAGA
dct	dct_Xmac_f01	ACTTCTTCGTCTGGCAGCAT
	dct_Xmac_r01	TTCCAAGCTCAGAAGGTGGT
cdkn2ab	cdkn2ab_Xipho_f01	ACGGGGTGAATAGTTTTGGA
	cdkn2ab_Xipho_r01	GCTTTTGTCCGTAACGTTTG
pdcd4a	pdcd4_xmac_f01	CACGTCCTTCCCATGAACTT
	pdcd4_xmac_r01	ACCAACACCCCACACGTTAT
mtap	mtap_Xmac_f01	GCTGCACTCATCTGGTGGTA
	mtap_Xmac_r01	CCATGGGGATGTGACAGAC
cxxc4	cxxc4_Xmac_f01	CTCAGCAGCCGTTACAGACA
	cxxc4_Xmac_r01	CTGCTGCAAACACCACAGTT
mitfa	mitfa_Xipho_f02	ATCCCAAAATGCTGGAGATG
	mitfa_Xipho_r02	CAGGTACTGCCTCACCTGCT
mitfb	mitfb_Xipho_f01	GTCCTCCCACTACCCACAAA
	mitfb_Xipho_r01	GAACCAAGCTTTCCCAGGTA

For quantification of transcript levels of different *xmrk* alleles in different *Xiphophorus* genotypes, qPCR analysis was performed using primers that amplify both *xmrk* allele classes, but do not bind to the proto-oncogene *egfrb*. To directly compare transcript levels of $xmrk^A$ and $xmrk^B$ in the same sample, allele specific primers were used. Unless noted otherwise, *xmrk* expression in the melanoma cell line PSM was set as reference (arbitrary value = 1), because *xmrk* is expressed on a relatively high level in this cell line. For quantification of *egfrb* transcript levels, primers specific for the proto-oncogene were used. The PCR products of the different *xmrk* and *egfrb* primers pairs are of approximately the same size and furthermore, have a similar G/C content and melting point, enabling direct comparison of the abundance of transcripts. To compare expression levels of two genes in the same sample, the fold change for *gene 1*, normalized the housekeeping gene *ef1a*, was calculated relative to expression of *gene 2* in the melanoma cell line PSM ($\Delta\Delta C_T = (C_{T,gene\ 1} - C_{T,ef1a})_{tissue\ x} - (C_{T,gene\ 2} - C_{T,ef1a})_{PSM\ cells}$). Specificity of *xmrk* and *egfrb* PCR primers was confirmed by performing PCR on cDNA from gills of *X. maculatus* (strain WLC 2065) and from melanoma tissues (WLC 1748, WLC 534) and on plasmid DNA containing the $xmrk^A$ coding sequence.

Protein isolation and Western blot analysis

Xiphophorus tissues and cell cultures were homogenized in lysis buffer (20 mM HEPES (pH 7.8), 500 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 0.1% deoxycholate, 0.5% Nonidet-P40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 200 µM Na_3VO_4, 1 mM PMSF, 100 mM NaF). 80 µg of protein was separated by SDS-PAGE and transferred to nitrocellulose membranes according to standard Western blotting protocols. Membranes were blocked in 5% (anti-β-actin) or 1.5% (anti-Xmrk, anti-Cdkn2ab) bovine albumin serum (BSA) in TBST (10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% Tween-20) for 60 min. Incubation with primary antibodies was carried out overnight at 4°C. Anti-β-actin (C-4) antibody was purchased from Santa Cruz Biotechnology. Anti-Xmrk is a rabbit polyclonal antiserum obtained by immunization with a polypeptide representing a C-terminal part of Xmrk (‘‘pep-mrk’’) (Malitschek et al., 1994). Anti-Cdkn2ab is a rabbit polyclonal antibody directed against bacterially-expressed recombinant *Xiphophorus* Cdkn2ab (p13) (Butler et al., 2007b). As secondary antibodies, horseradish peroxidase-coupled antibodies directed against mouse (Thermo Scientific) or rabbit IgG (Bio-Rad) were used.

Generation of BAC reporter constructs

Generation of bacterial artificial chromosome (BAC) reporter constructs was performed as described in (Nakamura et al., 2008), with the following modifications: The PCR amplification of the targeting fragment for homologous recombination was performed using Phusion High-Fidelity DNA Polymerase (NEB) and the following conditions: initial denaturing at 98°C for 30 sec, followed by 30 amplification cycles at 98°C for 20 sec, 58°C for 30 sec and 72°C for 2 min, and a final elongation step at 72°C for 5 min. The PCR products were gel-purified, digested with DpnI (Fermentas) at 37°C overnight and subsequently, the digested PCR products were purified again. For electroporation, EasyjecT (EquiBio) and 0.2 cm wide electroporation cuvettes (Bio-Rad) were used. Extraction and purification of BAC DNA was conducted using a customer-adapted protocol for the QIAGEN Plasmid Midi Kit (http://www.qiagen.com/literature/render.aspx?id=450). To generate deletion constructs of the full-length reporter BACs, the same protocol was used. For this purpose, the 5' overhang sequences of the primers were designed in such a way that insertion of the targeting fragment results in the intended deletion of a certain part of the original BAC construct.

All *Xiphophorus* BACs used in this work are derived from a genomic BAC library of *X. maculatus* (strain WLC 1274, Rio Jamapa) (Froschauer et al., 2002) from which contigs covering several megabases of the sex-determining region on the X and Y chromosomes had been assembled and sequenced. Genomic sequences of BAC clones containing mdl^{Sr}-$xmrk^{A}$ (BAC_A6), mdl^{Sd}-$xmrk^{B}$ (BAC_A3), and the Y-chromosomal *egfrb* allele (BAC_A4) were kindly provided by Prof. Jean-Nicolas Volff (IGFL, Lyon, France). For generation of the BAC reporter constructs BAC_A6_pLUC, BAC_A3_pLUC, and BAC_A4_pLUC, targeting DNA fragments for homologous recombination containing a firefly luciferase and a kanamycin resistance gene (firefly luciferase-poly(A)-KanR) were amplified by PCR using the pLUC+Km vector (a kind gift from Lisa Osterloh) and primers BAC_A6_pLUC_fw/rev, BAC_A3_pLUC_fw/rev, and BAC_A4_pLUC_fw/rev, respectively. Subsequently, these DNA fragments were inserted at the translational start sites of *xmrk* or *egfrb* by homologous recombination. The BAC_A3_pLUC_deldown deletion construct was generated by inserting a targeting DNA fragment containing an ampicillin resistance gene (AmpR) into BAC_A3_pLUC by homologous recombination. This DNA fragment was amplified by PCR using the pRL-TK vector (Promega) and the primer pair BAC_A3_pLUC_deldown_fr01. The pLUC+Km vector and primers BAC_A3_deldown_fr02 were used for PCR to obtain a linear

DNA fragment containing a kanamycin resistance gene (KanR), which was inserted by homologous recombination into BAC_A3_pLUC_deldown to generate the BAC_A3_pLUC_delup+down deletion construct. For construction of BAC_A3_pLUC_delup, a targeting DNA fragment containing an AmpR was amplified by PCR using the pRL-CMV vector (Promega) and primers BAC_A3_upstream_fr01. This PCR product was inserted into BAC_A3_pLUC (full-length) by homologous recombination.

The *Oikopleura dioica* BAC clone OIKO003 5xk14 (GenBank accession no. NG_007518.1) (Seo et al., 2001), which was a kind gift from Daniel Chourrout (Sars International Centre for Marine Molecular Biology, Bergen, Norway), was used to generate BAC reporter constructs containing the renilla luciferase under control of the *cmv* promoter. For generation of the full-length reporter construct (BAC_RLUC), a targeting DNA fragment (cmv::renilla luciferase-poly(A)-AmpR) was prepared by PCR using the pRL-CMV vector (Promega) and primers BAC_OIKO_RLUC_fr03. Subsequently, this DNA fragment was inserted in the *Oikopleura dioica* BAC clone by homologous recombination. To generate the BAC_RLUC_85kb deletion construct, a targeting DNA fragment was amplified by PCR using the pETM-30 vector (addgene) and primers BAC_RLUC_deldown_fr01. The DNA fragment, which contains a KanR, was inserted by homologous recombination into BAC_RLUC. The pLUC+Km vector and primers BAC_renilla_40kb_fr01 were used for PCR to obtain a linear DNA fragment containing an AmpR. This PCR product was inserted by homologous recombination into BAC_RLUC_85kb to generate the BAC_RLUC_40kb deletion construct.

Correctness of all BAC reporter constructs used for luciferase experiments was checked by Colony PCR and control digestion using different restriction enzymes. The parts of the finished BAC reporter constructs that are derived from PCR-amplified targeting fragments were additionally controlled by sequencing.

For BAC recombineering, the following primers were used:

primer name	sequence
BAC_A3_pLUC_fw	GGAGCAGCAGTCTGACCTGCCGGACTCTAGTTTCTAACCGGACCGTCTTCATGGAAGACGCCAAAAACATAAAGAAAGG
BAC_A3_pLUC_rev	CTGTCCGGGTCTGTGCTGCTGCAGCGGCTGATGCTGAGCAGCAGCAGCAGCTGCATGATTACGAATTCGAGCTCGGTACC
BAC_A6_pLUC_fw	GCAGCAGTCCGACCTGCCGGACTCTAGTTTCTAACCGGACCGTCTTCATGGAAGACGCCAAAAACATAAAGAAAGG
BAC_A6_pLUC_rev	CACCCTTTCTGTCCGGGTCTGTGCTGCAGCAGCGGCTGATGCTGAGCAGCAGCAGCAGCTGCATGATTACGAATTCGAGCTCGGTACC
BAC_A4_pLUC_fw	GGAGCAGCAGTCCGACCTGCCGGACTCTAGTTTCTAACCGGACCGTCTTCATGGAAGACGCCAAAAACATAAAGAAAGG
BAC_A4_pLUC_rev	GTGCTGCAGCAGCGGCCGATGCTCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCTGCCATGATTACGAATTCGAGCTCGGTACC
BAC_A3_pLUC_deldown_f01	GGGAGGTGTGGGAGGTTTT
BAC_A3_pLUC_deldown_r01	CGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGTTACCAATGCTTAATCAGTGAG
BAC_A3_deldown_f02	GTGCTAACCATGCTAACGTGACTCGGTGGGTGCTACGACCCGAAGTACCCACGGCAAGCTGCTAGACGTTGTGTCTCAA
BAC_A3_deldown_r02	GGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGGTACCGGGCCCCCCCTCGAG
BAC_A3_upstream_f01	GTGCTAACCATGCTAACGTGACTCGGTGGGTGCTACGACCCGAAGTACCCACGGACCCCTATTTGTTTATTTTTCTAAATAC
BAC_A3_upstream_r01	GGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGTTACCAATGCTTAATCAGTGAGGC
BAC_OIKO_RLUC_f03	CCACGTAAGAGAGTAAATGAAGAAAGAGAGTAGTTTAATTCTGACTGATTGTCAATATTGGCCATTAGCCATA
BAC_OIKO_RLUC_r02	GATGCGGATGATTCATATTAGAAGATTTACAAAAGCTGGACTATAGTTTTACCAATGCTTAATCAGTGAG
BAC_RLUC_deldown_f01	CGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGCCATATTCAACGGGAAAC
BAC_RLUC_deldown_r02	CTTTGAATATTATGACTTTATTTTAAATGTCATAGGCTCTCAATCAAGCTAGTTACGTTAGAAAAACTCATCGAGCATCAAA
BAC_renilla_40kb_f01	CTTGTTATTTTGATAATTGCGTTACCTATGATTCCTCCAAGGAAGATAATTGATCCATGAGTAAACTTGGTCTGACAGTTA
BAC_renilla_40kb_r01	CAAAATATAGGCCAAAGTATCATGGCGCGGCGGCTCCGGGACCGCGAAATGGTCCTTTCGGGGAAATGTGCGCGGA

Luciferase assay

For luciferase assays, A2 and PSM cells were seeded at a density of $2x10^5$ and $8x10^4$ cells per 12 well, respectively. On the next day (d0), cells were transfected using FuGENE HD Transfection Reagent (Roche), either in F 12 medium supplemented with 5% FCS (A2 cells) or in conditioned medium (PSM cells). Cells were cotransfected with firefly luciferase and renilla luciferase BAC reporter constructs at a ratio of 2:1 (copy numbers). For transfection of a 12 well, 1 µg of the respective firefly luciferase BAC reporter construct was used. After 16-18 hrs (d1), medium was changed. On d2, cells were transferred to a 6 cm plate. On d7, cells were harvest in 50 µl of 1x PLB (Promega). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and the TriStar LB941 microplate multimode reader (Berthold). For each experiment, cell were seeded and transfected in triplicates. Data are presented as mean ± standard deviation of three or four independently performed experiments and significance was determined using Welch's t-test (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Preparation of gDNA, PCR and sequencing

Genomic DNA from *Xiphophorus* cell cultures and pooled tissues was isolated using standard procedures. PCR amplification was performed using 10-100 ng gDNA and Phusion High-Fidelity DNA Polymerase (NEB), according to the supplier's instructions.

For analysis of *cdkn2ab* upstream regions in A2 and PSM cells, PCR amplification was performed using the following primers:

primer name	sequence
CDKN2X-Pro-F2	CGAAGCTTTGAAGGGTGATGT
CDKN2X-Pro-R2	CGGTGTGTGTCCGGGCTT
Xiph-CDKN2ab-prom-for	GCTTGCGAAGCTTTGAAGGG
Xiph-CDKN2ab-prom-rev	CGCCGATAAACTACTCACAGC
Xipho_cdkn2ab_Xmac_f01	GTGCACTGCAACTTGCTTTC
Xipho_cdkn2ab_Xmac_r01	TGAGATCAGCAAGGCAAGAA

After amplification, PCR products were separated on a 2 % agarose gel.

For sequencing of the putative promoter region of the *mdl*$^{Sp\text{-}type}$*-xmrk*^{A} allele from PSM cells, the following primer pairs were used:

primer name	forward primer	reverse primer
Seq_xmrkY_prom_fr01	GGATACATGCAGCCTCACCT	ATTTCCATTGCGTGTCCTTC
Seq_xmrkY_prom_fr02	GCAAAGCCAAAGGAGTGAAG	GAACAAACGACGGAACCTGT
Seq_xmrkY_prom_fr03	CTACATGGGCACCCTTCTGT	CTTTGCAGATCGAGGGTCAG
Seq_xmrkY_prom_fr06	GTCAAAATCGCGGTCAAAAT	GCAGCAGGATGAACTCGTTT
Seq_xmrkY_prom_f07r06	TGTTGAGCAAAACAGATAAATGG	GCAGCAGGATGAACTCGTTT

PCR products were purified and sequenced using the indicated primers.

For sequencing of the *mtap* cds and partial 3'UTR, the following primer pair was used:

primer name	forward primer	reverse primer
Seq_mtap_Xipho_fr01	CGGACGGATGTTAATGGTG	CGGAGGTGCATTATTCCTGT

PCR products were purified, cloned into pCR®-Blunt vector (Zero Blunt® PCR cloning Kit, Invitrogen) according to manufacturer's instructions and transformed into competent *E. coli*. For each genotype (WLC 1274 and WLC 1337) two clones were sequenced with primers M13 Forward and M13 Reverse.

To amplify the mitochondrial control region and the *cytochrome b* gene of the *Xiphophorus* cell line A2, the following primer pairs were used:

	primer name	sequence
mitochondrial control region	H693	GGCGGATACTTGCATGT
	L15513	CTRGGAGACCCNGAAAACTT
cytochrome b	H15982	CCTAGCTTTGGGAGYTAGG
	L14725	GAYTTGAARAACCAYCGTTG

PCR products were purified and sequenced using the indicated primers.

All sequences were visually inspected and each determined sequence was confirmed from two independent PCR and sequencing reactions. Sequence processing and alignments were performed using Clustal W (Thompson et al., 1994) as implemented in BioEdit (Hall, 1999) and VectorNTI (Invitrogen), followed by manual improvement.

Phylogenetic analysis

Sequences of the mitochondrial control region and the *cytochrome b* gene from A2 cells were determined in this study, sequences from various *Xiphophorus* species were obtained from (Meyer et al., 2006) (mitochondrial control region) and (Hrbek et al., 2007) (*cytochrome b*).

cytochrome b	***Xiphophorus* species**	**GenBank accession no.**
	Xiphophorus xiphidium	EF017549.1
	Xiphophorus clemenciae	EF017552.1
	Xiphophorus maculatus	EF017551.1
	Xiphophorus hellerii	EF017548.1
	Xiphophorus evelynae	EF017550.1
mitochondrial control region	***Xiphophorus* species**	**GenBank accession no.**
	Xiphophorus hellerii	DQ235834.1
	Xiphophorus maculatus	DQ235823.1
	Xiphophorus xiphidium	DQ235826.1
	Xiphophorus alvarezi	DQ235833.1
	Xiphophorus milleri	DQ235814.1
	Xiphophorus signum	DQ235832.1
	Xiphophorus multilineatus	DQ235829.1
	Xiphophorus montezumae	DQ235818.1
	Xiphophorus clemenciae	DQ235835.1
	Xiphophorus evelynae	DQ235827.1

Sequence alignments were obtained with Clustal W (Thompson et al., 1994) as implemented in BioEdit (Hall, 1999). For tree reconstruction, maximum-likelihood analysis was performed using MEGA 5.05 software (Tamura et al., 2011) according to the Tamura-Nei model of nucleotide substitutions. Bootstrapping values for the maximum-likelihood tree were obtained from 500 iterations.

Comparative analysis of genomic sequences

Comparative analysis of BAC_A6 (mdl^{Sr}-$xmrk^{A}$) and BAC_A3 (mdl^{Sd}-$xmrk^{B}$) insert sequences was performed with mVISTA at http://genome.lbl.gov/vista (Frazer et al., 2004) using the AVID (Bray, 2002) and the Shuffle-LAGAN (Brudno et al., 2003) alignment program. Screens for repetitive elements were performed with RepeatMasker as implemented in mVista. Sequences of all elements, with the exception of *xmrk* and *mc4r*, were obtained from (Volff et al., 2003) (GenBank accession nos. AY228504.1, AY298859.1). Positions of all elements in BAC insert sequences were determined in this study.

Results

Analysis of the transcriptional regulation of different *mdl-xmrk* allele combinations

Expression of the proto-oncogene *egfrb* in adult tissues of *X. maculatus* and *X. hellerii*

To analyze transcriptional regulation of the *xmrk* oncogene in more detail, I first performed a quantitative, comparative analysis of *xmrk* expression levels in a whole array of different melanoma and melanoma precursor lesions from various natural and mutant alleles. Furthermore, I determined transcript levels of the proto-oncogene *egfrb* in tissues of parental and hybrid genotypes. For *egfrb* expression pattern analysis in parental genotypes, the *X. hellerii* strain WLC 1337 (also known as hIII strain) and the *X. maculatus* strain WLC 1274 (Fig. 3a) were used, as these fish represent the crossing partners for the classical Gordon-Kosswig-Anders cross. QPCR analysis revealed that the proto-oncogene *egfrb* is ubiquitously expressed in tissues of *X. hellerii*, with gill and liver being the tissues with the highest transcript levels (Fig. 4a). In brain and eye *egfrb* mRNA was expressed on a low background level, whereas expression in skin was slightly higher. The *egfrb* expression pattern in tissues of *X. maculatus* resembled the one found in *X. hellerii*. *egfrb* was transcribed on a high level in gills, whereas transcription in brain, eye, liver, and skin was on a lower background level (Fig. 4b). To determine expression levels in the black pigment cell lineage of *X. maculatus*, *egfrb* transcript levels were measured not only in normal skin, which contains micromelanophores, but also in accurately cut out dorsal fin macromelanophore spots. However, expression of the proto-oncogene *egfrb* in macromelanophore spots was not significantly increased compared to normal skin ($p = 0.19323$) and is lower than expression in gills (Fig. 4b).

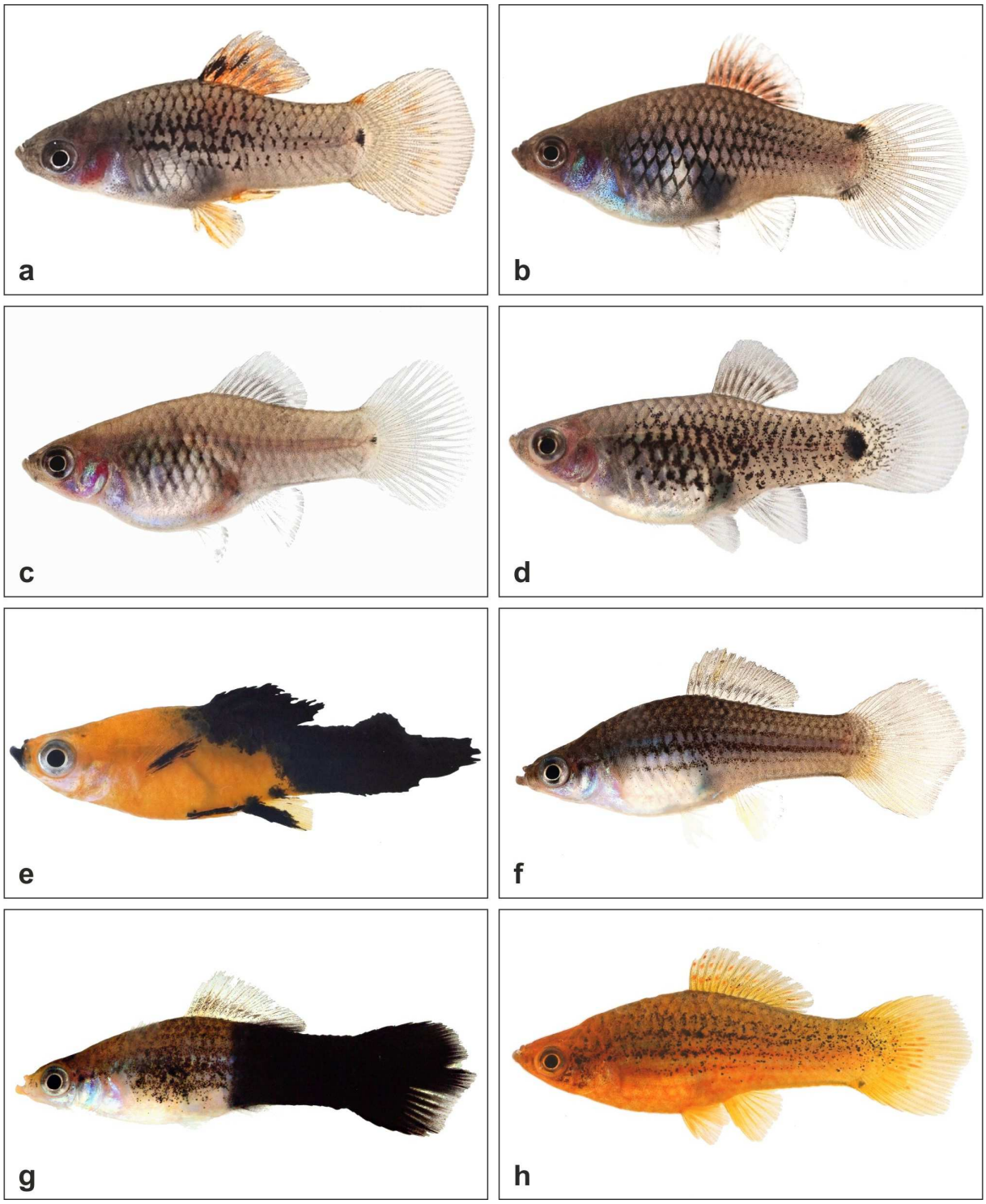


Fig. 3: Pigmentation phenotypes of parental *X. maculatus* and backcross hybrids. (a) *X. maculatus* (WLC 1274) male showing the Sd pattern (mdl^{Sd} phenotype) and the Sr pattern (mdl^{Sr} phenotype). (b) *X. maculatus* (WLC 1375) female exhibiting the Sr pattern. (c) *X. maculatus* (WLC 2065) female. Fish of this strain have neither *xmrk* nor *mdl* and do not exhibit macromelanophore patterns. (d) *X. maculatus* (WLC 591) female. These fish carry the mutant locus Sr'' and exhibit an enhanced Sr pattern due to an increased number of macromelanophores on the flanks of the body. (e) Backcross hybrid (WLC 407) carrying a malignant melanocytic melanoma in trunk, dorsal fin, and tail fin. (f) Backcross hybrid (WLC 1748) exhibiting a slightly enhanced Sr pattern compared to purebred *X. maculatus* (WLC 1375) (b). (g) Backcross hybrid (WLC 1748) carrying a malignant melanoma in trunk and dorsal fin. (h) Backcross hybrid (WLC 2044) showing, like WLC 1748 fish (f), a slightly enhanced Sr pattern. (modified from (Regneri and Schartl, 2012))

To test whether these transcripts are translated, I determined Egfrb protein levels in tissues of *X. maculatus* by Western blot analysis using a rabbit polyclonal antibody directed against Xmrk (Malitschek et al., 1994). Due to the high amino acid sequence similarity between Xmrk and Egfrb, the Xmrk antibody recognizes not only the *xmrk* oncogene product itself, but also that of its corresponding proto-oncogene *egfrb*. To be able to identify signals specific for Egfrb on the blot, I used the *X. maculatus* wildtype strain WLC 2065 (Fig. 3c) for Western blot analysis. This strain is devoid of *xmrk* and thus enables detection of Egfrb protein levels alone. In WLC 2065 fish, Egfrb protein was highly expressed in liver, expressed on a lower level in brain, eye, and skin, whereas no Egfrb protein was detected in gills (Fig. 4c). This was surprising as high levels of *egfrb* mRNA were found in gills of *X. maculatus* (Fig. 4b).

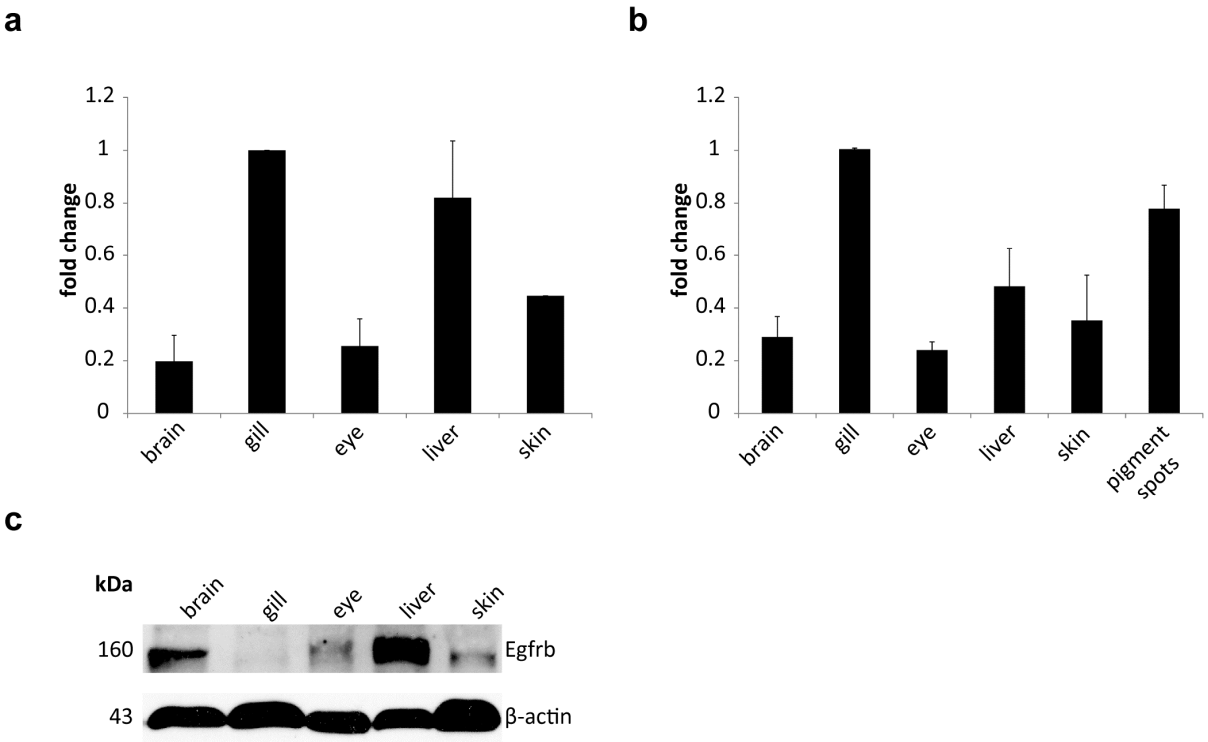


Fig. 4: Analysis of *egfrb* expression patterns. Quantitative real-time PCR analysis of *egfrb* transcript levels in different tissues of the parental fish strains *X. hellerii* (WLC 1337) (a) and *X. maculatus* (WLC 1274) (b). *egfrb* expression in gills was set as reference. (c) Western blot analysis of Egfrb protein levels in tissues of a *X. maculatus* strain (WLC 2065) devoid of *xmrk*. β-actin was used as loading control.

Expression of the tumorigenic *mdl*^{Sd}-*xmrk*^{B} allele

The *mdl*^{Sd}-*xmrk*^{B} allele (Fig. 2a), which is located on the X chromosome in WLC 1274 fish, is equivalent to the well-known spotted dorsal locus (spotted dorsal, Sd) from the classical Gordon-Kosswig-Anders cross. This allele induces the formation of several small macromelanophore spots in the dorsal fin of purebred wildtype platyfish that possess the *R* locus. In backcross hybrids that do not carry the *R* locus, the *mdl*^{Sd}-*xmrk*^{B} allele leads to

melanoma development with almost 100% penetrance and is therefore considered as highly tumorigenic. To determine whether the *R* locus has an effect on the transcriptional regulation of the *mdl*^{Sd}-*xmrk*^{B} allele, I compared transcription levels in parental platyfish and backcross hybrids. For expression analysis in parental fish, females of the *X. maculatus* strain WLC 1274 were used, because they are homozygous for the *mdl*^{Sd}-*xmrk*^{B} allele. Notably, qPCR analysis revealed that the abundance of *xmrk*^{B} and *egfrb* transcripts is on a comparable level in most tissues of *X. maculatus* (Fig. 5a). The *xmrk*^{B} oncogene was transcribed on a low background level in brain, whereas no transcription was detected in liver. In eye expression was slightly higher than in brain and comparable to expression in healthy skin of WLC 1274 female fish, which contains only micromelanophores. Interestingly, *xmrk*^{B} expression levels in accurately cut out dorsal fin macromelanophore spots were considerably higher, demonstrating that there is a robust *xmrk*^{B} transcription in macromelanophores already in the parental fish *X. maculatus*. Furthermore, I detected a robust expression of *xmrk*^{B} in gills. This was surprising as the gills of these fish contain no macromelanophores and we never observed any sign of hyperplasia or other proliferative disorders in gills of *X. maculatus* carrying the *xmrk*^{B} allele. As expected, *xmrk*^{B} expression in all tissues analyzed was considerably lower than expression in the melanoma-derived cell line PSM.

To analyze if these transcripts are translated and give rise to Xmrk^{B} protein, I performed Western blot analysis on tissues of WLC 1274 females using the same Xmrk antibody as for Egfrb in Fig. 4c. Interestingly, in contrast to the situation found for WLC 2065 (Fig. 4c), I detected a strong band in gills of WLC 1274 fish, indicating Xmrk^{B} protein expression in this tissue (Fig. 5b). Thus, Xmrk^{B} protein and not only *xmrk*^{B} mRNA is present in gills of *X. maculatus*. In all other tissues, Western blot analysis revealed comparable bands in WLC 1274 and WLC 2065. This finding, in combination with the weak *xmrk*^{B} transcription in brain, eye, liver, and skin of *X. maculatus* (Fig. 5a), indicates low levels of Xmrk^{B} protein in these tissues.

I then determined transcription of the highly tumorigenic *mdl*^{Sd}-*xmrk*^{B} allele in normal and melanoma tissues of backcross hybrids (WLC 407/534) carrying either a malignant melanoma (Fig. 3e) or a benign pigmented precursor lesion (classified as melanotic hyperpigmentation according to (Gimenez-Conti et al., 2001)). As expected, the tumorigenic *xmrk*^{B} allele was highly overexpressed in malignant melanomas of backcross hybrids (Fig. 5e), whereas expression in benign precursor lesions was considerably lower (Fig. 5d). To determine Xmrk^{B} protein expression in tissues of backcross hybrids carrying a malignant melanoma, Western

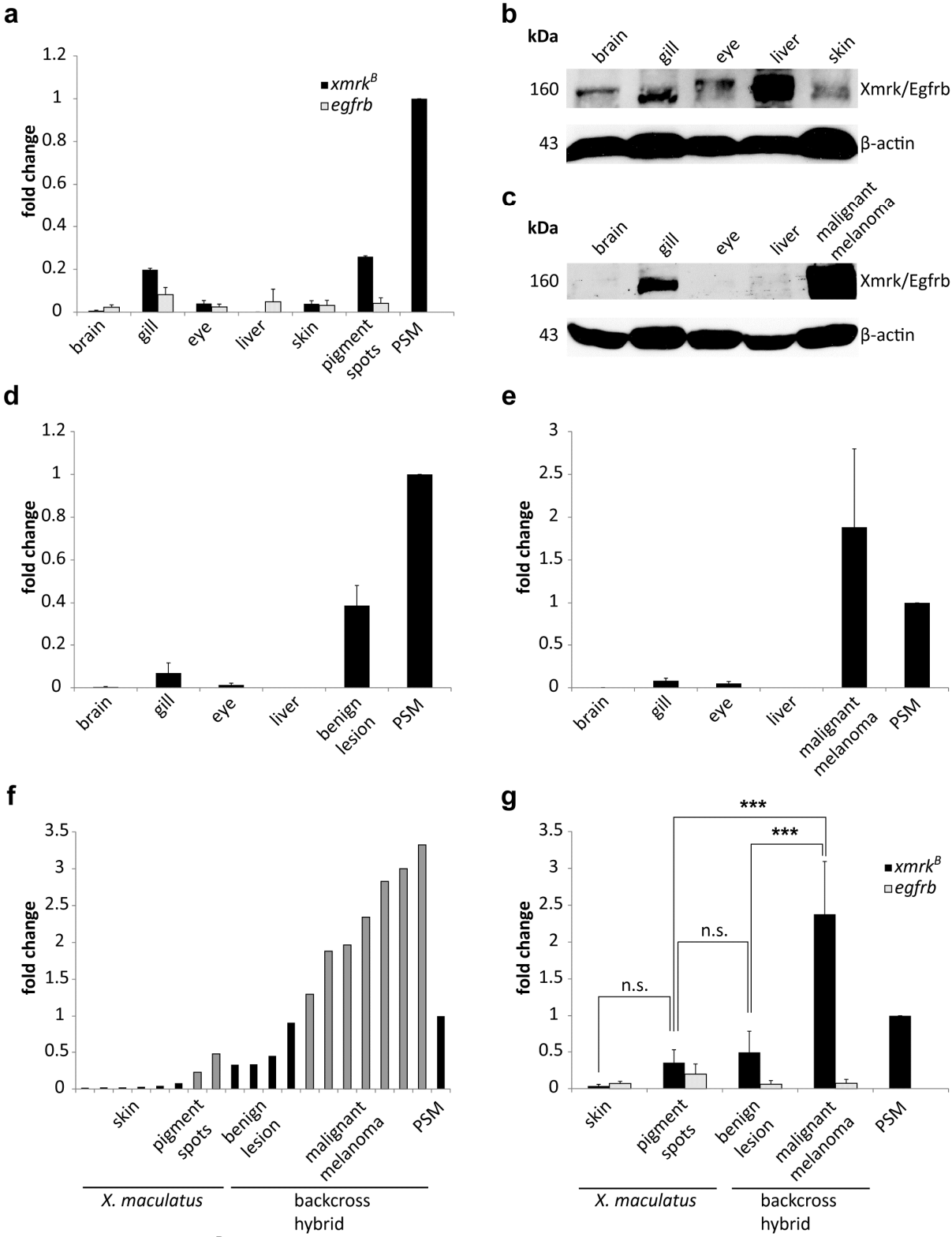


Fig. 5: Analysis of *xmrk*^{B} expression patterns in parental and hybrid genotypes. (a) Quantitative real-time PCR analysis of *xmrk*^{B} and *egfrb* mRNA expression in tissues of *X. maculatus* (WLC 1274). Western blot analysis of Egfrb and Xmrk^{B} protein levels in tissues of (b) *X. maculatus* (WLC 1274) and (c) tumor-bearing backcross hybrids (WLC 534). The Xmrk antibody recognizes Xmrk^{B}, as well as the proto-oncogene product Egfrb. β-actin was used as loading control. Quantitative real-time PCR analysis of *xmrk*^{B} transcript levels in non-tumorous tissues and (d) benign lesions or (e) malignant melanomas of backcross hybrids (WLC 407/534). (f) Comparison of *xmrk*^{B} transcript levels in skin (n = 6) and two independent preparations of macromelanophore spots of *X. maculatus* (WLC 1274), and in benign lesions (n = 4) and malignant melanomas (n = 7) of backcross hybrids (WLC 407/534). (g) Mean transcript levels of *xmrk*^{B} and *egfrb* in the same set of samples used for (f) and (Fig. 7 a, b). (modified from (Regneri and Schartl, 2012))

blot analysis was performed (Fig. 5c). Since the proto-oncogene *egfrb* is transcribed only on a low background level in *Xiphophorus* melanomas (Fig. 5g), the strong band on the blot indicates overexpression of $Xmrk^{B}$ protein in malignant melanomas.

In contrast to the clear overexpression of $xmrk^{B}$ in melanoma tissues of backcross hybrids, the $xmrk^{B}$ mRNA expression pattern in normal non-tumorous tissues was comparable in backcross hybrids and *X. maculatus*. The highest transcription level was detected in gills, followed by transcription in eye, whereas transcription in brain was only on a very low background level (Fig. 5d, e). Expression in skin was not analyzed in tumor-bearing backcross hybrids because of the risk of contamination with transformed pigment cells. These data clearly demonstrate that $xmrk^{B}$ transcription in non-pigment cells is not increased in backcross hybrids compared to purebred parental platyfish.

To further analyze whether the *R* locus has an influence on $xmrk^{B}$ transcription in the macromelanophore lineage, I directly compared $xmrk^{B}$ expression levels in healthy skin and macromelanophore spots of *X. maculatus* with expression in several benign precursor lesions and malignant melanomas of backcross hybrids (Fig. 5f, g). As already shown in Fig. 5a, $xmrk^{B}$ expression was only on a background level in normal skin, whereas it was clearly increased in macromelanophore spots of parental fish. Interestingly, $xmrk^{B}$ expression in benign melanocytic lesions of backcross hybrids was comparable to expression in macromelanophore spots of *X. maculatus*. Expression in malignant melanomas, in contrast, was significantly increased compared to pigment spots ($p = 0.00026$) and benign lesions ($p = 0.00021$) and was even higher than expression in the melanoma cell line PSM. However, it becomes evident that the level of $xmrk^{B}$ transcription varied to some extent between individual melanoma samples and that there is a seamless transition in expression levels from the more benign precursor lesions to malignant melanomas (Fig. 5f).

To determine whether the increase in $xmrk^{B}$ expression levels in malignant melanomas versus benign precursor lesions is due to higher $xmrk^{B}$ transcription in melanoma cells or only the consequence of an increased proportion of cells of the melanocyte lineage in malignant melanomas compared to benign lesions, $xmrk^{B}$ transcript levels in these tissues were normalized to expression of the melanocyte marker gene *dct* (*dopachrome tautomerase*) (Kelsh, 2000; Steel et al., 1992). The *dct* gene was expressed on a high level in melanoma tissues (expression levels are comparable to those of *xmrk*, data not shown) and expression was only slightly increased in a subset of malignant melanomas compared to benign lesions (Fig. 6a, b). In normal healthy skin *dct* was expressed only on a low background level

correlating with the relatively low number of melanocytes in this tissue. When being normalized to expression of *dct*, the abundance of *xmrk*^{B} transcripts was still significantly increased in malignant melanomas compared to benign melanocytic lesions ($p = 0.04075$) (Fig. 6c, d), indicating that the increase in *xmrk*^{B} transcription in malignant melanomas is indeed absolute and not only due to a higher proportion of melanocytes in this tissue. Thus, a clear correlation between the level of *xmrk*^{B} expression and the development of a malignant melanoma exists.

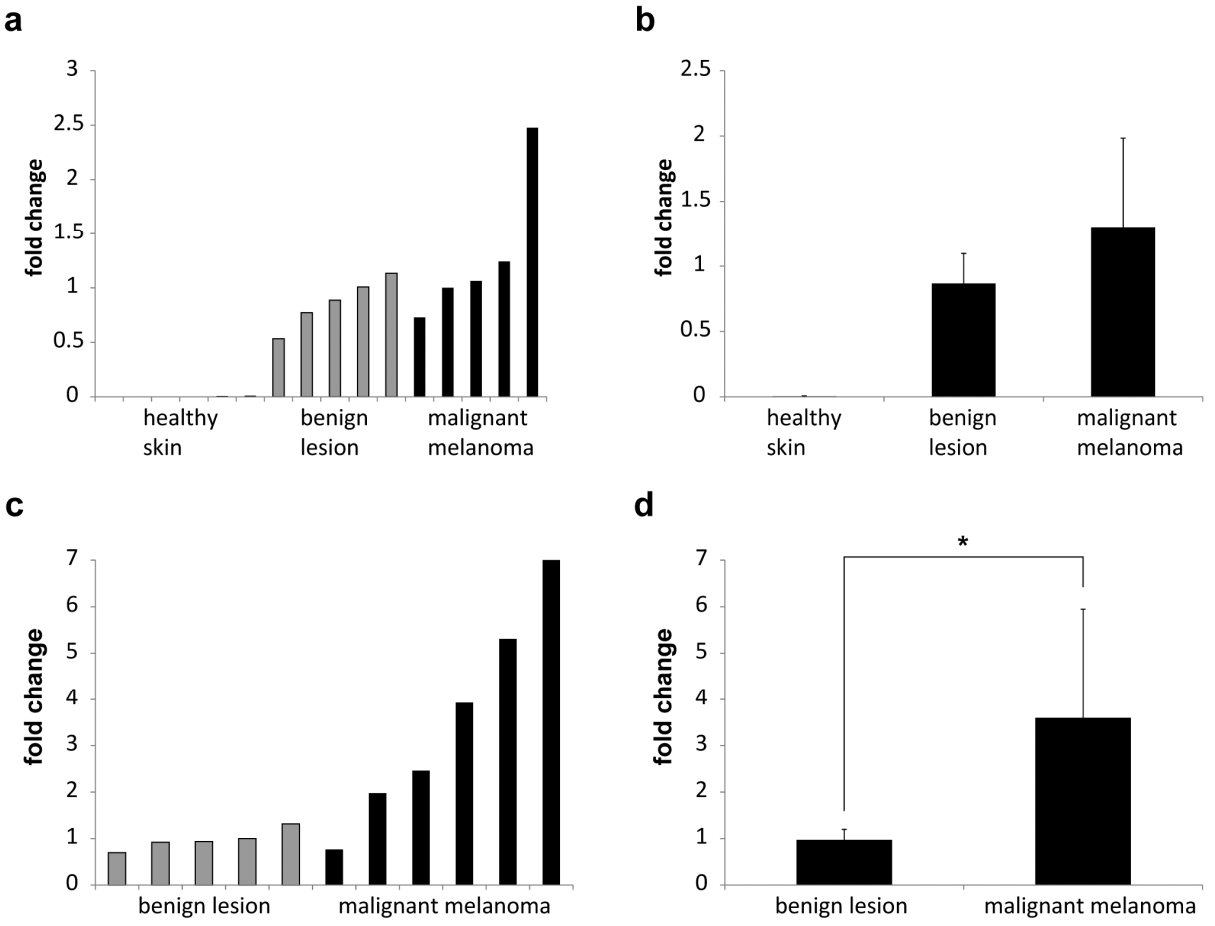


Fig. 6: *xmrk*^{B} mRNA expression in melanoma tissues of backcross hybrids normalized to expression of a melanocyte specific gene. (a) Quantitative real-time PCR analysis of transcript levels of the melanocyte marker gene *dct* in healthy skin (n = 6) of *X. maculatus* (WLC 1274) and in benign lesions (n = 5) and malignant melanomas (n = 5) of backcross hybrids (WLC 407/534). *dct* expression in malignant melanoma 2 was set as reference. (b) Mean expression levels of *dct* in the same set of samples used for (a). (c) *xmrk*^{B} transcript levels in benign lesions (n = 5) and malignant melanomas (n = 6) of backcross hybrids (WLC 407/534) normalized to expression of *dct*. Benign lesion 4 was used as reference. (d) Mean transcript levels of *xmrk*^{B} normalized to *dct* in the same set of samples used for (c). (modified from (Regneri and Schartl, 2012))

Expression of the proto-oncogene *egfrb* in *xmrk*^{B}-expressing tissues of the black pigment cell lineage

To investigate if transcription of the proto-oncogene *egfrb* is also increased in melanoma tissues, qPCR with primers specific for *egfrb* was performed using the same panel of cDNAs as for Fig. 5f and g. In skin of parental platyfish *egfrb* was transcribed on a background level, whereas transcription in pigment spots was slightly increased (Fig. 7a, b), but was still lower than expression of *xmrk*^{B} in this tissue (Fig. 5g). Interestingly, in contrast to the situation found for *xmrk*^{B}, expression of *egfrb* was on a low level in benign melanocytic lesions and malignant melanomas and was not increased in melanoma tissues of backcross hybrids compared to normal skin and macromelanophore spots of *X. maculatus* (Fig. 7a, b). Taken together, these findings show that *xmrk*^{B} and *egfrb* are differentially regulated and that only transcription of *xmrk*^{B} is increased in the melanocyte lineage of *R*-free backcross hybrids.

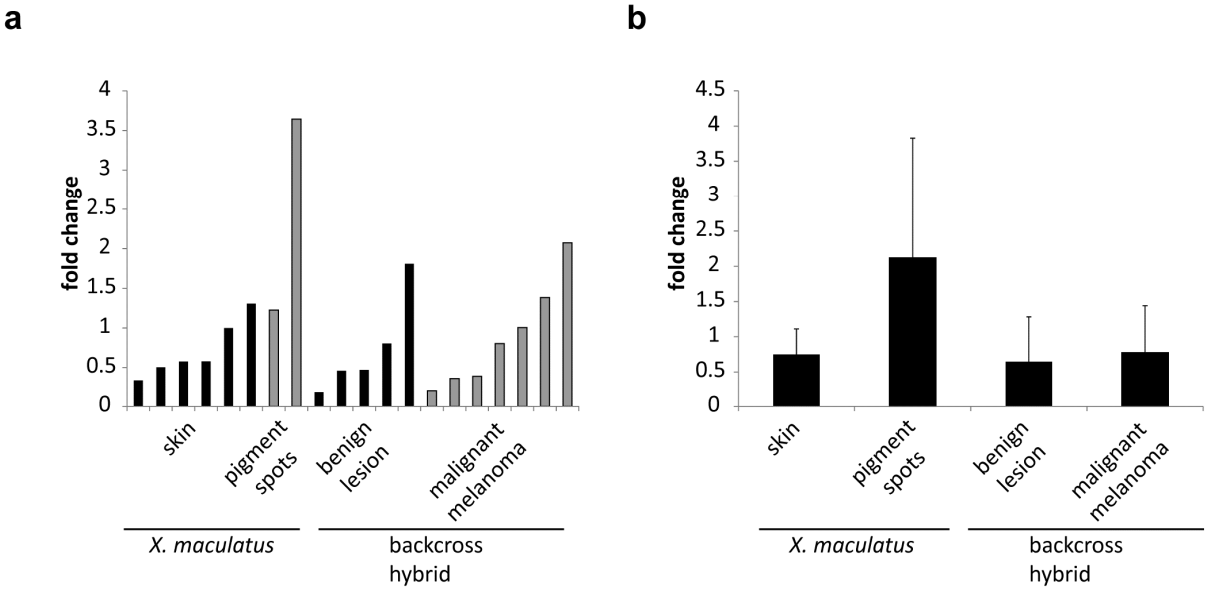


Fig. 7: Quantitative real-time PCR analysis of *egfrb* expression levels. (a) Comparison of *egfrb* transcript levels in skin (n = 6) and two independent preparations of macromelanophore spots of *X. maculatus* (WLC 1274), and in benign lesions (n = 5) and malignant melanomas (n = 7) of backcross hybrids (WLC 407/534). Skin 5 was used as reference. (b) Mean transcript levels of *egfrb* in the same set of samples used for (a).

Expression of the non-tumorigenic *mdl^{Sr}-xmrk^{A}* allele

The *mdl^{Sr}-xmrk^{A}* allele (Fig. 2b), which is located on the Y chromosome in WLC 1274 fish, is, in contrast to the highly tumorigenic *mdl^{Sd}-xmrk^{B}* allele, not tumorigenic and does not even lead to melanosis in hybrid crosses. The molecular reason for the differential tumorigenic potential of different *mdl-xmrk* allele combinations has not been determined so far. However, considering a pigment cell specific release of the transcriptional control after loss of *R* as initiating event in tumor formation, it was tempting to assume that differential transcriptional regulation might account for these differences in tumorigenicity. To test the hypothesis that the transposon (*piggyBac-like* element) insertion in its putative promoter region (Volff et al., 2003) might have altered the transcriptional regulation of the *mdl^{Sr}-xmrk^{A}* compared to the *mdl^{Sd}-xmrk^{B}* allele, I determined the *xmrk^{A}* expression pattern in tissues of *X. maculatus* and backcross hybrids. For expression analysis in purebred parental fish, females of the *X. maculatus* strains WLC 1375 (Fig. 3b) and WLC 1750 were used. In the WLC 1375 strain an intragenic A/B-allele chimeric *xmrk* (*xmrk$^{A/B}$*) is under control of the *mdl^{Sr}-xmrk^{A}* promoter. This allele is located on the X chromosome (Fig. 2e). The WLC 1750 strain has the wildtype *mdl^{Sr}-xmrk^{A}* allele on the X chromosome (instead on the Y as in the above studied WLC 1274 strain) (Fig. 2d). Both *xmrk* crossover alleles produce the wildtype Sr pattern and are not tumorigenic (Gutbrod and Schartl, 1999; Schartl and Meierjohann, 2010). Female fish of both strains are homozygous for the crossover alleles, which makes them suitable for studying transcriptional regulation of the *mdl^{Sr}-xmrk^{A}* allele. QPCR analysis revealed that *xmrk^{A}* is transcribed on a relatively low background level in brain and eye of *X. maculatus*, whereas no expression was observed in liver (Fig. 8a). As already seen for *xmrk^{B}*, the highest *xmrk^{A}* expression was found in gills. In skin a robust expression of *xmrk^{A}* was detected correlating with the presence of macromelanophores that form a striped pattern in the skin of these fish. Taken together, the expression patterns of *mdl^{Sr}-xmrk^{A}* and *mdl^{Sd}-xmrk^{B}* are similar in tissues of *X. maculatus*.

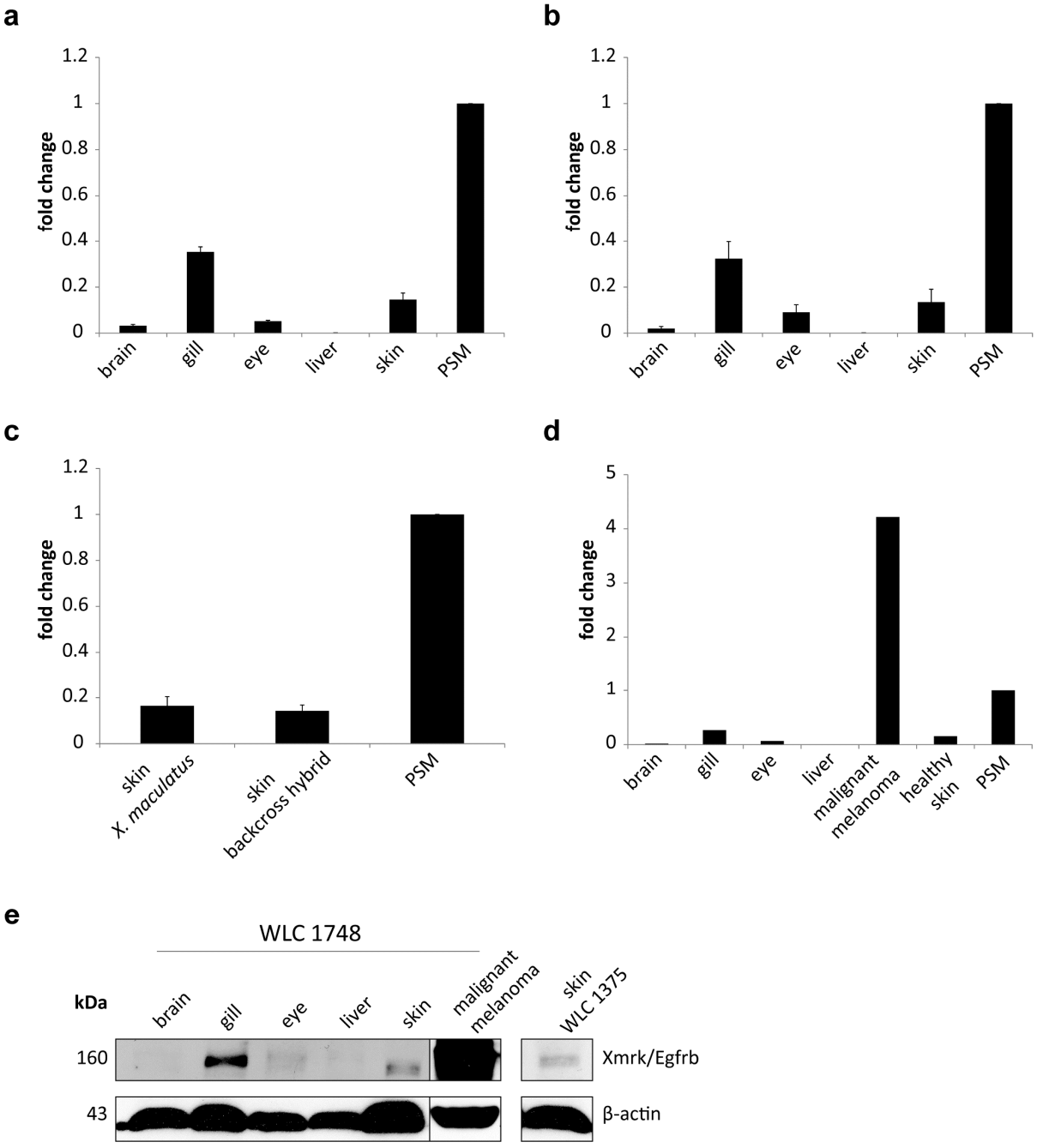


Fig. 8: Analysis of *xmrk*^{A} expression in parental and hybrid genotypes. Quantitative real-time PCR analysis of *xmrk*^{A} mRNA expression levels in different tissues of (a) *X. maculatus* (WLC 1375/1750) and (b) backcross hybrids (WLC 1748). (c) Comparison of *xmrk*^{A} transcript levels in skin of *X. maculatus* (WLC 1375/1750, n = 8) and of backcross hybrids (WLC 1748, n = 8). (d) *xmrk*^{A} transcript levels in different tissues of a single backcross hybrid (WLC 1748) carrying a sporadic melanoma. Skin was obtained from a healthy WLC 1748 fish. (e) Western blot analysis of Egfrb and Xmrk^{A} protein levels in tissues of a healthy backcross hybrid (WLC 1748), in a sporadic melanoma of a WLC 1748 fish, and in healthy skin of *X. maculatus* (WLC 1375). The Xmrk antibody recognizes Xmrk^{A}, as well as the proto-oncogene product Egfrb. β-actin was used as loading control. (modified from (Regneri and Schartl, 2012))

To directly compare transcription levels of the above mentioned alleles in *X. maculatus*, I used tissues of WLC 1274 males, as they are heterozygous for mdl^{Sd}-$xmrk^{B}$ and mdl^{Sr}-$xmrk^{A}$. QPCR analysis was performed using primers that specifically amplify one *xmrk* allele class, but do not bind to the other one. As expected from the previous qPCR analyses using allele nonspecific *xmrk* primers (Fig. 5a, 8a), the expression patterns of mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$ were similar in tissues of purebred parental platyfish (Fig. 9). Interestingly, in all tissues analyzed with the exception of dorsal fin macromelanophore spots, $xmrk^{A}$ was transcribed on a slightly higher level than $xmrk^{B}$, which, however, did not reach significance. As aforementioned, the mdl^{Sr}-$xmrk^{A}$ allele induces the development of a reticulate macromelanophore pattern in the skin, whereas the mdl^{Sd}-$xmrk^{B}$ allele triggers the development of distinct macromelanophore spots in the dorsal fin. Notably, the increase of $xmrk^{A}$ and $xmrk^{B}$ transcript levels in skin and pigment spots, respectively, correlates perfectly with the predominant location of macromelanophore development.

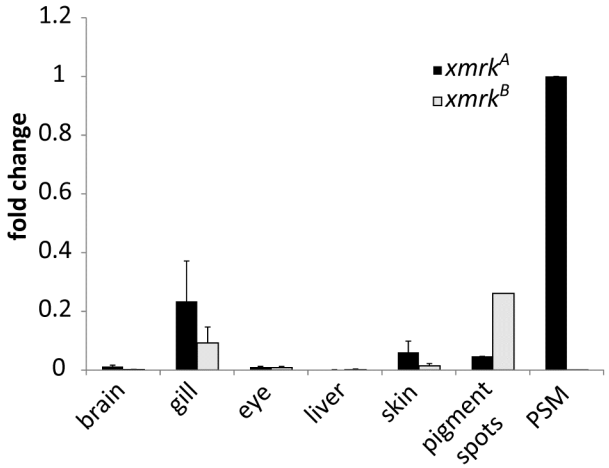


Fig. 9: Comparison of $xmrk^{A}$ and $xmrk^{B}$ transcript levels in tissues of *X. maculatus*. Quantitative real-time PCR analysis of mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$ mRNA expression levels in *X. maculatus* males (WLC 1274) using allele specific primer pairs. Expression of $xmrk^{A}$ in the melanoma cell PSM was set as reference.

Next, a quantitative analysis of mdl^{Sr}-$xmrk^{A}$ expression levels in backcross hybrids (WLC 1748) was conducted. $xmrk^{A}$ transcription levels in all tissues analyzed, including skin, were on a comparable level in backcross hybrids (Fig. 8b) and parental platyfish (Fig. 8a). To further investigate transcriptional regulation of $xmrk^{A}$ in the melanocyte lineage, I directly compared transcript levels in skin of backcross hybrids and *X. maculatus*. In contrast to $xmrk^{B}$, which was clearly overexpressed in the absence of *R*, transcription of $xmrk^{A}$ in the black pigment cell lineage was not increased in backcross hybrids compared to parental platyfish (Fig. 8c). To compare $Xmrk^{A}$ protein expression in skin of these fish, Western blot analysis was performed (Fig. 8e). In healthy backcross hybrids (WLC 1748) $Xmrk^{A}$ protein

was robustly expressed in gills and expressed on a lower level in skin. Notably, a similar expression level in skin was also observed in parental platyfish (WLC 1375). In all other tissues only very faint bands were detected on the blot. Thus, similar to *xmrk*^{A} mRNA expression, Xmrk^{A} protein expression is not increased in skin of backcross hybrids compared to skin of *X. maculatus*. Hence, *xmrk*^{A} and *xmrk*^{B} are differentially regulated in melanophores, which might account for the differences in their tumorigenic potential.

Backcross hybrids carrying the *mdl*^{Sr}*-xmrk*^{A} allele are generally not cancer-prone and develop only a slightly enhanced Sr pattern. However, in rare cases WLC 1748 fish develop a sporadic malignant melanoma later in life. The molecular reason for melanoma development in these fish has not been determined so far, but transcriptional activation of *xmrk*^{A} by a so far unknown mechanism could be one explanation. To test if elevated levels of *xmrk*^{A} mRNA and protein are present in these tumors, expression in sporadic melanomas was compared to expression in skin of healthy WLC 1748 individuals. Interestingly, qPCR analysis revealed a massive overexpression of *xmrk*^{A} mRNA in melanoma samples compared to healthy skin (Fig. 8d), whereas *xmrk*^{A} transcript levels in brain, gill, eye, and liver were not increased in tumor-bearing fish compared to healthy siblings (Fig. 8b, d). By performing Western blot analysis on sporadic melanoma samples, I could furthermore show that not only *xmrk*^{A} mRNA but also Xmrk^{A} protein is present at high levels in sporadic melanomas of backcross hybrids carrying the *mdl*^{Sr}*-xmrk*^{A} allele (Fig. 8e). These data indicate that the Xmrk^{A} protein, when expressed above a certain threshold level, is able to elicit melanoma development comparable to Xmrk^{B}.

Expression of Sr'' and DrLi (mut) mutant alleles

To further investigate transcription driven by the *mdl*^{Sr}*-xmrk*^{A} promoter region, I additionally determined the expression pattern of the Sr'' and the DrLi (mut) allele. The Y-chromosomal Sr'' locus (*mdl*^{Sr}*-xmrk*^{Amut}) (Fig. 2c), which originates from the *mdl*^{Sr}*-xmrk*^{A} allele, is highly tumorigenic due to a so far uncharacterized mutation in the *xmrk*^{A} gene. Transcription of this mutant *xmrk* allele is under control of the wildtype *mdl*^{Sr}*-xmrk*^{A} promoter region (Gutbrod and Schartl, 1999; Schartl and Meierjohann, 2010). For expression analysis, females of the *X. maculatus* strain WLC 591 were used (Fig. 3d). As this strain has a W/Y sex determination system, female WLC 591 fish harbor one copy of the Sr'' locus, but have no wildtype *mdl-xmrk* allele. QPCR analysis revealed that the expression pattern of the mutant *mdl*^{Sr}*-xmrk*^{Amut} allele (Fig. 10a) resembles the one found for the non-tumorigenic wildtype *mdl*^{Sr}*-xmrk*^{A} allele in *X. maculatus* (Fig. 8a). In skin transcription of the *mdl*^{Sr}*-xmrk*^{Amut} allele was slightly higher

than expression of the wildtype allele, which is in line with an increased number of macromelanophores in the skin of WLC 591 fish (Gutbrod and Schartl, 1999). However, it has not been determined whether this increase is statistically significant.

Furthermore, *xmrk* expression was determined in the backcross hybrid strain WLC 2044 (Fig. 3h). In this strain, a chimeric A/B-allele *xmrk* is under control of the wildtype *mdl*^{Sr}-*xmrk*^{A} promoter region (*mdl*^{Sr}-*xmrk*$^{A/B}$) (Fig. 2f). This mutant allele, designated DrLi (mut), is non-tumorigenic (Gutbrod and Schartl, 1999). The DrLi (mut) allele is comparable to the X-chromosomal *mdl*^{Sr}-*xmrk*$^{A/B}$ allele from the WLC 1375 strain (Fig. 2e), but originates from a separate crossover event. The *xmrk* expression pattern in the backcross hybrid strain WLC 2044 (Fig. 10b), which is homozygous for the DrLi (mut) allele, was comparable to the one found for the *mdl*^{Sr}-*xmrk*$^{A/B}$ allele in *X. maculatus* (WLC 1375) (Fig. 8a). The expression pattern was also similar to that of the wildtype *mdl*^{Sr}-*xmrk*^{A} allele in backcross hybrids (WLC 1748) (Fig. 8b). Thus, these data further confirm that transcription of the non-tumorigenic *mdl*^{Sr}-*xmrk*^{A} allele in the black pigment cell lineage is not increased in backcross hybrids compared to parental platyfish.

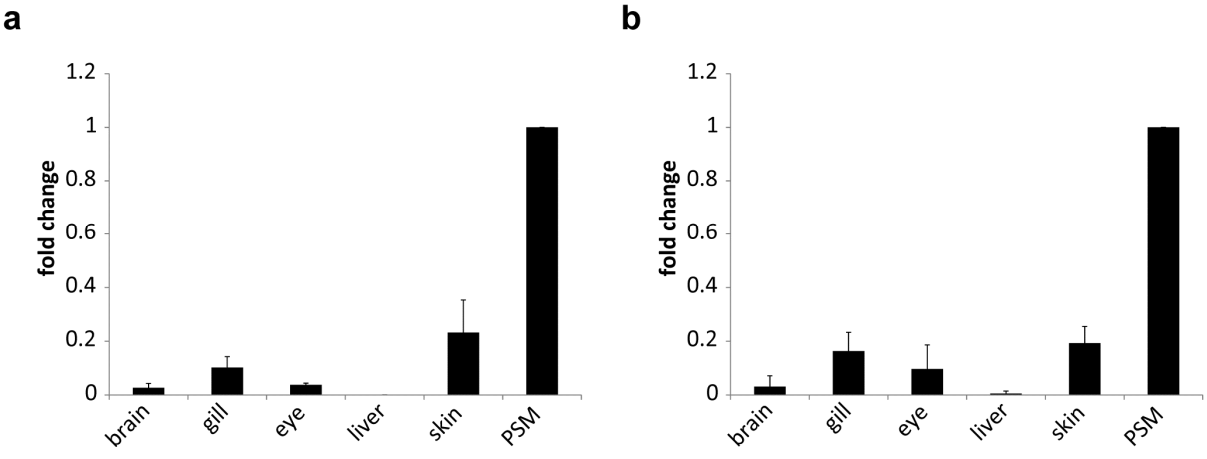


Fig. 10: Expression pattern analysis of the Sr'' and the DrLi (mut) mutant allele. (a) Quantitative real-time PCR analysis of *xmrk* transcript levels in tissues of *X. maculatus* females (WLC 591) carrying the mutant locus Sr''. Data are presented as mean ± standard deviation of only two independent reverse-transcribed RNA samples. (b) *xmrk* mRNA expression levels in tissues of backcross hybrids (WLC 2044) carrying the mutant locus DrLi (mut).

Comparison of *mdl*^{Sr}-*xmrk*^{A} and *mdl*^{Sd}-*xmrk*^{B} expression in gills of *X. maculatus* and backcross hybrids

The previous qPCR analyses clearly demonstrated that both *xmrk* alleles are transcribed at considerable levels in gills of *X. maculatus* and backcross hybrids. Moreover, robust Xmrk^{A} and Xmrk^{B} protein expression was detected in the gills of these fish by Western blot analysis. To determine whether transcription of *xmrk* in gills is influenced by the crossing-conditioned elimination of the regulator locus *R*, a direct comparison of expression levels of both *xmrk* alleles in parental versus hybrid fish was performed by qPCR analysis. For the non-tumorigenic *xmrk*^{A} allele, similar transcript levels were detected in gills of *X. maculatus* (n = 10) and backcross hybrids (n = 9) (Fig. 11a). Expression of the tumorigenic *xmrk*^{B} allele, in contrast, was significantly decreased in gills of backcross hybrids (n = 11) compared to gills of parental platyfish (n = 8) (p = 0.00476) (Fig. 11b). The reason for this difference in expression levels could not be evaluated in the context of the experiments performed in this study.

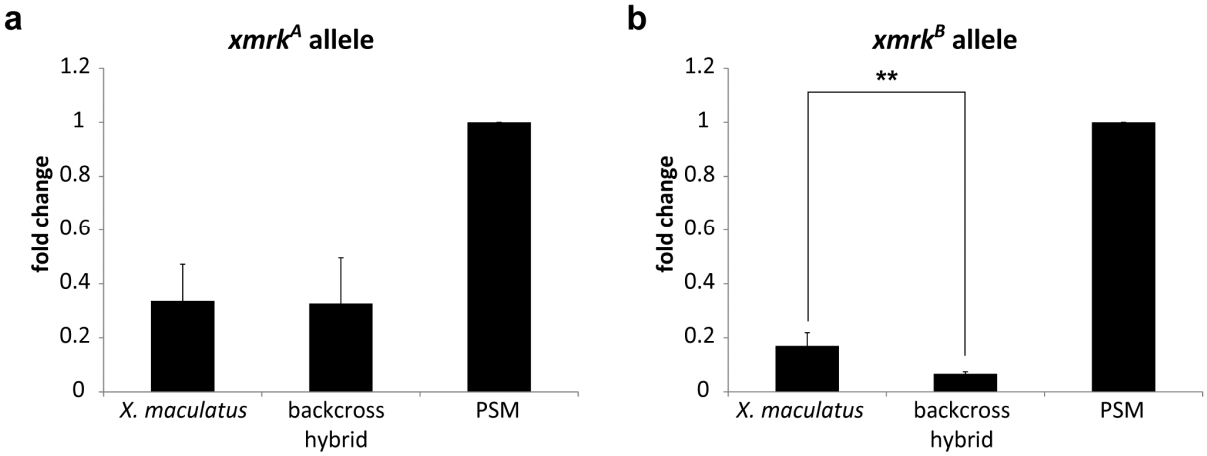


Fig. 11: Comparison of *xmrk*^{A} and *xmrk*^{B} transcript levels in gills of *X. maculatus* and backcross hybrids. (a) Quantitative real-time PCR analysis of *xmrk*^{A} mRNA levels in gills of the parental fish *X. maculatus* (WLC 1375/1750, n = 10) and of backcross hybrids (WLC 1748, n = 9). (b) Analysis of *xmrk*^{B} transcript levels in gills of *X. maculatus* (WLC 1274, n = 8) and of backcross hybrids (WLC 407/534, n = 11). (modified from (Regneri and Schartl, 2012))

Structural analysis of the proximal 5' region of different *mdl-xmrk* alleles

The data from the expression studies indicated that differential transcriptional regulation in the black pigment cell lineage determines the tumorigenic potential of the mdl^{Sr}-$xmrk^{A}$ and the mdl^{Sd}-$xmrk^{B}$ allele. To investigate whether structural differences in the putative *xmrk* promoter region (e.g. the *piggyBac-like* insertion in the mdl^{Sr}-$xmrk^{A}$ upstream region) might be causative for these transcriptional differences, I compared the 5' flanking regions of the tumorigenic mdl^{Sd}-$xmrk^{B}$ and the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele. In addition, I included the tumorigenic $mdl^{Sp\text{-}type}$-$xmrk^{A}$ allele from the *Xiphophorus* melanoma cell lines PSM in the analysis. This class A *xmrk* allele is highly tumorigenic because it leads to melanoma development already in F1 hybrids between *X. maculatus* and *X. hellerii* (Wakamatsu, 1981) and induces pigment cell tumors in transgenic medakas with high penetrance (Schartl et al., 2009). Furthermore, the $xmrk^{A}$ oncogene is highly overexpressed in PSM cells (Fig. 5 and (Dimitrijevic et al., 1998)). For structural analysis of the putative $mdl^{Sp\text{-}type}$-$xmrk^{A}$ promoter region, PCR products covering the proximal 4.4 kb of the 5' flanking region were sequenced and aligned to mdl^{Sd}-$xmrk^{B}$ and mdl^{Sr}-$xmrk^{A}$ upstream sequences (Fig. S3). A schematic drawing of the 5' flanking regions is shown in Fig. 12. Interestingly, the putative promoter regions of the tumorigenic $mdl^{Sp\text{-}type}$-$xmrk^{A}$ and the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele are highly similar. The *piggyBac-like* DNA transposon, which is integrated into the *D* locus sequence of the mdl^{Sr}-$xmrk^{A}$ allele, is present at the same position in the $mdl^{Sp\text{-}type}$-$xmrk^{A}$ allele from PSM cells, whereas it is absent in the 5' region of the mdl^{Sd}-$xmrk^{B}$ allele (Fig. 12). Furthermore, the *piggyBac-like* insertion shows a high sequence similarity between the two $xmrk^{A}$ alleles (Fig. S3). Taken together, these findings strongly indicate that the *piggyBac-like* insertion is not the leading cause for the differential transcriptional control of mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$. Besides the *piggyBac-like* insertion, a 134 bp $xmrk^{A}$ specific fragment was found approximately 3.8 kb upstream of the translational start side in the mdl^{Sr}-$xmrk^{A}$ 5' flanking region. Apart from these insertions, the proximal 4.4 kb of the mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$ 5' regions differ only by 25 nucleotide exchanges and two microindels of 6 bp and 7 bp length (Fig. S3).

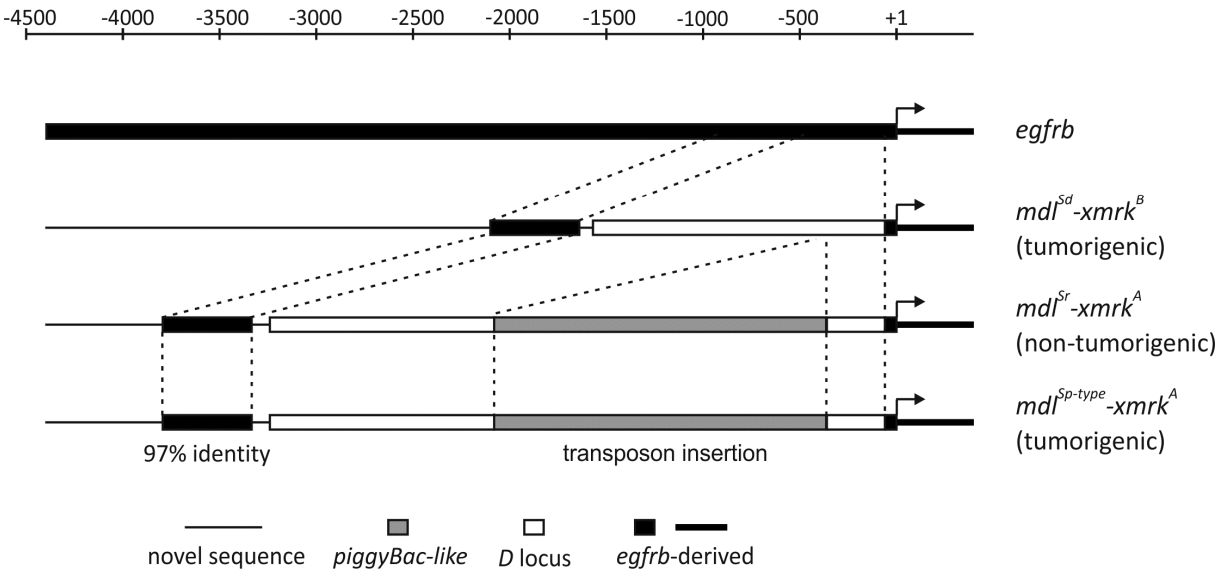
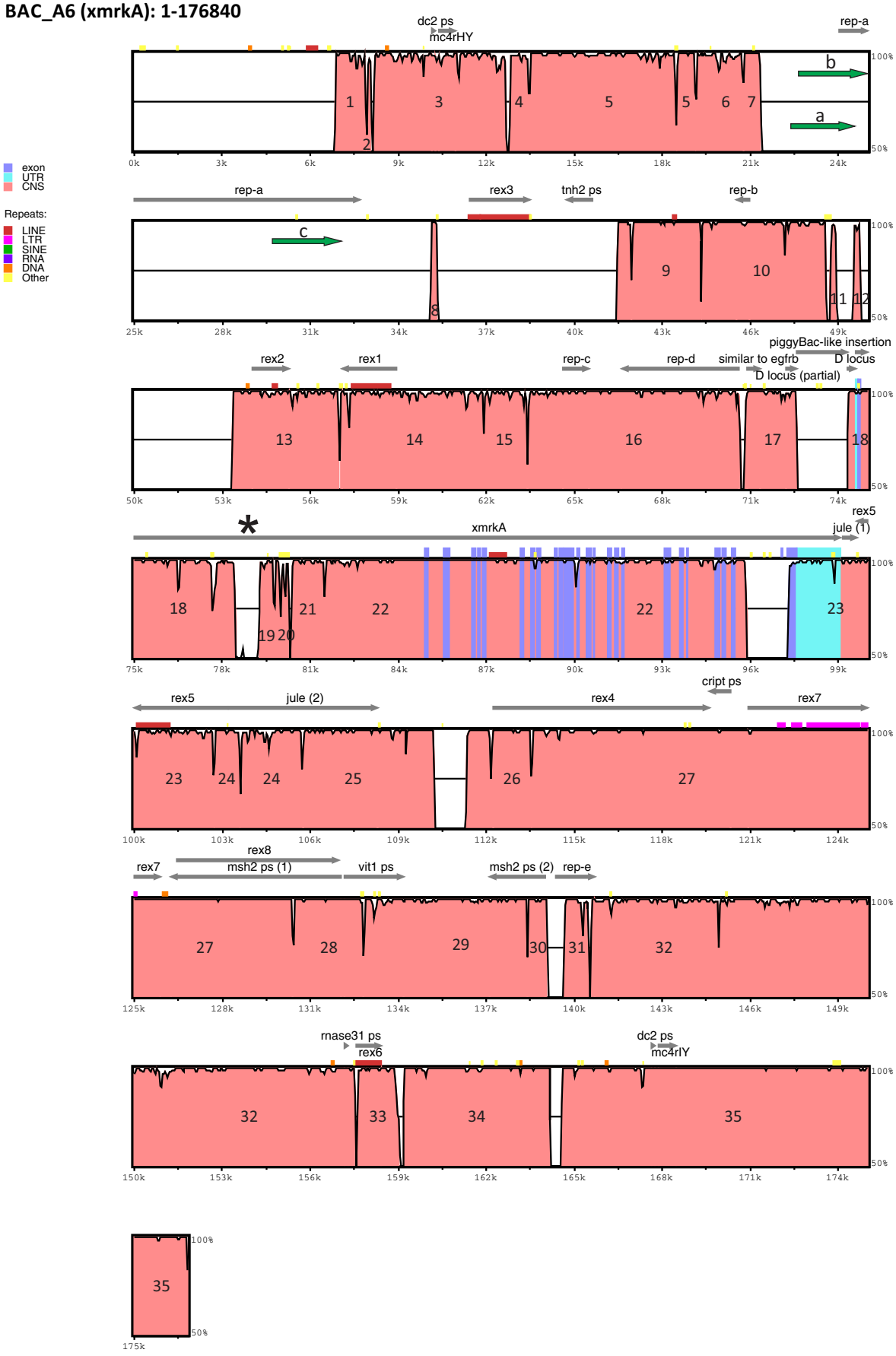


Fig. 12: Schematic drawing of the 5' flanking regions of *egfrb* and different *mdl-xmrk* alleles in *X. maculatus*. A *piggyBac-like* DNA transposon (gray box) is integrated into the *D* locus sequence (white box) of the tumorigenic $mdl^{Sp\text{-}type}$-$xmrk^{A}$ (from PSM cells) and the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele, but is not present at the same position in the *D* locus sequence of the tumorigenic mdl^{Sd}-$xmrk^{B}$ allele. Black boxes indicate the presence of a segment with more than 97% nucleotide identity to the *egfrb* promoter sequence within the *xmrk* promoter region. (modified from (Regneri and Schartl, 2012))

Comparative structural analysis of the mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$ genomic regions

To search for additional structural differences that could be involved in the differential transcriptional control of these *mdl-xmrk* alleles, I compared the genomic insert sequences of *Xiphophorus* BAC clones (Froschauer et al., 2002) containing either the tumorigenic mdl^{Sd}-$xmrk^{B}$ (BAC_A3) or the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele (BAC_A6). The comparative sequence analysis was performed with the online tool mVISTA using the global alignment program AVID. It was shown previously that the *xmrk* oncogene is located in a highly unstable genomic region, where it is encircled by diverse categories of transposable elements, other repetitive sequences, and pseudogenes (Froschauer et al., 2001; Volff et al., 2003). These studies focused however mainly on the genomic regions flanking the mdl^{Sr}-$xmrk^{A}$ allele. To analyze if the identified DNA elements are conserved between the two *mdl-xmrk* alleles, all elements were annotated in the VISTA plots. Interestingly, most repetitive elements and pseudogenes are present at the same position in $xmrk^{A}$ and $xmrk^{B}$ flanking regions and often show a high sequence similarity (Figure 13a, b). This is in line with previous findings from Volff and colleagues (Volff et al., 2003). However, despite several highly conserved regions, the VISTA plots clearly demonstrate that additional allele specific genomic rearrangements have taken place in the *xmrk* flanking regions (Fig. 13a, b).

a **BAC_A6 (xmrkA): 1-176840**



b **BAC_A3 (xmrkB): 1-200000**

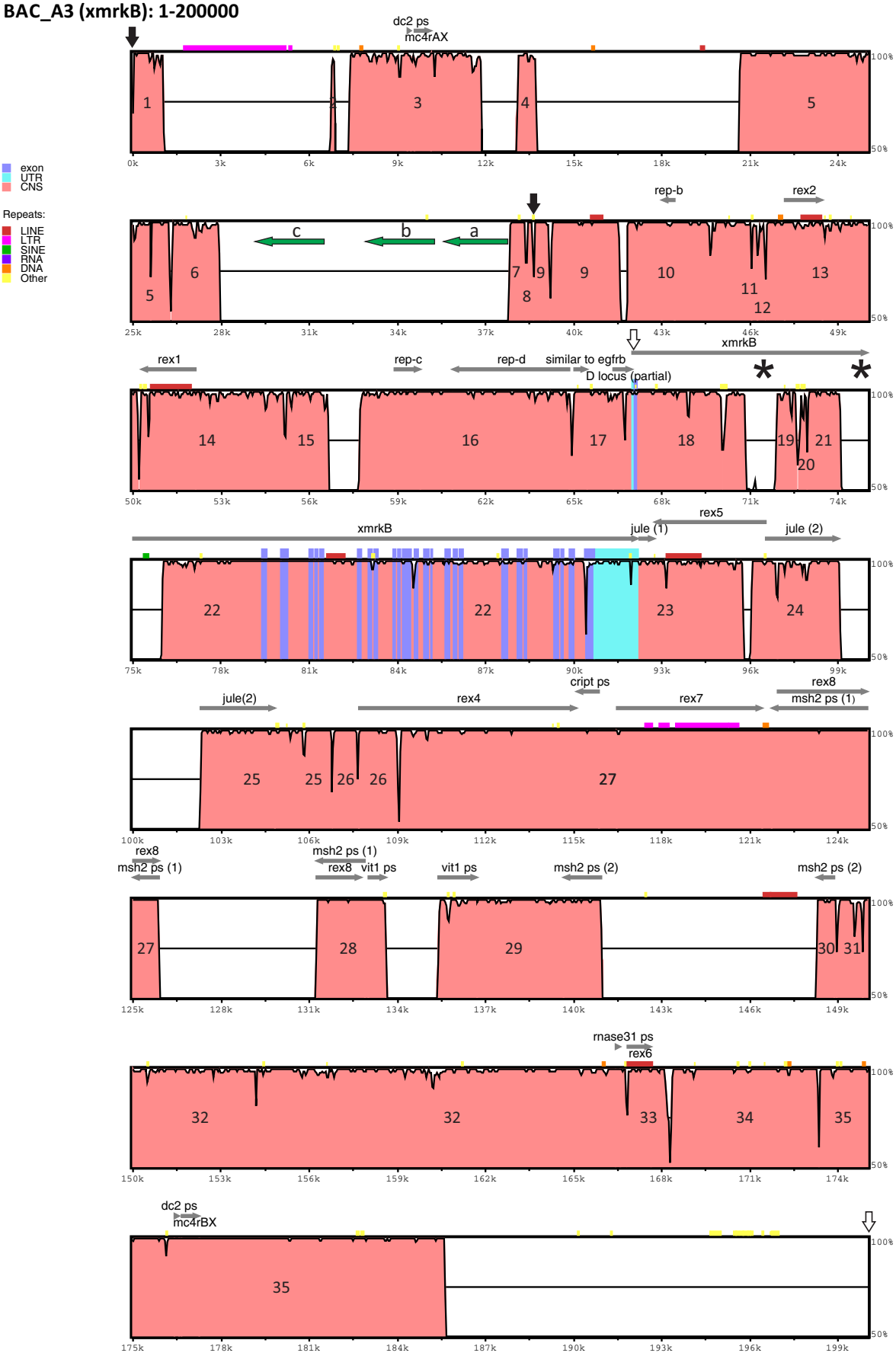


Fig. 13: mVISTA plots showing an AVID alignment of *mdl*^{Sd}-*xmrk*^{B}- (from BAC_A3) and *mdl*^{Sr}-*xmrk*^{A}- (from BAC_A6) containing genomic regions. The level of conservation (vertical axis) is displayed either in the coordinates of the *mdl*^{Sr}-*xmrk*^{A} containing sequence (a) or in the coordinates of the *mdl*^{Sd}-*xmrk*^{B} containing sequence (b). Conserved sequences (70% identity over 50 bp length) are highlighted under the curve with blue indicating a conserved exon, turquoise a conserved untranslated region, and red conserved non-coding sequences. Exons of pseudogenes are not annotated. Correlation of conserved regions in the two Vista plots is facilitated by numbers. Green arrows indicate directions and positions of regions showing high similarity between BAC_A3 and BAC_A6 insert sequences that were detected using the Shuffle-LAGAN alignment program. Asterisks highlight structural differences in the first intron of *xmrk*. Sequences of repetitive elements (*rep* clusters and *rex* retrotransposons) and pseudogenes were obtained from (Volff et al., 2003) (GenBank accession no. AY228504.1, AY298859.1). Deletion sites used to generate BAC_A3_pLUC (*xmrk*^{B}) deletion constructs are marked by vertical arrows in (b) (black arrows for BAC_A3_delup, white arrows for BAC_A3_deldown).

As expected, the transcribed regions of the *xmrk*^{A} and *xmrk*^{B} oncogenes are highly similar (Fig. 13a, b, Fig. S1). Apart from the well described deletion in the *mdl*^{Sd}-*xmrk*^{B} allele (Adam et al., 1991), the only large structural differences were found in intron 1 (highlighted by asterisks in Fig. 13a, b). A fragment (780 bp in *xmrk*^{A}, 1015 bp in *xmrk*^{B}) showing no sequence similarity between the two alleles was detected on the plots. Furthermore, a 1.9 kb fragment was identified in intron 1 of *xmrk*^{B}, which is not present at the same position in the *xmrk*^{A} allele.

The genomic region flanking *xmrk* on its 5' side contains numerous allele specific rearrangements, especially in the more distal part of the sequence analyzed in this study. As described previously (Volff et al., 2003), a *piggyBac-like* DNA transposon is integrated into the *D* locus sequence of the non-tumorigenic *mdl*^{Sr}-*xmrk*^{A} allele (Fig. 13a). Moreover, the Vista plots showed a 1 kb *xmrk*^{B} specific fragment approximately 9.4 kb (Fig. 13b) and a 3.5 kb *xmrk*^{A} specific fragment approximately 21 kb upstream of the *xmrk* start codon (Fig. 13a). Apart from these differences, the proximal part of the *xmrk* 5' flanking region (from conserved region 9 onwards) is relatively well conserved between *mdl*^{Sd}-*xmrk*^{B} and *mdl*^{Sr}-*xmrk*^{A}. This genomic region comprises the *D* locus, the *xir*-associated repeat clusters *rep-d*, *rep-c*, and *rep-b*, and furthermore, the non-long terminal repeat (non-LTR) retrotransposons *rex1* (Volff et al., 2000) and *rex2*. Interestingly, in the region distal thereof several large (up to 10 kb) allele specific fragments were identified (Fig. 13a, b). To further investigate these non-conserved regions, I performed an additional Vista plot analysis using the Shuffle-LAGAN instead of the AVID alignment program, as the former is able to detect rearrangements and inversions (Vista plots not shown). With this analysis, I detected conserved sequence elements (92% - 95% nucleotide identity) within these "non-conserved" regions (indicated by green arrows in Fig. 13a, b). Interestingly, these sequence elements are present in the flanking regions of both *xmrk* alleles, but at different positions and in inverted

orientation. Further sequence analyses revealed *xir*-like sequences (the 461 bp repeat as defined by (Nanda et al., 2000)) within these conserved sequence elements. The *xir* repeat, which has been proposed to correspond to the LTR of a *Xiphophorus* retrovirus (Roushdy et al., 1999), is amplified in the *xmrk* region (Nanda et al., 2000). Thus, these findings strongly indicate that additional rearrangements have taken place in this region. The *rep-a* cluster, the non-LTR retrotransposon *rex3* (Volff et al., 1999), and the pseudogene *tnh2* are absent in the mdl^{Sd}-$xmrk^{B}$ flanking region (Fig. 13b), whereas copies of the *mc4r* gene (*mc4rAX* allele upstream of $xmrk^{B}$, *mc4rHY* allele upstream of $xmrk^{A}$) are present 5' of both *xmrk* alleles.

The genomic region flanking *xmrk* on its 3' side contains less structural variations between the two *mdl-xmrk* alleles (Fig. 13a, b). The Vista plots showed four large insertions in the mdl^{Sd}-$xmrk^{B}$ flanking region, which disrupt the *Ty3/gypsy*-like retrotransposons *jule* (Volff et al., 2001) and *rex8*, as well as the *msh2* and *vit1* pseudogenes (Fig. 13b). In addition, a 1.1 kb $xmrk^{A}$ specific fragment located approximately 11 kb downstream of the polyadenylation site of $xmrk^{A}$ was identified (Fig. 13a). However, apart from these insertions, the *xmrk* 3' flanking region shows a high level of similarity between the two *xmrk* alleles. The *xir*-associated repeat cluster *rep-e* is truncated in the $xmrk^{B}$ genomic region and thus is not annotated (Fig. 13b).

Taken together, the VISTA plots clearly demonstrate that there are numerous structural differences between the mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$ flanking regions, especially in the distal part of the *xmrk* 5' region analyzed here. As the *xmrk* region is rich in transposable elements and other kinds of repetitive sequences, many of these structural differences might represent additional allele specific transposition events. These sequence variations are of great interest, as they might contain distal regulatory elements that contribute to the differential transcriptional regulation of the mdl^{Sr}-$xmrk^{A}$ and the mdl^{Sd}-$xmrk^{B}$ allele.

Transcriptional activity of *mdl*^{Sr}-*xmrk*^{A} and *mdl*^{Sd}-*xmrk*^{B} reporter constructs

The expression analyses performed in this study enable an indirect comparison of *xmrk* transcription levels in tumorous and healthy tissues of different *Xiphophorus* genotypes. To directly quantify the transcriptional activity of different *mdl-xmrk* alleles in melanoma versus non-melanoma cell lines, a luciferase assay was conducted using BAC clones that contain the genomic regions where *egfrb*, *mdl*^{Sr}-*xmrk*^{A}, and *mdl*^{Sd}-*xmrk*^{B} are located as reporter constructs. BAC reporter constructs are powerful tools to analyze transcriptional activity of gene promoters because they include, in contrast to conventional plasmid based reporter systems, distal regulatory sequences and thus are able to recapitulate the endogenous regulation of the gene. The *xmrk* and *egfrb* BAC reporter constructs were generated by inserting the firefly luciferase gene together with a selection marker at the translational start of *xmrk*^{A}, *xmrk*^{B}, and *egfrb* in the respective BAC clones (Fig. 14a). These BAC reporter constructs were transiently transfected in the melanoma cell line PSM and the non-melanoma cell line A2 and luciferase activity was measured 7 days after transfection. As internal control, I generated a BAC reporter construct containing the renilla luciferase gene under control of the *cmv* promoter (BAC_RLUC), which was then cotransfected. A BAC reporter construct was used as internal control because due to their size BACs have a lower transfection efficiency than plasmids and furthermore expression of reporter genes within BACs occurs at lower levels, requiring several days before it can be evaluated (Montigny et al., 2003).

In the non-melanoma cell line A2, the luciferase assay revealed a relatively high transcriptional activity of the reporter construct containing the proto-oncogene *egfrb* (BAC_A4_pLUC), whereas the transcriptional activity of the two *xmrk* reporter constructs (BAC_A3_pLUC and BAC_A6_pLUC) was significantly lower in this cell line (Fig. 14c) ($p = 0.02029$ and $p = 0.02883$, respectively). The high transcriptional activity of *egfrb* in the luciferase assay is consistent with high levels of endogenous *egfrb* transcripts found in A2 cells by semi-quantitative RT-PCR analysis (Fig. 15). A2 cells, which are derived from *X. hellerii* (Fig. S4), are devoid of *xmrk* and of the regulator locus *R* (Fig. S5). However, as A2 cells are a non-pigment cell line, only a low background activity of the *xmrk* reporter constructs was expected in this cell line. In line with this, endogenous expression of *xmrk* could not be detected by semi-quantitative RT-PCR analysis in the *X. maculatus* fibroblast cell line SdSr24 (Fig. 15). In the melanoma cell line PSM, both *xmrk* reporter constructs (BAC_A3_pLUC and BAC_A6_pLUC) showed a significantly higher transcriptional activity ($p = 0.01206$ and $p = 0.00691$, respectively) than the reporter construct containing the proto-oncogene *egfrb* (Fig. 14b). However, the differences in transcription strength between *xmrk*

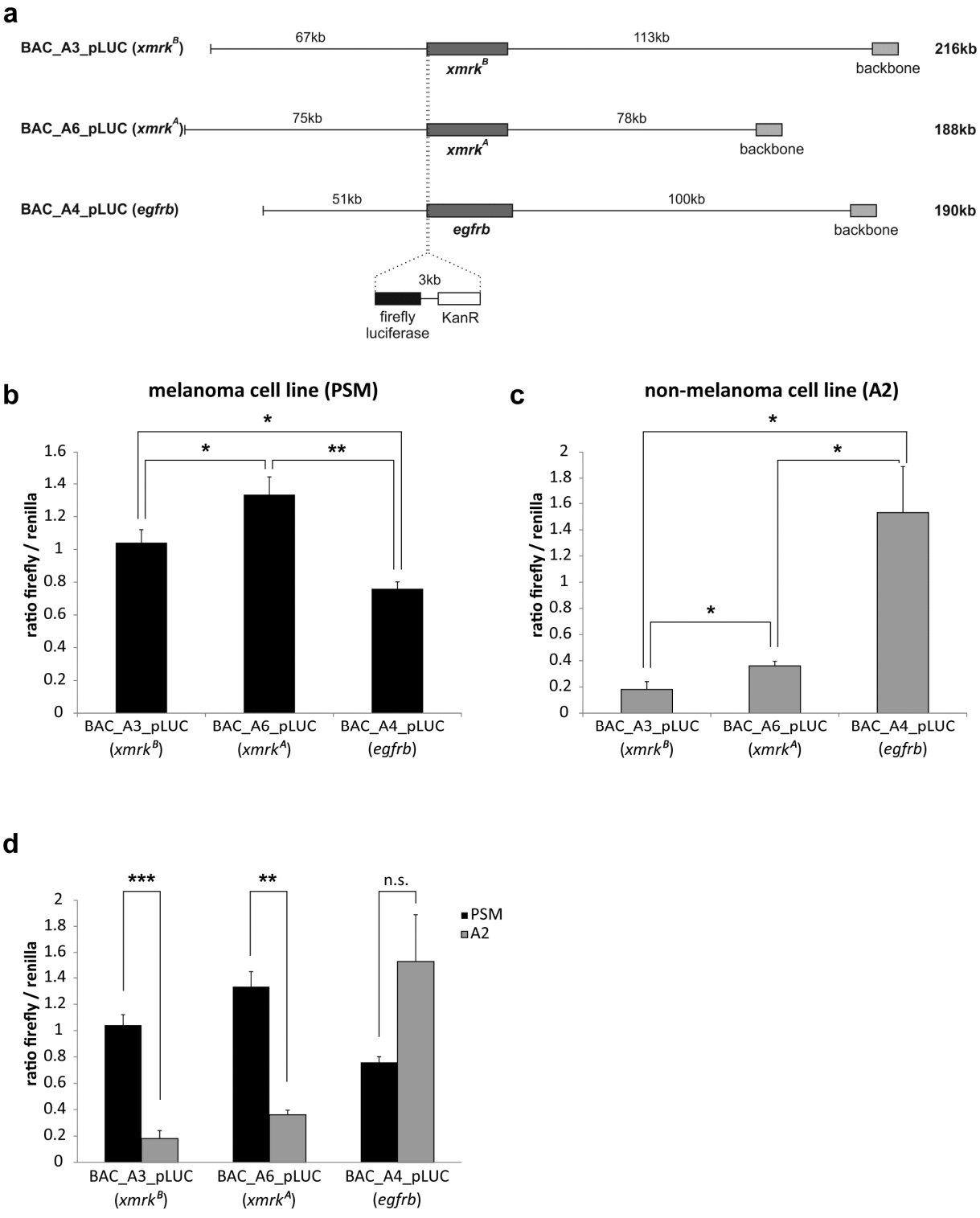


Fig. 14: Analysis of the transcriptional activity of *xmrk* and *egfrb* BAC reporter constructs in *Xiphophorus* melanoma and non-melanoma cell lines. (a) Schematic drawing of the $xmrk^B$ (BAC_A3_pLUC), $xmrk^A$ (BAC_A6_pLUC), and *egfrb* (BAC_A4_pLUC) firefly luciferase reporter constructs. These reporter constructs were generated by inserting the firefly luciferase together with a selection marker at the translation start sites of *xmrk* and *egfrb*. Transcriptional activity of $xmrk^B$, $xmrk^A$, and *egfrb* firefly luciferase reporter constructs was determined (b) in the *Xiphophorus* melanoma cell line PSM and (c) in the non-melanoma cell line A2. (d) Comparison of the transcriptional activity of firefly luciferase reporter constructs in the melanoma cell line PSM and the non-melanoma cell line A2. Activity of the firefly luciferase was normalized to activity of a cotransfected *cmv::renilla luciferase* control BAC (BAC_RLUC).

and *egfrb* reporter constructs were not as distinct as expected from expression studies in cell lines and *Xiphophorus* melanoma tissues. In the melanoma cell line PSM, endogenous expression of *egfrb* could not be detected by semi-quantitative RT-PCR analysis, whereas the *xmrk* oncogene was highly expressed (Fig. 15). In this regard, it has to be considered that the melanoma cell line PSM still contains one copy of the chromosomal region where *R* is located (Fig. S5). As expected, the transcriptional activity of both *xmrk* reporter constructs was significantly higher in the melanoma than in the non-melanoma cell line (Fig. 14d) ($p = 0.00019$ and $p = 0.00238$ for BAC_A3_pLUC and BAC_A6_pLUC, respectively). The *egfrb* reporter construct, in contrast, was transcriptionally less active in the melanoma cell line PSM than in the embryonic fibroblast cell line A2. Thus, this luciferase assay showed for the first time a melanoma cell specific transcriptional activation of *xmrk* by its flanking regions.

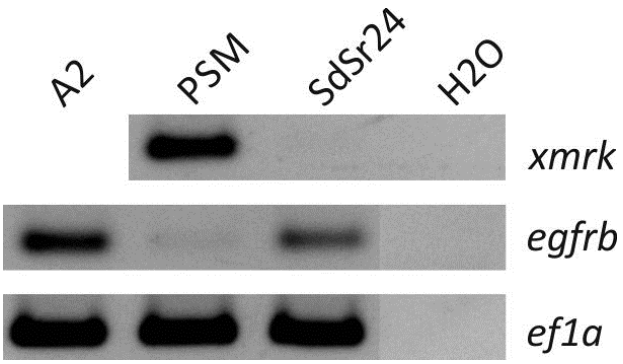


Fig. 15: Endogenous expression of *xmrk* and *egfrb* in *Xiphophorus* cell lines. Transcript levels of the *xmrk* oncogene, the proto-oncogene *egfrb*, and the housekeeping gene *ef1a* in A2, PSM, and SdSr24 cells were determined by semi-quantitative RT-PCR analysis. A2 cells are devoid of *xmrk*. PCR amplification was run for 35 cycles.

Surprisingly, the data from the luciferase assay indicate that in the melanoma cell line PSM the non-tumorigenic $xmrk^{A}$ allele has a significantly higher transcriptional activity than the tumorigenic $xmrk^{B}$ allele (Fig. 14b) ($p = 0.02645$). This was unexpected as the data from the expression studies suggest that only the highly tumorigenic mdl^{Sd}-$xmrk^{B}$ allele is transcriptionally activated in melanoma cells, whereas transcription of the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele is not influenced by crossing-conditioned elimination of *R*. For this reason, a significantly lower transcriptional activity of the $xmrk^{A}$ than of the $xmrk^{B}$ reporter construct was expected in the melanoma cell line. Thus, the *in vitro* activity of the reporter constructs, especially the activity of the *xmrk* reporter constructs, does not completely reproduce the transcript levels of their endogenous counterparts.

Analysis of a deletion series of the *mdl*^{Sd}-*xmrk*^{B} reporter construct

By using BAC clones as reporter constructs for luciferase assays, I was able to show for the first time a melanoma cell specific transcriptional activation of the *xmrk*^{B} oncogene by its flanking regions. To narrow down the location of distal regulatory elements important for transcriptional control of *xmrk*^{B}, a deletion series of the *xmrk*^{B} reporter construct (BAC_A3_pLUC) was generated by BAC recombineering. This approach should provide evidence how different parts of the *xmrk* flanking region influence transcriptional activity.

The Vista plots demonstrated that the distal part of the *xmrk* 5' flanking region (upstream of conserved region 9, Fig. 13b) contains several large rearrangements and is less conserved than the regions 3' thereof. To determine whether distal regulatory elements important for transcriptional control of *xmrk*^{B} are located in this region, this part of the BAC_A3_pLUC reporter construct was deleted by homologous recombination (black arrows in Fig. 13b mark deletion sites). As shown in Fig. 16a, the newly generated BAC_A3_pLUC_delup reporter construct contains the proximal part of the *xmrk*^{B} 5' region (28 kb), the *xmrk*^{B} gene itself, and 113 kb 3' flanking sequence. To test if transcriptional regulatory elements are present downstream of the proximal promoter in intron or 3' flanking sequences, I generated the BAC_A3_pLUC_deldown construct. This construct contains the full-length *xmrk*^{B} 5' region (67 kb), but the *xmrk* gene and the regions downstream thereof are deleted (Fig. 16a) (white arrows in Fig. 13b mark deletion sites). In addition, the BAC_A3_pLUC_delup+down reporter construct was generated, which only contains the proximal part (28 kb) of the *xmrk*^{B} 5' sequence (Fig. 16a). The BAC_A3_pLUC full-length and deletion constructs were transiently transfected in PSM and A2 cells and luciferase activity was measured 7 days after transfection. To exclude erroneous luciferase data due to differences in the transfection rates between BAC_A3_pLUC full-length and deletion constructs, I additionally generated deletion constructs of the same size of the renilla luciferase control BAC (BAC_RLUC), which were then cotransfected.

As already shown in Fig. 14d, the full-length *xmrk*^{B} reporter construct (BAC_A3_pLUC) had a significantly higher transcriptional activity in the melanoma cell line PSM than in the non-melanoma cell line A2 (Fig. 16d) ($p = 2.22x10^{-06}$), which mirrors the endogenous expression levels of *xmrk* in these cell lines (Fig. 15). Interestingly, deletion of the distal part of the *xmrk*^{B} 5' flanking region (BAC_A3_pLUC_delup) significantly decreased transcriptional activity in melanoma cells (Fig. 16b) ($p = 0.00121$), whereas the transcriptional activity in the non-melanoma cell line A2 was slightly increased (Fig. 16c), hinting at pigment

cell specific distal enhancer elements in the deleted 39 kb fragment. However, BAC_A3_pLUC_delup still had a 1.8 fold higher transcriptional activity in the melanoma than in the non-melanoma cell line (Fig. 16d) ($p = 0.02501$) (compared to 6.5 fold for the full-length construct). Deletion of the *xmrk*^{B} gene and the region downstream thereof (BAC_A3_pLUC_deldown), in contrast, significantly increased the transcriptional activity in PSM cells (Fig. 16b) ($p = 0.00232$), suggesting silencer elements in this region that repress transcription in pigment cells. In the non-melanoma cell line A2, the BAC_A3_pLUC_deldown as well as the BAC_A3_pLUC_delup+down deletion construct showed a significantly lower transcriptional activity than the full-length reporter construct (Fig. 16c) ($p = 0.02502$ and 0.02983, respectively). This finding hints at distal enhancer elements important for transcriptional regulation of *xmrk*^{B} in this non-pigment cell line in *xmrk*^{B} introns or 3' flanking sequences. Interestingly, in the melanoma cell line PSM the BAC_A3_pLUC_delup+down and the full-length reporter construct showed a comparable transcriptional activity (Fig. 16b), indicating that the combinatorial activities of putative enhancer and silencer elements in the deleted regions might have a compensatory effect. As already shown for the full-length reporter construct, BAC_A3_pLUC_deldown and BAC_A3_pLUC_delup+down showed a considerably higher (55.8 and 21.7 fold, respectively) transcriptional activity in PSM than in A2 cells (Fig. 16d) ($p = 0.00065$ and $p = 0.00027$, respectively). This strongly suggests that the full-length *xmrk*^{B} 5' region (67 kb), but also the proximal 28 kb alone, are able to induce pigment cell specific transcriptional activation. Notably, removal of the distal 39 kb of the *xmrk*^{B} 5' region from BAC_A3_deldown, similar to removal of this region from the full-length reporter construct, resulted in a significantly reduced transcriptional activity in the melanoma cell line PSM (Fig. 16b) ($p = 0.00171$), whereas it had no effect on the transcriptional activity in A2 cells (Fig. 16c). This suggests that this part of the *xmrk*^{B} 5' region contains distal enhancer elements, which contribute to transcriptional activation of *xmrk*^{B} in melanoma cells. However, the melanoma cell specific activity of the BAC_A3_pLUC_delup+down construct strongly indicates that the proximal part of the *xmrk*^{B} 5' region alone is sufficient to induce pigment cell specific transcriptional activation of the *xmrk*^{B} oncogene.

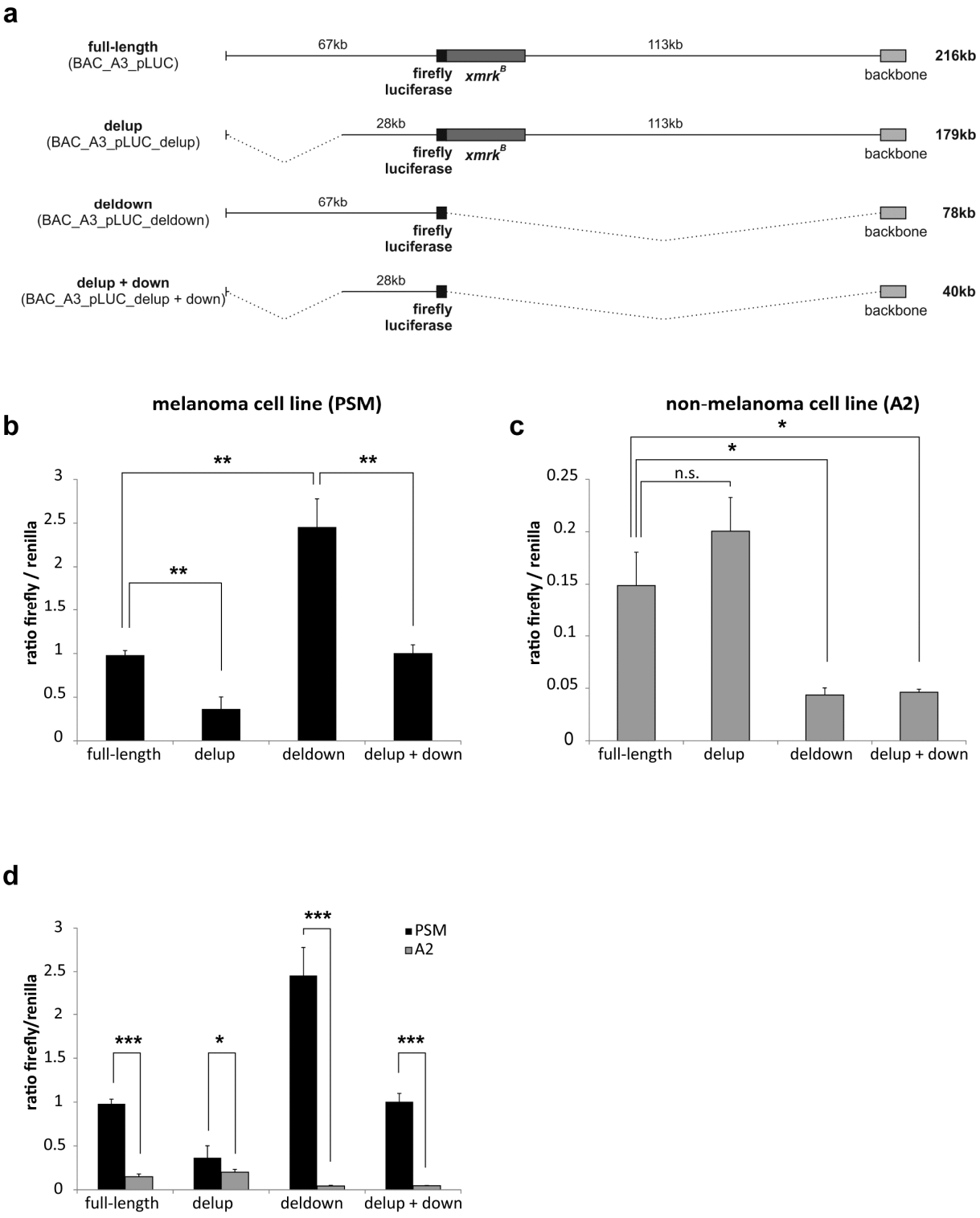


Fig. 16: Analysis of the transcriptional activity of BAC_A3_pLUC full-length and deletion constructs in *Xiphophorus* melanoma and non-melanoma cell lines. (a) A deletion series of the *xmrk*^{B} firefly luciferase BAC reporter construct (BAC_A3_pLUC) was generated by BAC recombineering. The transcriptional activity of the full-length and different deletion constructs was measured in (b) the *Xiphophorus* melanoma cell line PSM and (c) the non-melanoma cell line A2. (d) Comparison of the transcriptional activity of BAC_A3_pLUC full-length and deletion constructs in the melanoma cell line PSM and the non-melanoma cell line A2. For normalization, firefly luciferase reporter constructs were cotransfected with *cmv::renilla luciferase* control BACs. BAC_A3_pLUC and BAC_A3_delup were cotransfected with BAC_RLUC, BAC_A3_deldown was cotransfected with BAC_RLUC_85kb and BAC_A3_delup+down was cotransfected with BAC_RLUC_40kb.

Analysis of candidates for the *R* locus-encoded gene

So far, only one candidate gene for the *R* locus has been postulated based on genetic linkage studies and analyzed further, namely the *cdkn2ab* gene. Recently, the full genomic sequence of *Xiphophorus maculatus* has been published (Schartl et al., 2013) and is now available on the Ensembl genome browser (http://www.ensembl.org/Xiphophorus_maculatus/Info/Index?db=core). This now allows the identification of genes in proximity to *cdkn2ab* (Fig. 17) and to evaluate if they are critical constituents of the tumor suppressor locus *R*. Three of these candidate genes, namely *mtap*, *pdcd4a*, and *cxxc4*, were analyzed for concordant expression in *Xiphophorus* melanoma tissues versus pigment spots and normal skin. Furthermore, I analyzed *cdkn2ab* mRNA and protein expression in *Xiphophorus* tissues in more detail.

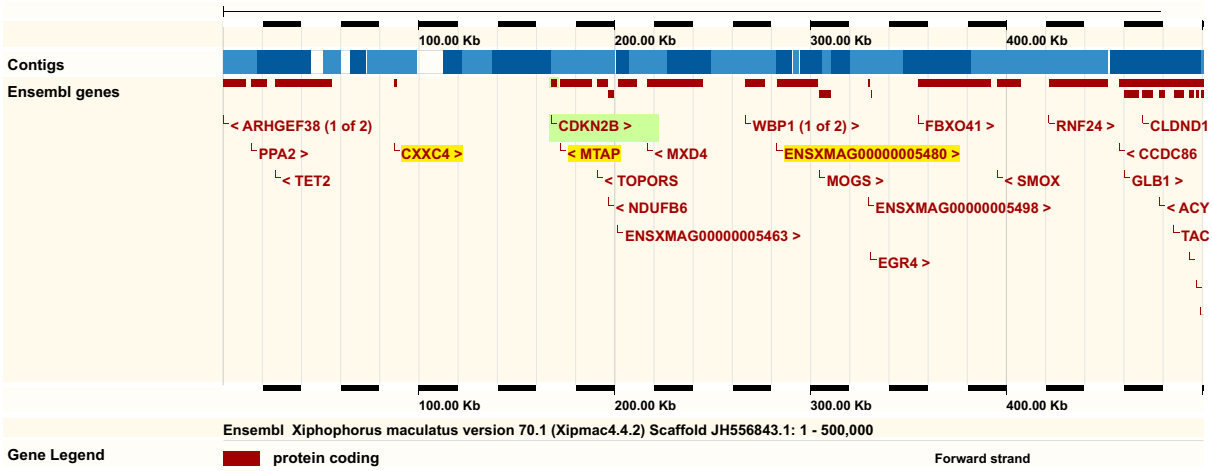
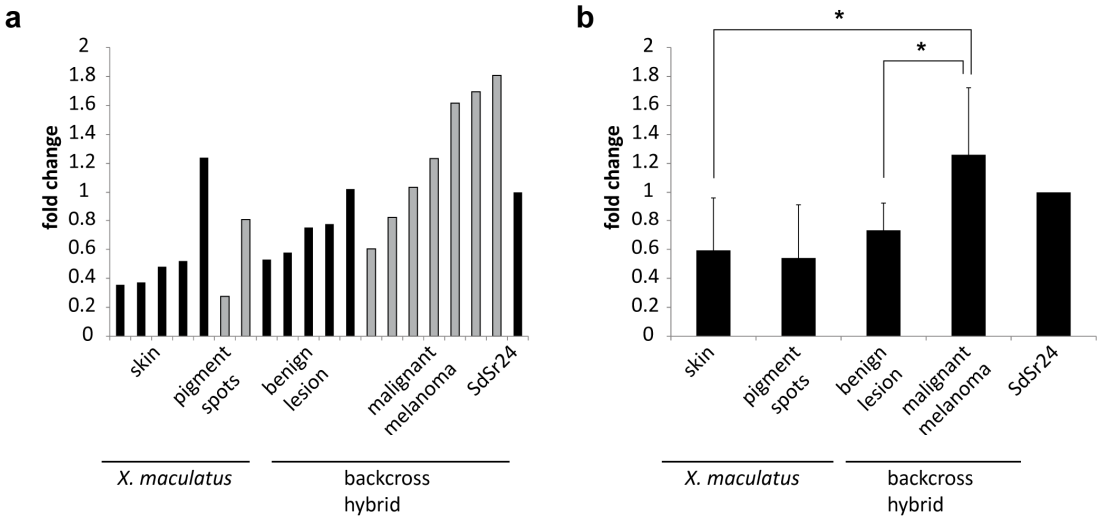


Fig. 17: The newly identified *R* locus candidate genes *mtap*, *pdcd4a* (ENSXMAG00000005480), and *cxxc4* are located in close proximity to *cdkn2ab* in the *Xiphophorus maculatus* genome. 500 kb of scaffold JH556843.1 from the *Xiphophorus* genome assembly Xipmac4.4.2 (Ensembl 70) are shown. *cdkn2ab* is highlighted in green, whereas new potential *R* locus genes analyzed in this study are highlighted in yellow.

mtap

The *mtap* gene is located in close proximity to *cdkn2ab* in the *Xiphophorus* genome (Fig. 17). Concordantly, in the human genome *MTAP* is located in immediate vicinity to the tumor suppressor genes *CDKN2A* and *CDKN2B* on chromosome 9p21, a region that is often affected by deletions in malignant melanoma (Sini et al., 2007). It has been shown that MTAP protein expression is significantly reduced in primary malignant melanomas and melanoma metastases compared to benign nevi and primary melanocytes (Meyer et al., 2009; Wild et al., 2006). Moreover, expression of MTAP was reported to be inversely correlated with progression of melanoma and invasive potential (Behrmann et al., 2003; Stevens et al., 2009), making *mtap* a promising candidate for the crucial *R* locus-encoded gene. To determine whether *mtap* expression is reduced in melanoma tissues of *Xiphophorus*, *mtap* transcript levels in macromelanophore spots and normal skin of *X. maculatus* and in several benign lesions and malignant melanomas of backcross hybrids were compared. QPCR analysis revealed however that *mtap* mRNA levels are not decreased in malignant melanomas. In contrast, I even detected a 2 fold increased expression in malignant melanoma tissues of backcross hybrids compared to skin and macromelanophore spots of purebred platyfish (Fig. 18a, b). To investigate if Mtap is mutationally inactivated in tumor-bearing backcross hybrids, *mtap* mRNA sequences from *X. maculatus* (WLC 1274) and *X. hellerii* (WLC 1337) were determined and compared (Fig. 18c, d) (backcross hybrids have the *X. hellerii* allele). No inactivating mutations were found in the *X. hellerii* Mtap protein compared to the *X. maculatus* Mtap sequence from the Ensembl database (Fig. 18c). But surprisingly, a Gly to Ser amino acid exchange at position 157 was detected in one of the two *X. maculatus* sequences determined in this study. However, as only two clones were sequenced, this exchange could be a PCR-induced sequence artifact. No sequence variations were found in the *mtap* 5' UTR (data not shown). Interestingly, several sequence differences in the *mtap* 3' UTR were detected between the *X. maculatus* and the *X. hellerii* allele (Fig. 18d). However, whether these sequence variations have an impact on mRNA stability and consequently on Mtap protein levels remains to be elucidated. The high expression level of *mtap* in malignant melanomas of *Xiphophorus*, together with the absence of inactivating mutations in the *X. hellerii* protein, speaks against a tumor-suppressing activity of this gene in the *Xiphophorus* melanoma model. Thus, these data strongly indicate that *mtap* is not the critical constituent of the *R* locus.



c **Mtap protein**

```
X. maculatus ensembl  MASSVPVKIGIIGGSGLDDPDILEGRTERYVDTPYGKPSDALILGKIKNVECVLLARHGRQHTIMPSNVNYQANIWALRE 80
X. maculatus clone 1  ..................................................................................... 80
X. maculatus clone 2  ..................................................................................... 80
X. hellerii clone 1    ..................................................................................... 80
X. hellerii clone 2    ..................................................................................... 80

X. maculatus ensembl  EGCTHLVVTTACGSLREEIQPGDIVIIDQFIDRTTKRPQTLYDGQPTSPPGVCHIPMAEPFCNKTREVLVEVARSLGVKC 160
X. maculatus clone 1  .....................................................................................  160
X. maculatus clone 2  .....................................................................................S... 160
X. hellerii clone 1    .....................................................................................  160
X. hellerii clone 2    .....................................................................................  160

X. maculatus ensembl  HVRGTMLTIEGPRFSSRAESLMFRQWGADVINMTTVPEVVLAKEAGLCYASIAMATDYDCWKEHEEAVCVDNVLKTMKEN 240
X. maculatus clone 1  ..................................................................................... 240
X. maculatus clone 2  ..................................................................................... 240
X. hellerii clone 1    ..................................................................................... 240
X. hellerii clone 2    ..................................................................................... 240

X. maculatus ensembl  ANKASSILLTAIPQISQMDWTQTTKNLKSMAQSSVMLPKH* 281
X. maculatus clone 1  ..................................*  281
X. maculatus clone 2  ..................................*  281
X. hellerii clone 1    ..................................*  281
X. hellerii clone 2    ..................................*  281
```

d ***mtap* 3‘ UTR (partial)**

```
X. maculatus ensembl  GTGGGCGTAGAAGACATGAAGCAGAGAAGAGGGGGGGGGGGGGG--AACAGACACCACGTGCACGTATCCCAGCACAGAC 78
X. maculatus clone 1  ........................................---.................................... 77
X. maculatus clone 2  ..........................................GG.................................... 80
X. hellerii clone 1    .......................A..........AAAAAAA---.................................... 77
X. hellerii clone 2    .......................A..........AAAAAAA---.................................... 77

X. maculatus ensembl  GGCGGACTCTGACTCTGTCTGTGGATTCATTATCGGTGAATCACAAGAAAAGTGCCTCACAGGAATAATGCACCTCCG 156
X. maculatus clone 1  ..................................................................................... 155
X. maculatus clone 2  ..................................C...................................... 158
X. hellerii clone 1    ..................................................T..................................... 155
X. hellerii clone 2    ..................................................T..................................... 155
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Fig. 18: Evaluation of *mtap* as potential candidate for the crucial *R* locus gene. (a) Quantitative real-time PCR analysis of *mtap* mRNA expression levels in skin (n = 5) and pigment spots (n = 2) of *X. maculatus* (WLC 1274), and in benign melanocytic precursor lesions (n = 5) and malignant melanomas (n = 7) of backcross hybrids (WLC 534/407). *mtap* expression in the embryonic fibroblast cell line SdSr24 was set as reference. (b) The bar chart shows mean *mtap* expression levels in the indicated tissues. The same set of samples as for (a) was used. ($p = 0.01997$ and $p = 0.02524$ for normal skin and benign lesions, respectively). (c, d) Comparison of *mtap* sequences from *X. hellerii* and *X. maculatus*. Partial *mtap* mRNA sequences of *X. hellerii* and *X. maculatus* were determined and compared to the *X. maculatus mtap* sequence from the Ensembl database (Gene ID ENSXMAG00000005446). Alignments of Mtap protein and partial *mtap* 3’ UTR sequences are depicted in (c) and (d), respectively. Sequence variations are highlighted in gray. Dots indicate conserved sites.

pdcd4a

PDCD4, the human ortholog of *pdcd4a*, is known as a potential tumor suppressor gene since several years (reviewed in (Lankat-Buttgereit and Göke, 2009)) and has recently been implicated in melanoma development and progression in a single study (Guo, 2013). Interestingly, PDCD4 was found to be downregulated in a variety of tumors, including different cutaneous lesions (Matsuhashi et al., 2007) and melanoma specimens (Guo, 2013).

As a first approach to test if the potential tumor suppressor gene *pdcd4a* is a promising candidate for the crucial *R* locus-encoded gene, *pdcd4a* transcript levels in *Xiphophorus* melanoma tissues were determined by qPCR analysis. Interestingly, I detected a strong downregulation of *pdcd4a* expression levels in malignant melanomas and benign lesions of backcross hybrids compared to normal skin ($p = 0.00394$ and 0.00126, respectively) and pigment spots of *X. maculatus* (Fig. 19a, b). This makes *pdcd4a* a promising new candidate for the crucial *R* locus-encoded gene, which should be further investigated.

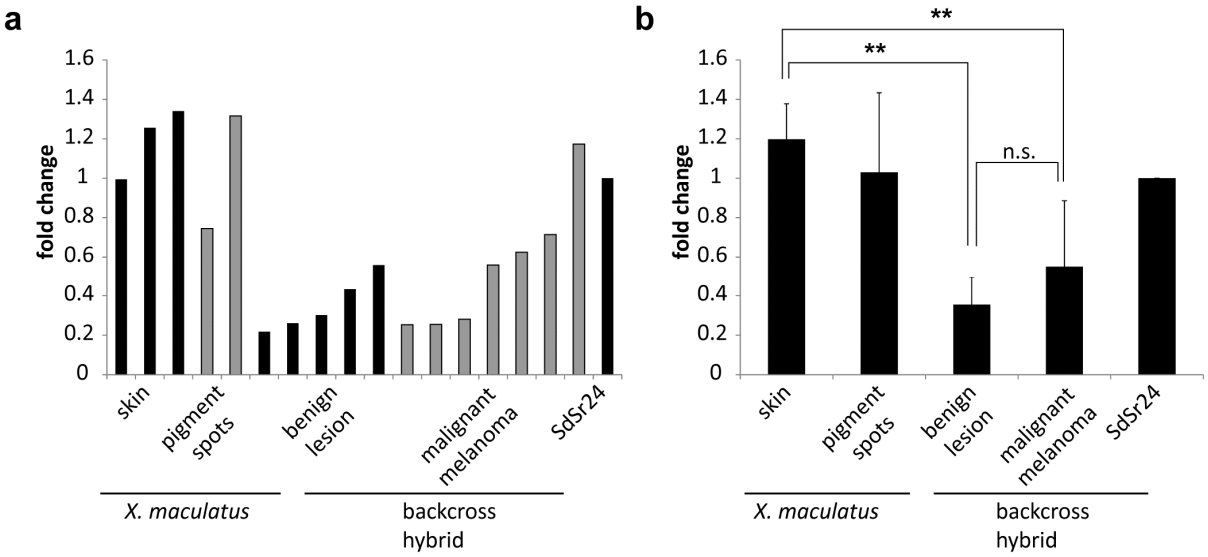


Fig. 19: Comparison of *pdcd4a* transcript levels in the macromelanophore lineage of *Xiphophorus* by quantitative real-time PCR analysis. (a) *pdcd4a* mRNA levels in skin (n = 3) and pigment spots (n = 2) of *X. maculatus* (WLC 1274), and in benign lesions (n = 5) and malignant melanomas (n = 7) of backcross hybrids (WLC 534/407) are shown. The *Xiphophorus* embryonic fibroblast cell line SdSr24 was used as reference. (b) Mean *pdcd4a* transcript levels in the same set of samples used for (a).

cxxc4

Next, I tested the potential of *cxxc4* (*cxxc finger protein 4*, alias *idax*) to be a critical constituent of the tumor suppressor locus *R*. The human ortholog of this gene, *CXXC4*, acts as a negative regulator of Wnt signaling by interaction with DVL1 (Hino et al., 2001). The Wnt signaling pathway is important for melanocyte development and deregulation of this pathway has been implicated in malignant transformation of melanocytes (reviewed in (Lucero et al., 2010; O'Connell and Weeraratna, 2009)). Thus far, *CXXC4* has not been associated with melanoma development, but it has been reported that decreased levels of *CXXC4* promote a more aggressive phenotype in renal cell carcinoma (Kojima et al., 2008). Hence, *cxxc4* is an interesting new candidate for the crucial *R* locus-encoded gene. To analyze whether this gene plays an essential role for melanoma development in *Xiphophorus*, I at first determined *cxxc4* mRNA expression levels in tissues of *X. maculatus* (WLC 1274) and tumor-bearing backcross hybrids (WLC 407). QPCR analysis revealed that *cxxc4* is abundantly expressed in brain and eye, whereas expression in gill, liver, skin, and melanoma could hardly be detected (ct values > 32) (Fig. 20a, b). Thus, *cxxc4* is expressed, if at all, only on a very low background level in the macromelanophore lineage, speaking against a tumor-suppressing activity of this gene in the *Xiphophorus* melanoma model.

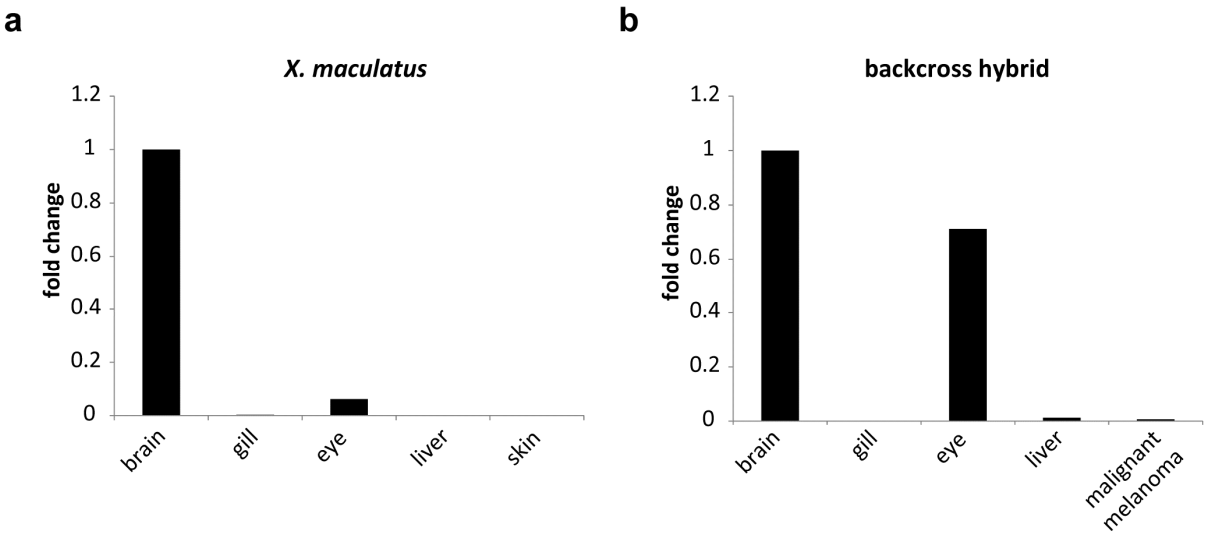


Fig. 20: Analysis of *cxxc4* mRNA expression in *Xiphophorus* tissues. Quantitative real-time PCR analysis of *cxxc4* transcript levels in different tissues of (a) parental platyfish (WLC 1274) and (b) tumor-bearing backcross hybrids (WLC 407). *cxxc4* mRNA expression levels are shown relative to expression in brain.

cdkn2ab

Previous studies have demonstrated that the *R* locus candidate gene *cdkn2ab* is significantly overexpressed in *Xiphophorus* melanomas compared to skin and other non-tumorous tissues (Kazianis et al., 2000). Furthermore, a positive correlation between *xmrk* and *cdkn2ab* expression levels was shown in *Xiphophorus* melanoma tissues (Butler et al., 2007b).

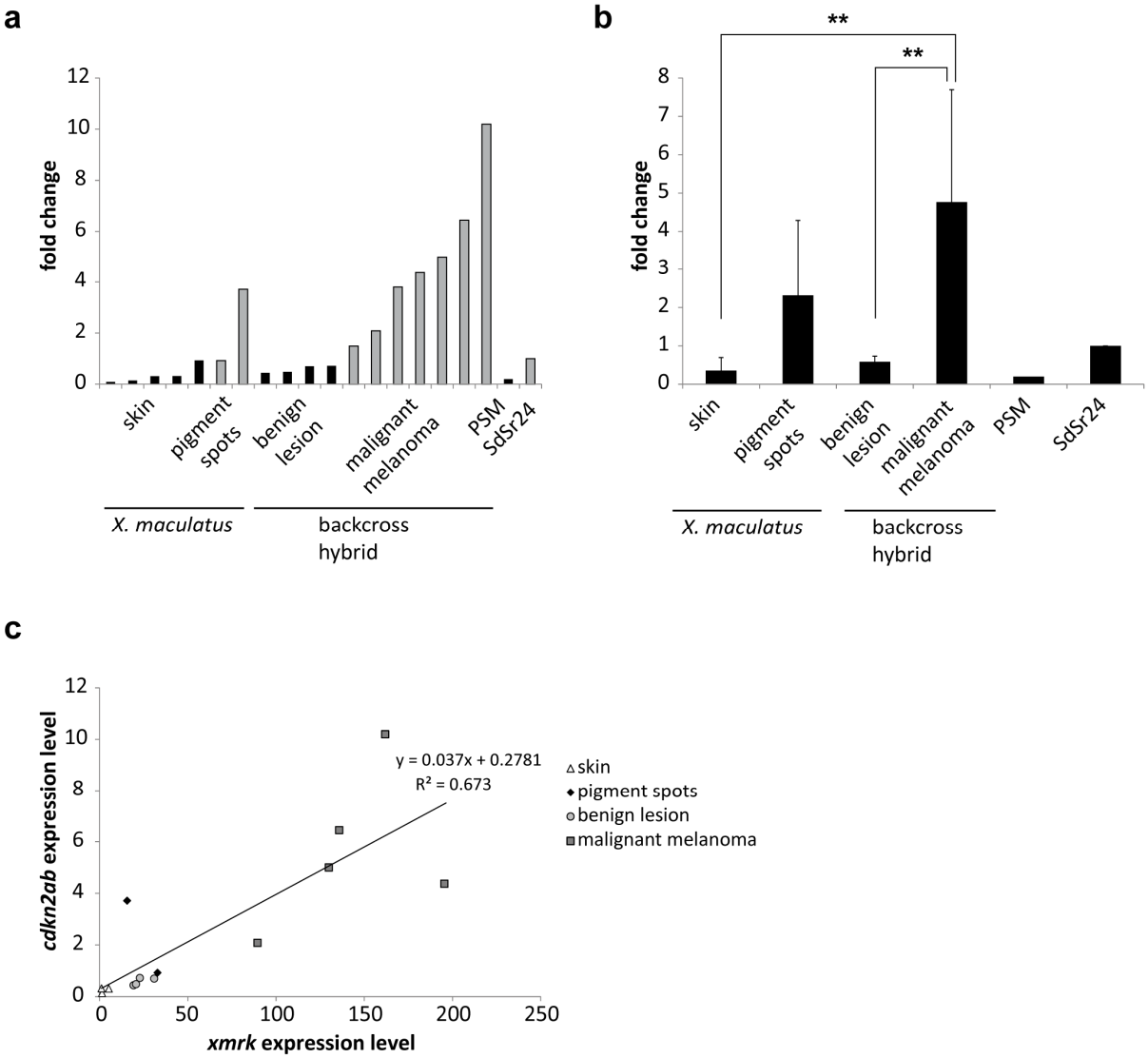


Fig. 21: Transcript levels of *cdkn2ab* in the macromelanophore lineage of *Xiphophorus*. (a) Quantitative real-time PCR analysis of *cdkn2ab* mRNA expression levels in skin (n = 5) and pigment spots (n = 2) of *X. maculatus* (WLC 1274), in benign melanocytic precursor lesions (n = 4) and malignant melanomas (n = 7) of backcross hybrids (WLC 534/407), and in the *Xiphophorus* melanoma cell line PSM. *cdkn2ab* expression in the embryonic fibroblast cell line SdSr24 was set as reference. (b) Mean *cdkn2ab* transcript levels in the indicated tissues. The same set of samples as for (a) was used. (c) Correlation of *cdkn2ab* (Y axis) and $xmrk^{B}$ (X axis) mRNA expression levels in skin (n = 3), in two independent preparations of macromelanophore spots, and in individual benign precursor lesions (n = 4) and malignant melanomas (n = 5). Quantitative real-time PCR was used to determine the relative abundance of *cdkn2ab* and $xmrk^{B}$ transcripts in the indicated samples. Transcript levels are shown relative to expression of *cdkn2ab* in SdSr24 cells.

To further investigate mRNA and protein expression of *cdkn2ab* in the *Xiphophorus* melanoma system, I at first determined *cdkn2ab* transcript levels in the macromelanophore

lineage in more detail. To this end, *cdkn2ab* mRNA levels in normal skin and accurately cut out dorsal fin macromelanophore spots of *X. maculatus* and in several benign melanocytic lesions and malignant melanomas of backcross hybrids were determined by qPCR analysis (Fig. 21a, b). As expected from previous data, *cdkn2ab* transcript levels were significantly increased in malignant melanomas of backcross hybrids compared to normal skin of *X. maculatus* ($p = 0.00700$). A slight increase in malignant melanomas compared to macromelanophore spots was detected, which was however not statistically significant, as the two pigment spot samples varied considerably in their *cdkn2ab* expression levels. Notably, *cdkn2ab* transcript levels were also significantly enhanced in malignant melanomas compared to benign lesions ($p = 0.00910$), whereas *cdkn2ab* expression in benign lesions and normal skin of *X. maculatus* was on similar level. Thus, comparable to the situation found for $xmrk^{B}$ (Fig. 5f, g), *cdkn2ab* transcription is strongly increased in malignant tumors, whereas expression in benign melanocytic precursor lesions is not increased compared to the macromelanophore lineage of purebred platyfish (Fig. 21a, b). Moreover, as already seen for $xmrk^{B}$ (Fig. 5f), *cdkn2ab* expression levels varied considerably between individual malignant melanoma samples (Fig. 21a). To investigate whether *cdkn2ab* and *xmrk* mRNA expression levels are correlated in these tissues, I directly compared the abundance of *xmrk* and *cdkn2ab* transcripts in skin, pigment spots, and individual melanoma samples (Fig. 21c). The result of this analysis indicates that $xmrk^{B}$ and *cdkn2ab* transcript levels are indeed positively correlated in the *Xiphophorus* macromelanophore lineage.

Next, I determined *cdkn2ab* mRNA and protein expression levels in tissues of *X. maculatus* and backcross hybrids carrying either the tumorigenic mdl^{Sd}-$xmrk^{B}$ or the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele. QPCR analysis revealed that *cdkn2ab* is transcribed in most tissues of parental platyfish and backcross hybrids, but at considerably different levels (Fig. 22). Strikingly, high *cdkn2ab* mRNA levels were found in gills, where *xmrk* is also transcribed on a considerable level. In eye and brain *cdkn2ab* transcription levels were considerably lower, whereas *cdkn2ab* expression in liver could hardly be detected. In normal healthy skin of parental *X. maculatus* (Fig. 22a, c) and tumor-free backcross hybrids carrying the mdl^{Sr}-$xmrk^{A}$ allele (Fig. 22d) only a low background level expression of *cdkn2ab* was found. In contrast, *cdkn2ab* was strongly overexpressed in malignant melanomas of backcross hybrids carrying the mdl^{Sd}-$xmrk^{B}$ allele (Fig. 22b). Surprisingly, the *cdkn2ab* expression pattern in *Xiphophorus* tissues resembles that one found for the *xmrk* oncogene. A comparable *cdkn2ab* mRNA expression pattern was also detected in the *X. maculatus* strain WLC 2065 that is

devoid of *xmrk* (Fig. 22e), indicating that, at least in healthy *Xiphophorus* tissues, *cdkn2ab* transcript levels are independent of the *xmrk* oncogene.

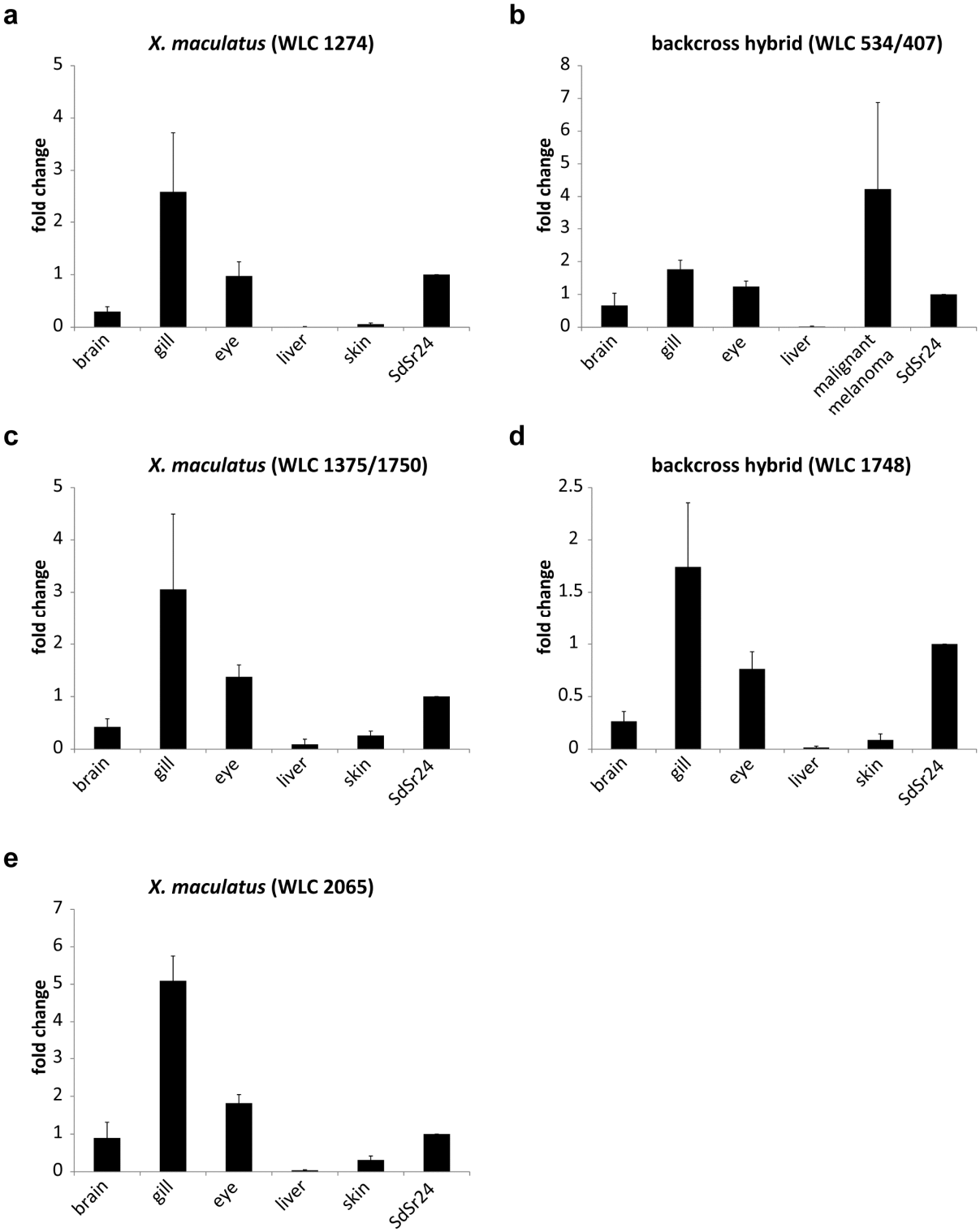


Fig. 22: Analysis of *cdkn2ab* expression in parental and hybrid *Xiphophorus* genotypes. Quantitative real-time PCR analysis of *cdkn2ab* mRNA expression levels in different tissues of (a) purebred platyfish (WLC 1274) and (b) tumor-bearing backcross hybrids (WLC 534/407) carrying the tumorigenic mdl^{Sd}-$xmrk^{B}$ allele. *cdkn2ab* transcript levels in (c) *X. maculatus* (WLC 1375/1750) and (d) tumor-free backcross hybrids (WLC 1748) carrying the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele. (e) *cdkn2ab* transcript levels in tissues of the *X. maculatus* strain WLC 2065. Fish of this strain are devoid of *xmrk* and do not exhibit macromelanophore patterns. *cdkn2ab* expression in SdSr24 cells was set as reference.

To determine whether Cdkn2ab protein and not only *cdkn2ab* mRNA is overexpressed in *Xiphophorus* melanomas, Western blot analysis was performed on melanoma and healthy tissues of parental and hybrid *Xiphophorus* genotypes (Fig. 23). Consistent with previous studies (Butler et al., 2007b), Cdkn2ab protein levels were highly upregulated in melanoma tissues compared to normal healthy skin. High Cdkn2ab levels were found not only in melanomas of backcross hybrids carrying the tumorigenic mdl^{Sd}-$xmrk^{B}$ allele (Fig. 23f) but also in two sporadic melanomas of WLC 1748 fish that carry the mdl^{Sr}-$xmrk^{A}$ allele (Fig. 23e). In normal tissues of parental *X. maculatus* and backcross hybrids Cdkn2ab protein was expressed abundantly in gills, expressed on a slightly lower level in skin, whereas only very faint bands were detected in brain, eye, and liver (Fig. 23a-f). Taken together, these data clearly demonstrate that *cdkn2ab* is massively overexpressed in *Xiphophorus* melanomas on the mRNA as well as on the protein level. Furthermore, I could show that *cdkn2ab* and *xmrk* transcript levels are indeed positively correlated in melanoma tissues of *Xiphophorus*. Interestingly, these analyses revealed that *xmrk* and *cdkn2ab* are expressed in the same tissues in *Xiphophorus*.

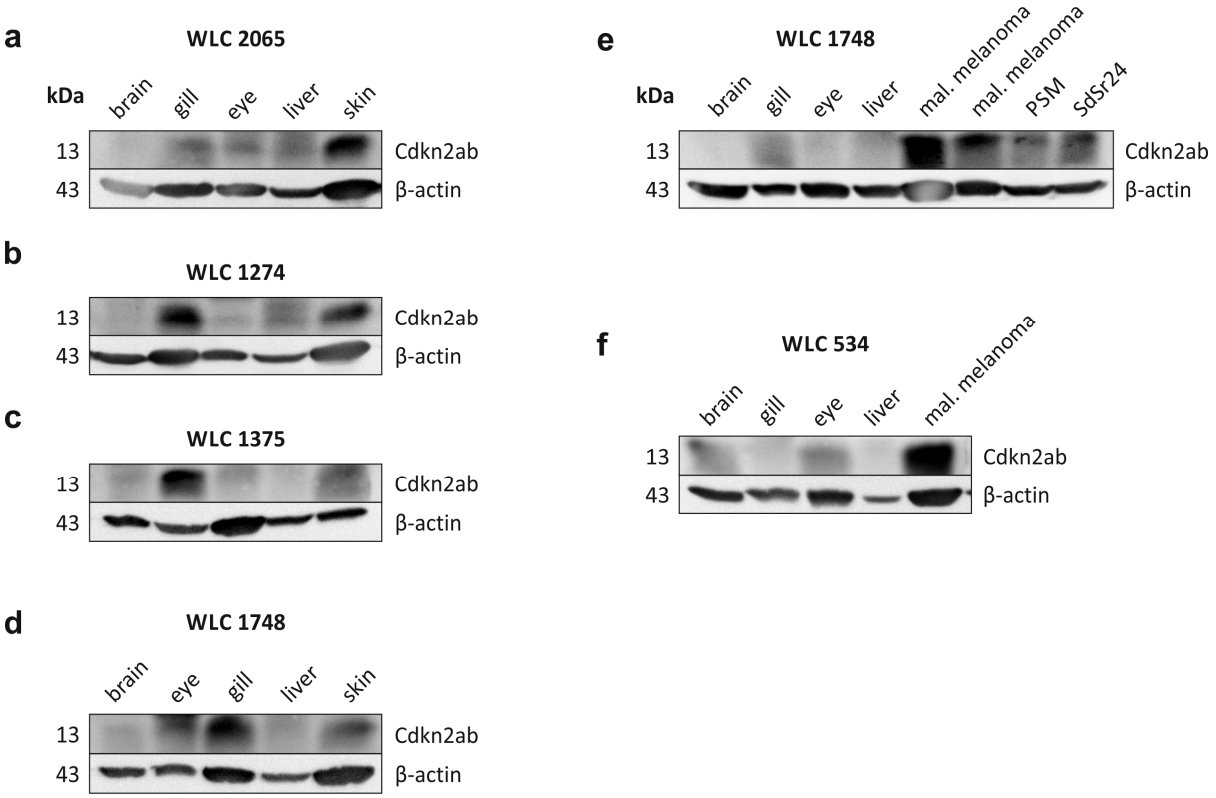


Fig. 23: Analysis of Cdkn2ab protein expression levels in parental and hybrid *Xiphophorus* genotypes. Western blot analysis of Cdkn2ab protein levels in tissues of different *X. maculatus* strains that are (a) either devoid of *xmrk* (WLC 2065), (b) carry the mdl^{Sd}-$xmrk^{B}$ allele (WLC 1274), or (c) carry an mdl^{Sr}-$xmrk^{A/B}$ mutant allele (WLC 1375). (d) Cdkn2ab protein levels in tissues of a tumor-free backcross hybrid (WLC 1748) carrying the mdl^{Sr}-$xmrk^{A}$ allele and (e) in tissues of a WLC 1748 individual with a sporadic melanoma. (e) furthermore shows Cdkn2ab protein levels in a sporadic melanoma of another WLC 1748 fish and in the *Xiphophorus* cell lines PSM and SdSr24. (f) Western blot analysis of Cdkn2ab protein levels in a tumor-bearing backcross hybrid (WLC 534) carrying the mdl^{Sd}-$xmrk^{B}$ allele. β-actin was used as loading control.

Discussion

Transcriptional regulation of *mdl-xmrk* allele combinations

As a first approach to test the hypothesis that the tumor-suppressive effect of the *R* locus on *xmrk* is exerted by directly or indirectly downregulating *xmrk* expression on the transcriptional level, a comparative analysis of expression levels of various *mdl-xmrk* allele combinations in healthy and tumorous *Xiphophorus* tissues was performed in this study. Expression of the highly tumorigenic mdl^{Sd}-$xmrk^{B}$ allele, which is the well-known spotted dorsal (Sd) allele from the classical Gordon-Kosswig-Anders cross, has been analyzed previously in several Northern blot and semi-quantitative RT-PCR based expression studies (Mäueler et al., 1993; Schartl et al., 1999; Woolcock et al., 1994). In this study, these earlier results were confirmed on a more precise quantitative level by performing qPCR analyses. I found that $xmrk^{B}$ expression in macromelanophore spots of purebred *X. maculatus* animals is comparable to expression in the majority of benign lesions, whereas expression is considerably increased in all malignant melanomas. By normalizing $xmrk^{B}$ expression to the melanocyte marker gene *dct*, I furthermore confirmed that this increase is absolute and not due to an increased proportion of melanocytes in malignant melanomas. Since cells of the macromelanophore lineage are considered to be the cellular progenitors of the *xmrk*-induced melanomas, these data indicate that a certain threshold of $xmrk^{B}$ transcripts is necessary for transformation to the full neoplastic phenotype. By Western blot analysis, I furthermore detected high $Xmrk^{B}$ protein expression levels in malignant melanomas. Thus, these data confirm that there is a positive correlation between the level of $xmrk^{B}$ mRNA and protein expression and the development of a malignant melanoma. Moreover, I found that $xmrk^{B}$ expression levels in normal healthy tissues are not increased in backcross hybrids compared to purebred animals. At least in gills, $xmrk^{B}$ transcription is even decreased in backcross hybrids. These data indicate that there is a pigment cell specific regulation of $xmrk^{B}$ transcript levels. Moreover, by using an $xmrk^{B}$ BAC as reporter construct for luciferase assays, this study showed for the first time a melanoma cell specific transcriptional activation of $xmrk^{B}$ by its flanking regions. The transcriptional activity of the $xmrk^{B}$ BAC reporter construct was significantly higher in the melanoma cell line PSM than in the non-melanoma cell line A2. Taken together, these findings indicate that the tumorigenic mdl^{Sd}-$xmrk^{B}$ allele is controlled by a pigment cell specific promoter region driving high level expression of *xmrk* exclusively in pigment cells in the absence of *R*, thereby supporting the hypothesis that melanoma

development in *Xiphophorus* is regulated by a tight transcriptional control of the *xmrk* oncogene.

In this study, I furthermore determined the expression pattern of the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele to test the hypothesis that differential transcriptional control triggers its low tumorigenic potential compared to mdl^{Sd}-$xmrk^{B}$. I found that $xmrk^{A}$ and $xmrk^{B}$ show a comparable expression pattern in healthy tissues of purebred fish and backcross hybrids. But interestingly, in sharp contrast to the massive overexpression of $xmrk^{B}$ in pigment cells of backcross hybrids, $xmrk^{A}$ mRNA and protein expression levels are not increased in skin of backcross hybrids compared to skin of *X. maculatus*, thereby strongly supporting the hypothesis that altered transcriptional regulation in the black pigment cell lineage is responsible for the low tumorigenicity of the mdl^{Sr}-$xmrk^{A}$ allele. This hypothesis is further supported by the finding that the chimeric A/B-allele *xmrk* alleles DrLi (mut) and 30^{84}B, which are under control of the mdl^{Sr}-$xmrk^{A}$ promoter region, are non-tumorigenic (Gutbrod and Schartl, 1999) and furthermore show a similar expression pattern as the wildtype mdl^{Sr}-$xmrk^{A}$ allele. However, as this hypothesis is not in line with the unexpected finding that the $xmrk^{A}$ and $xmrk^{B}$ BAC reporter constructs show a comparable transcriptional activity in luciferase assays, alternative reasons for differences in the oncogenic potential of *mdl-xmrk* alleles have to be considered as well.

As already discussed in a previous publication (Regneri and Schartl, 2012), another possible reason for differences in tumorigenicity between *mdl-xmrk* allele combinations are sequence variations in the *xmrk* coding sequence. As the tumorigenic potential of coding sequences of different *mdl-xmrk* alleles has not been compared directly so far, one could speculate that, due to the presence of exon 25 in their coding sequence (Fig. S1), class A *xmrk* alleles might have a lower tumorigenic potential as class B alleles. In addition, the mature Xmrk proteins differ by several single amino acid exchanges (Fig. S2), which could also alter the tumorigenic potential of the protein. There is evidence, however, strongly suggesting that coding sequence variations are not the leading cause for differences in tumorigenicity between *mdl-xmrk* alleles. First, the chimeric A/B-allele *xmrk* allele DrLi (mut), which shows the same expression pattern as the wildtype mdl^{Sr}-$xmrk^{A}$ allele, is non-tumorigenic in hybrid crosses. Because the crossover site is located between intron 1 and exon 15, the 3' part of this mutant allele is derived from mdl^{Sd}-$xmrk^{B}$. As a result, the DrLi (mut) allele is devoid of exon 25. This strongly indicates that neither the presence or absence of exon 25 nor amino acid exchanges in the C-terminal part of the Xmrk protein determine the tumorigenic potential.

Second, many transgenic experiments have demonstrated that the full-length *xmrk*^{A} cDNA from the melanoma cell line PSM has a high tumorigenic potential (Schartl et al., 2009; Winkler et al., 1994; Winnemoeller et al., 2005; Wittbrodt et al., 1992), suggesting class A *xmrk* alleles to be equally tumorigenic as class B alleles. Third, the finding that spontaneous melanoma formation in backcross hybrids carrying the *mdl*^{Sr}*-xmrk*^{A} allele is paralleled by high *xmrk*^{A} mRNA and protein expression levels strongly suggests that the Xmrk^{A} protein, if highly expressed, is able to transform pigment cells. The tumor-inducing overexpression of *xmrk*^{A} in these hybrids could be due to somatic mutations or alterations in epigenetic modifications in the *xmrk* promoter region. Given the relatively high incidence of such melanoma – approximately 1% – the latter possibility seems to be more likely. Environmental factors inducing transcriptional activation of *xmrk* have also been discussed as reason for spontaneous tumor development in purebred wildtype fish of *X. variatus* with *mdl*^{Pu}*-xmrk*^{C} (punctatus-2, Pu-2) and *X. cortezi* with *mdl*^{Sc}*-xmrk*^{D} (spotted caudal, Sc) (Fernandez and Morris, 2008; Schartl et al., 1995), as high levels of Xmrk protein were also found in these melanomas. Taken together, the data presented here strongly suggest differential transcriptional regulation as reason for the non-tumorigenicity of the *mdl*^{Sr}*-xmrk*^{A} allele, as the Xmrk^{A} protein, if overexpressed, is able to induce tumor development. Hence, these data further support the initial hypothesis that differential transcriptional regulation, and not differences in the cds, determines the tumorigenicity of *mdl-xmrk* alleles.

Consequently, it has to be taken into account that the *in vitro* activity of the *xmrk* BAC reporter constructs may fail to duplicate the endogenous *xmrk* expression patterns. As aforementioned, the *xmrk*^{A} and *xmrk*^{B} BAC reporter constructs showed a similar transcriptional activity in the melanoma cell line PSM, whereas a significantly lower activity of the *xmrk*^{A} BAC reporter construct was expected based on the expression data. In the melanoma cell line PSM, the *xmrk*^{B} reporter construct furthermore showed an only 1.4 fold higher transcriptional activity than the reporter construct containing the proto-oncogene *egfrb*. In contrast, endogenous expression of *egfrb* is on a very low background level in melanoma tissues and PSM cells, whereas *xmrk* is highly overexpressed. There are several possible explanations why the *in vitro* activity of the *xmrk* and *egfrb* BAC reporter constructs might not completely reproduce the transcript levels of their endogenous counterparts. First, important regulatory elements might be missing in the *xmrk* or *egfrb* BAC reporter constructs. Hypothetically, this could result in incomplete activation of the *xmrk* reporter constructs in the melanoma cell line PSM. Second, the cell lines used to assay the reporter gene activity could play a role. The melanoma cell line PSM is derived from a F1 hybrid between *X. maculatus*

and *X. hellerii* and thus should still harbor one copy of the regulator locus *R* (Wakamatsu, 1981) (Fig. S5). Backcross hybrids developing malignant melanoma from the mdl^{Sd}-$xmrk^{B}$ allele, in contrast, are devoid of *R*. Because it is assumed that the transcriptional control of *xmrk* is only partly released in presence of one *R* allele, it is conceivable that a remaining *R* copy results in a reduced transcriptional activation of the $xmrk^{B}$ BAC reporter construct in this cell line. Third, additional mechanisms, e.g. epigenetic modifications, could be essential for transcriptional regulation of *xmrk*. Interestingly, evidence indicating a crucial role of aberrant DNA methylation in transcriptional deregulation of *xmrk* in melanoma cells was presented earlier by Altschmied et al. (Altschmied et al., 1997). In addition, differences in the histone modification pattern at the *xmrk* promoter were found in melanoma versus non-melanoma cells (Master thesis, Janine Regneri). Hence, there is accumulating evidence for a crucial role of epigenetic modifications in transcriptional regulation of *xmrk*. Hypothetically, differences in the status of epigenetic modifications at the *xmrk* promoter could also play a role for the differential transcriptional activity of mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$ in pigment cells of backcross hybrids. In that case, the unexpected finding that $xmrk^{A}$ and $xmrk^{B}$ BAC reporter constructs show a comparable transcriptional activity in the melanoma cell line PSM could be at least partly explained by the absence of such epigenetic modifications in the transiently transfected BAC DNA. Hence, the impact of epigenetic modifications for *xmrk* transcriptional regulation should be addressed in further studies. Moreover, to further substantiate the hypothesis that differential transcriptional control is responsible for the differences in tumorigenicity between *mdl-xmrk* alleles, the tumorigenic potential of the mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$ coding sequences should be compared directly. As a first approach to do so, it would be reasonable to establish stable cell lines that express the $xmrk^{A}$ or $xmrk^{B}$ cds from a ubiquitous promoter and to compare proliferation rate, colony forming ability, and invasive potential of these cells using cell culture assays. To compare the oncogenic potential of the $xmrk^{A}$ and $xmrk^{B}$ cds *in vivo*, future studies should generate transgenic medakas ectopically expressing $xmrk^{A}$ or $xmrk^{B}$ from a pigment cell specific promoter (e.g. *mitfa* promoter) and monitor melanoma formation in these fish.

Taken together, the data presented in this study strongly indicate that the tumorigenic mdl^{Sd}-$xmrk^{B}$ allele is controlled by a pigment cell specific promoter region driving high level expression of *xmrk* exclusively in the melanocyte lineage in the absence of *R*. The data from the expression study furthermore strongly suggest that differential transcriptional control triggers the tumorigenic potential of different *mdl-xmrk* alleles. Hence, there is accumulating evidence indicating transcriptional regulation of *xmrk* to be causative for melanoma

development, thereby supporting the hypothesis that *R* is involved in transcriptional regulation of *xmrk* and consequently acts upstream of the *xmrk* signal.

In previously published expression studies, no or only weak *xmrk* expression was detected in normal healthy tissues of *Xiphophorus* (Schartl et al., 1999; Woolcock et al., 1994). In contrast to that, using the more sensitive qPCR technology I detected robust mRNA and protein expression of mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$ in gills of purebred *X. maculatus* and of backcross hybrids. High level expression of the *xmrk* oncogene so far was assumed to be restricted to the black pigment cell lineage. However, the gills of these fish contain no macromelanophores. Hence, as already discussed previously (Regneri and Schartl, 2012), the high $xmrk^{A}$ and $xmrk^{B}$ expression levels in this tissue were an unexpected finding. Since the *xmrk* promoter region is only poorly characterized so far, one can only speculate what causes this transcriptional activation. The putative *xmrk* promoter region contains a segment with more than 97% nucleotide identity to the *egfrb* promoter sequence upstream of the *D* locus-derived sequence (Fig. 12, S3). Moreover, *xmrk* and *egfrb* intronic sequences show 96.8% nucleotide identity on average (Volff et al., 2003). Since the proto-oncogene *egfrb* is expressed on a moderate level in gills of *X. maculatus*, these conserved regions might contain transcription factor binding sites that are involved in transcriptional activation of *xmrk* in this organ. Interestingly, despite the relatively high levels of *xmrk* mRNA and protein, signs of hyperplasia or other proliferative disorders were never observed in gills of *X. maculatus* and backcross hybrids carrying mdl^{Sd}-$xmrk^{B}$ or mdl^{Sr}-$xmrk^{A}$ compared to platyfish without any *xmrk*. A possible explanation why *xmrk* expression in gills is not connected to tumorigenesis might be that a certain threshold level of *xmrk* transcripts is necessary to elicit neoplastic transformation. This is in accordance with studies on the transforming capacity of *xmrk* in transgenic fish, where high levels of the oncogene are required (Schartl et al., 2009; Winkler et al., 1994; Winnemoeller et al., 2005). Since such a threshold level is probably tissue specific, the critical level might not be reached in the gills of these fish. Alternatively, the high c*dkn2ab* mRNA and protein levels found in gills of *X. maculatus* and backcross hybrids might play a role. As the protein product of *cdkn2ab* is a putative tumor suppressor and regulator of the G1/S checkpoint, a tight control of the cell cycle might inhibit *xmrk*-induced proliferation and subsequent neoplastic transformation in this organ. However, as high levels of *cdkn2ab* mRNA and protein were also found in *Xiphophorus* melanomas, this scenario is rendered less likely. Another possible explanation for the lack of transforming capacity of *xmrk* in gills might be the absence of specific cellular targets of the *xmrk* signal transduction pathway, which are involved in melanoma formation in pigment cells.

From an evolutionary perspective, a prominent question is how the *xmrk* gene, which is potentially deleterious to its carriers, could persist over millions of years in a highly unstable region of the *Xiphophorus* genome (Schartl, 1995; Volff et al., 2003). The only known function of *xmrk* is its role as a highly potent oncogene and so far no positively selected feature can be ascribed to *xmrk*. The high level of Xmrk protein in gills of healthy purebred animals, which obviously has no adverse effect, could point to a so far unknown physiological function of *xmrk* in gills (Regneri and Schartl, 2012). However, every natural population of *Xiphophorus* looked at so far is polymorphic for the presence of *xmrk* and furthermore, there are even populations or entire species of the genus *Xiphophorus* where the gene has been lost (Weis and Schartl, 1998). Hence, as discussed previously (Volff et al., 2003), a possible physiological function of *xmrk* cannot be vital, but might be beneficial only under certain conditions.

One hypothesis that has been proposed to explain the evolutionary persistence of *xmrk* (Meierjohann and Schartl, 2006; Schartl, 2008) is based on the finding that the *xmrk* oncogene and the macromelanophore-determining locus *mdl* are closely linked (Weis and Schartl, 1998). The so-called "hitchhiking" hypothesis argues that certain alleles of a non-selected gene can become more frequent if they are linked to another gene, which is under positive selection (Charlesworth, 1994; Stephan et al., 2006). As the *mdl*-encoded pigment spots have been assigned a function in facilitating the finding of conspecific individuals for shoaling behavior (Franck et al., 2001), the *mdl* locus is probably under positive selection. Consequently, *mdl* might help to carry *xmrk* along. Another hypothesis favors sexual selection as mechanism that maintains the pattern associated *xmrk* oncogene (Fernandez and Morris, 2008). For mdl^{Sc}-$xmrk^{D}$ from *X. cortezi* it was shown that in some populations females prefer males with larger caudal fin macromelanophore spots.

As positively selected functions can be ascribed to the macromelanophore pigment patterns, another very attractive hypothesis to explain the evolutionary persistence of *xmrk* would be a direct role of the oncogene in macromelanophore pigment pattern formation in purebred *Xiphophorus* fish. However, such a function of *xmrk* has been controversially discussed. The occurrence of macromelanophore patterns in fish that are devoid of *xmrk* indicates that the *xmrk* oncogene is not necessary for the development of this cell type. Hence, it has been postulated that *xmrk* and *mdl* are separate genetic entities (Weis and Schartl, 1998). However, the absence of macromelanophores in platyfish carrying a loss of function mutant (*lof-1*) of the mdl^{Sd}-$xmrk^{B}$ allele strongly contradicts this hypothesis. In these fish a transposable

element is inserted into the *xmrk*^{B} gene, which disrupts the open reading frame and leads to a nonfunctional, prematurely terminated Xmrk^{B} protein (Schartl et al., 1999; Wittbrodt et al., 1989). As the consequence of this gene disruption is not only a loss of the ability to develop melanoma in hybrid crosses but also a loss of the ability to form macromelanophore patterns in purebred fish, these findings strongly indicate that *xmrk* is necessary for the formation of macromelanophores. The robust *xmrk*^{B} expression in macromelanophores already in *R*-bearing purebred animals found in the study presented here is in line with this reasoning, as it indicates that the transcriptional activity of the *xmrk* oncogene in black pigment cells of purebred animals might induce the formation of these cells. It was shown that ectopic expression of an inducible version of Xmrk ("HERmrk") in murine melanocytes leads to formation of senescent, multinucleated giant cells (Leikam et al., 2008). Moreover, recent data from our lab have demonstrated that ectopic expression of *xmrk* in melanocytes of transgenic medakas (from the pigment cell specific *mitfa* promoter) (Schartl et al., 2009), which normally do not develop macromelanophores, induces the formation of enlarged, multinucleated melanocytes in the fins of these fish (unpublished data, Katja Maurus). As these cells resemble macromelanophores and share their most important morphological features, these data strongly suggest that *xmrk* expression in the black pigment cell lineage is not only necessary but also sufficient for the development of macromelanophores. Hence, these findings indicate that *xmrk* and *mdl* might be the same genetic entity, with *mdl* representing distal regulatory elements in the *xmrk* upstream region that control tempo-spatial expression of the *xmrk* oncogene. In that case, the heterogeneity of the macromelanophore pigment patterns in *Xiphophorus* could be explained by different combinations of distal enhancer and silencer elements in the *xmrk* flanking regions. However, as this hypothesis does not explain the occurrence of macromelanophore pigment patterns in certain *Xiphophorus* species that are devoid of *xmrk* (e.g. *X. hellerii* and *X. variatus)* (Weis and Schartl, 1998), this point cannot be clarified at present.

Functional and structural characterization of the putative *xmrk* promoter region

Although a pigment cell specific transcriptional activation of *xmrk* is considered as precondition for melanoma formation in *Xiphophorus* backcross hybrids, the putative *xmrk* promoter region is only poorly characterized so far and no pigment cell specific activity of *xmrk* promoter fragments was detected in previous reporter gene assays ((Baudler et al., 1997); and unpublished data, Lisa Osterloh). By using an $xmrk^B$ BAC as reporter construct, this study is the first to show a melanoma cell specific transcriptional activation of $xmrk^B$ by its flanking regions, thereby demonstrating that the *xmrk* oncogene is indeed controlled by a pigment cell specific promoter region. Moreover, as a first step to functionally characterize the $xmrk^B$ promoter region, the transcriptional activity of different $xmrk^B$ BAC deletion constructs was analyzed in this study. I could demonstrate that the proximal 28 kb of the $xmrk^B$ 5' flanking region alone are sufficient to induce pigment cell specific transcriptional activation of $xmrk^B$. Furthermore, the data indicate that additional pigment cell specific enhancer elements are located in the more distal part of the $xmrk^B$ 5' sequence analyzed here, as this region increases transcriptional activity in the melanoma, but not in the non-melanoma cell line. The $xmrk^B$ gene and the region 3' thereof, in contrast, reduce transcriptional activity in the melanoma cell line. Hence, the data presented here indicate that the regulatory elements important for transcriptional activation of $xmrk^B$ in pigment cells are located upstream of the oncogene. This conclusion is further supported by the finding that the chimeric A/B-allele *xmrk* allele DrLi (mut) is non-tumorigenic and shows the same *xmrk* expression pattern as the wildtype mdl^{Sr}-$xmrk^A$ allele in hybrid crosses. If we consider differential transcriptional regulation as reason for the low tumorigenic potential of the mdl^{Sr}-$xmrk^A$ allele, this finding suggests that the regulatory elements driving high level expression of $xmrk^B$ in melanocytes in the absence of *R* are located upstream of exon 15 of the *xmrk* gene.

As aforementioned, previous studies comparing reporter gene expression in melanoma and non-melanoma cells using up to 4.5 kb $xmrk^B$ 5' flanking sequence found no melanoma cell specific regulation ((Baudler et al., 1997); and unpublished data, Lisa Osterloh). Both studies detected a robust transcriptional activation of the reporter gene constructs, which, however, was not specific for the melanoma cell line PSM, but was also found in the non-melanoma cell line A2. The transcriptional activation of the $xmrk^B$ BAC reporter constructs, in contrast, was significantly higher in the melanoma than in the non-melanoma cell line. Because different reporter gene assay systems were used in the three studies, the transcriptional

activities of the various promoter fragments cannot be compared directly. Hence, different scenarios are conceivable. Based on their data, Baudler and colleagues postulated the existence of additional silencer elements positioned outside the 0.7 kb fragment analyzed in their study, which lead to transcriptional inactivation of *xmrk*^{B} in non-pigment cells (Baudler et al., 1997). The melanoma cell specific activity of the *xmrk*^{B} BAC reporter constructs detected here would consequently be the result of a reduced transcriptional activity in the non-melanoma cell line A2 due to additional silencer elements in the longer promoter fragments. Alternatively, the ubiquitous transcriptional activity of the shorter promoter fragments could represent a basal background activity of the proximal promoter of *xmrk*^{B}. In that case, the melanoma cell specific activity of the *xmrk*^{B} BAC reporter constructs could be explained by one or several pigment cell specific distal enhancer elements located outside the proximal promoter region that drive high level expression of *xmrk*^{B} exclusively in the black pigment cell lineage. In this case, one could furthermore speculate that the restricted transcriptional activity of *xmrk*^{B} in pigment cells of purebred *X. maculatus* might be due to one or several additional distal silencer elements, which attenuate the activating effect of the pigment cell specific enhancer element(s) in presence of the regulator locus *R*. Alternatively, the *R* locus-encoded gene(s) might directly or indirectly downregulate transcription factors involved in transcriptional activation of *xmrk* in pigment cells. In summary, it is conceivable to assume that transcription of *xmrk* is controlled by the combinatorial action of distal silencer and enhancer elements and that the regulator locus *R* might act through such a regulatory element. To identify the transcription factors and regulatory elements involved in *xmrk* transcriptional regulation, additional *xmrk*^{B} deletion constructs have to be generated and tested in reporter gene assays. As the proximal part (28 kb) of the *xmrk*^{B} 5' flanking region is sufficient to induce pigment cell specific activation comparable to the full-length reporter construct, further studies should focus on this region.

The expression data presented in this study indicate altered transcriptional regulation in the black pigment cell lineage as reason for the low tumorigenic potential of *mdl*^{Sr}-*xmrk*^{A} compared to *mdl*^{Sd}-*xmrk*^{B}. Hence, sequence variations in the regions controlling *xmrk* transcription are of great interest as they might explain these transcriptional differences. A *piggyBac-like* insertion in the putative promoter region of the *mdl*^{Sr}-*xmrk*^{A} allele was described earlier (Volff et al., 2003) and has subsequently been discussed as potential reason for the low tumorigenic potential of this allele (Schartl and Meierjohann, 2010). In this study, I could demonstrate the presence of a *piggyBac-like* insertion at the same position in the putative promoter region of the tumorigenic *mdl*$^{Sp\text{-}type}$-*xmrk*^{A} allele from the melanoma cell

line PSM, which strongly contradicts the hypothesis that this insertion alone is responsible for the low tumorigenic potential of the *mdl*^{Sr}-*xmrk*^{A} allele. I furthermore detected an additional insertion in the putative *mdl*^{Sr}-*xmrk*^{A} promoter region approximately 3.8 kb upstream of the translational start, which is not present at the same position in the 5' region of the highly tumorigenic *mdl*^{Sd}-*xmrk*^{B} allele (Fig. S3). This 134 bp fragment comprises two additional copies of a repetitive sequence, three copies of which are already present in the promoter region of all three *mdl-xmrk* alleles analyzed in this study. Interestingly, the repetitive sequence contains putative Sox9 and Lef1 transcription factor binding sites (Fig. S3) (unpublished data, Lisa Osterloh, Janine Regneri). Moreover, a protein-bound site located directly adjacent to the putative Lef1 binding site in the repetitive sequence was detected by DNase footprints using the *xmrk*^{B} promoter region as template, indicating that the Lef1 binding site might be functional (unpublished data, Lisa Osterloh). Transcription factors of the Tcf/Lef family (Tcfs) are the downstream effectors of the Wnt signaling pathway. This pathway is important for melanocyte development by induction of *mitf* transcription via β-catenin and Tcf/Lef1 (Dorsky et al., 1998; Saito et al., 2003; Shibahara et al., 2001). Moreover, deregulation of the Wnt pathway has been implicated in malignant transformation of melanocytes and melanoma progression (reviewed in (Lucero et al., 2010; O'Connell and Weeraratna, 2009)). Sox9, a member of the Sox family of transcription factors, plays, together with Sox10, a pivotal role for melanocyte development and normal function by regulating transcription of *mitf* and other pigment cell specific genes, such as *tyr* and *dct*. In how far Sox9 contributes to melanocyte transformation and melanoma progression is only beginning to be addressed. SOX9 expression is upregulated in the majority of human melanoma samples, but interestingly, high SOX9 levels are associated with a less proliferative, more differentiated phenotype (reviewed in (Harris et al., 2010)). Based on these data, it is tempting to assume that the amplification of the repetitive sequence might have altered the transcriptional regulation of the *mdl*^{Sr}-*xmrk*^{A} allele, resulting in a very low tumorigenic potential. However, the presence of three additional copies of the repetitive sequence in the promoter region of the tumorigenic *mdl*$^{Sp\text{-}type}$-*xmrk*^{A} allele renders this reasoning less likely (Fig. S3). Besides Sox9 and Lef1 binding sites, putative binding sites for other transcription factors known to be involved in pigment cell specific transcription (e.g. Pax3 and Mitf) were detected in the proximal 4.4 kb *xmrk* 5' region analyzed in this study (unpublished data, Lisa Osterloh). However, none of the promising binding sites for pigment cell specific transcription factors shows sequence variations between the *mdl*^{Sd}-*xmrk*^{B} and *mdl*^{Sr}-*xmrk*^{A} promoter sequences. The protein-bound sites detected by DNase footprints in the *xmrk*^{B}

promoter region overlap with putative binding sites for instance for Stat factors, Nudr, E2fs, Rebb1, Lef1, and Brn2 (unpublished data, Lisa Osterloh). However, none of these sites is mutated in the promoter region of the non-tumorigenic *mdl*^{Sr}-*xmrk*^{A} allele. Taken together, these data give no evidence that sequence variations in the proximal 4.4 kb of the *mdl*^{Sr}-*xmrk*^{A} 5' region are responsible for the altered transcriptional regulation and thus for the low tumorigenic potential of this allele. However, even though the putative binding sites may not trigger transcriptional differences between *mdl-xmrk* alleles, they might play a pivotal role for transcriptional regulation of the *xmrk* oncogene in general. Thus, it would be reasonable in further studies to confirm the protein-bound sites by electrophoretic mobility shift assays (EMSA) and to validate the functionality of promising predicted binding sites by reporter gene assays. However, as the 2.5 kb *xmrk*^{B} promoter sequence analyzed here (corresponds approximately to 4.4 kb in the *xmrk*^{A} promoter sequence) showed no pigment cell specificity in previous reporter assays (unpublished data, Lisa Osterloh), these analyses should be repeated in future studies with larger promoter fragments showing melanoma cell specific regulation in such assays.

Besides the two insertions in the proximal 4.4 kb of the *mdl*^{Sr}-*xmrk*^{A} upstream region, numerous structural differences between the *mdl*^{Sr}-*xmrk*^{A} and *mdl*^{Sd}-*xmrk*^{B} flanking regions were detected in this study by Vista plot analysis. The data from the luciferase experiments performed here indicate that the regulatory elements important for melanoma cell specific transcriptional activation of *xmrk*^{B} are located in the *xmrk* 5' flanking region. In line with this, the expression data from the chimeric A/B-allele *xmrk* alleles suggest that the sequence variations leading to differential transcriptional regulation of *mdl*^{Sr}-*xmrk*^{A} and *mdl*^{Sd}-*xmrk*^{B} are located upstream of exon 15 of the *xmrk* gene. Hence, the structural differences in the first intron and in the *xmrk* 5' flanking region are of great interest for further studies, as they might play a role for the differential transcriptional regulation of these *mdl-xmrk* alleles. However, in reporter assays the *xmrk*^{A} and *xmrk*^{B} BAC reporter constructs, whose genomic insert sequences were compared here, showed a similar transcriptional activity in melanoma as well as in non-melanoma cells. As the reason for this surprising finding has not been determined so far, it cannot be excluded that the sequence variations detected in this study are not responsible for the differential transcriptional regulation of the tumorigenic *mdl*^{Sd}-*xmrk*^{B} and the non-tumorigenic *mdl*^{Sr}-*xmrk*^{A} allele.

Taken together, the data presented in this study strongly suggest that the tumorigenic *mdl*^{Sd}-*xmrk*^{B} oncogene is controlled by a pigment cell specific promoter region. Moreover, the

data indicate that the regulatory elements driving high level expression of *xmrk*^{B} in melanoma cells are located upstream of the *xmrk* oncogene. However, additional work is required to identify the specific DNA elements and relevant transcription factors involved in *xmrk* transcriptional regulation and to understand the exact molecular mechanism leading to tumor-inducing overexpression of *xmrk* exclusively in melanocytes in the absence of the regulator locus *R*.

Analysis of *R* locus candidate genes

In the last decades, numerous studies have been performed to define the molecular identity of the crucial *R* locus-encoded gene and to understand its mode of action. It has been proposed that *cdkn2ab* is the crucial tumor-suppressing gene of the *R* locus, because in genetic linkage studies it showed the best association with the *R*-mediated effect on melanoma formation in certain crosses (Kazianis et al., 1998). As Cdkn2ab is a potential cell cycle inhibitor and tumor suppressor, it should act downstream of the Xmrk signal and downregulate proliferation of transformed melanocytes. Such a downstream effect could be mediated by differences in the transcriptional regulation between *X. maculatus* and *X. hellerii cdkn2ab* alleles. Interestingly, several sequence differences are evident in the putative *cdkn2ab* promoter region (Kazianis et al., 1999). Concordantly, differences in expression levels between the respective *cdkn2ab* alleles were detected in UVB-induced melanomas of rare *cdkn2ab* heterozygous BC_1 hybrids. In these melanomas, but also in most non-tumorous tissues of these fish, the *X. maculatus cdkn2ab* allele was found to be expressed at a higher level than the *X. hellerii* allele (Kazianis et al., 1999). Based on this finding, a hypothetical model was postulated in which the weaker *cdkn2ab* expression in backcross hybrids homozygous for the *X. hellerii* allele leads to deregulation of the G1/S checkpoint and loss of control of melanocyte proliferation, which finally results in melanoma formation in these fish (Nairn et al., 2001). However, a subsequent study demonstrated strongly increased *cdkn2ab* expression in melanomas from heterozygous and homozygous backcross hybrids (Butler et al., 2007b). In line with this, I found high *cdkn2ab* mRNA and protein expression levels in spontaneous melanomas of mdl^{Sd}-$xmrk^{B}$ carrying backcross hybrids, which are homozygous for the *X. hellerii cdkn2ab* allele, whereas expression in macromelanophore spots and skin of purebred platyfish carrying the *X. maculatus* allele was clearly reduced. In normal healthy tissues I also found no evidence for differences in *cdkn2ab* mRNA and protein expression levels between *X. hellerii* and *X. maculatus* alleles, which is in sharp contrast to the data published by Kazianis et al. (Kazianis et al., 1999). The similar *cdkn2ab* expression level in skin of purebred platyfish and backcross hybrids carrying the mdl^{Sr}-$xmrk^{A}$ allele indicates that the respective *cdkn2ab* alleles also show a comparable transcriptional activity in the macromelanophore lineage. Taken together, these data render the hypothesis proposed by Nairn and colleagues unlikely, as no evidence for a reduced expression of the *X. hellerii* allele was found. However, the finding that *cdkn2ab* expression in all benign lesions analyzed here was decreased compared to expression in one of the two *X. maculatus* pigment spot samples could serve as evidence for a weaker expression of the *X. hellerii cdkn2ab* allele. As

aforementioned, the two pigment spot samples showed striking differences in *cdkn2ab*, but also in *xmrk*, *pdcd4a*, and *mtap* expression levels, which renders this reasoning less likely. Alternatively, an effect of *cdkn2ab* on Xmrk downstream signaling could be due to functional differences in the Cdkn2ab proteins resulting from subtle differences in the amino acid sequence between the *X. maculatus* and the *X hellerii* allele, but so far no inactivating mutations were identified (Butler et al., 2007a; Kazianis et al., 1999). Hypothetically, *cdkn2ab* could also act upstream of *xmrk* and control *xmrk*-induced melanoma formation by a tight transcriptional control of the oncogene. However, such a biochemical function has not been described for this gene so far. Hence, the functional evidence supporting *cdkn2ab* as candidate for the crucial *R* locus-encoded gene is scarce.

As Cdkn2ab is a potential cell cycle inhibitor, its overexpression in a highly proliferative tissue like malignant melanoma is noteworthy. Interestingly, *xmrk* and *cdkn2ab* are both upregulated in malignant melanomas compared to benign lesions and macromelanophore spots, indicating that there might be a correlation between the expression levels of these two genes in the macromelanophore lineage. Moreover, I found that *cdkn2ab* and *xmrk* are expressed in the same tissues in purebred and hybrid fish. Hence, one could speculate that *cdkn2ab* expression might be induced by Xmrk downstream signaling in these tissues. However, the presence of comparable *cdkn2ab* expression levels in tissues of the *X. maculatus* strain WLC 2065, which is devoid of *xmrk*, strongly contradicts this hypothesis. These data rather indicate that, at least in normal healthy tissues, *cdkn2ab* expression levels are independent of *xmrk* and consequently Xmrk downstream signaling does not regulate expression of *cdkn2ab*. Moreover, the cell cycle regulator gene *cdkn2ab* has not been described as direct target of RTK signaling so far.

Alternatively, mechanisms of the cell to prevent neoplastic transformation in presence of strong oncogenic signaling might be responsible for upregulation of *cdkn2ab* expression in melanoma cells of *Xiphophorus*. As already proposed by Butler and colleagues, *cdkn2ab* might be induced in a futile attempt of the cell to maintain proper G1/S checkpoint regulation in presence of the strong proliferative stimulus provided by Xmrk (Butler et al., 2007b). In this regard, the phenomenon of oncogene-induced senescence (OIC) in human nevi is interesting (reviewed in (Peeper, 2011)). Benign nevi frequently contain oncogenic versions of BRAF (e.g. $BRAF^{V600E}$) or, in some cases, NRAS, but still remain in a growth arrested state, rarely progressing to melanoma (Bauer et al., 2006; Mooi and Peeper, 2006; Pollock et al., 2002). This can be at least partly explained by the fact that sustained oncogene expression

triggers premature senescence in these melanocytes, which prevents malignant transformation. Interestingly, OIC is associated with induction of senescence markers, among them $p16^{INK4a}$, which is one of the human orthologs of Cdkn2ab (Gray-Schopfer et al., 2006; Michaloglou et al., 2005). As Xmrk activates the Ras/Raf/Erk1/2 signaling pathway, it is conceivable that Xmrk, similar to $BRAF^{V600E}$ in human nevi, might promote oncogene-induced senescence in *Xiphophorus* melanocytes, thereby leading to induction of *cdkn2ab* expression. Evidence that Xmrk, if highly expressed, is indeed able to induce premature senescence in melanocytes comes from cell culture experiments using murine melanocytes ectopically expressing an EGF-inducible version of Xmrk ("HERmrk") (Leikam et al., 2008). Thus, the robust *cdkn2ab* expression in cells of the macromelanophore lineage, which also abundantly express the *xmrk* oncogene, could be explained by this mechanism. Although the mechanism of senescence escape in human melanoma development is not completely understood so far, additional genetic or epigenetic changes are presumably required for progression from this benign state of cell senescence to malignancy (Ceol et al., 2011; Dankort et al., 2009; Dovey et al., 2009; Patton et al., 2005). Moreover, progression to malignant melanoma is frequently associated with downregulation or loss of $p16^{INK4a}$ (Gray-Schopfer et al., 2006; Ibrahim and Haluska, 2009), whereas *Xiphophorus* melanomas highly express *cdkn2ab* mRNA and protein. Because Xmrk is on top of numerous signal transduction pathways, its oncogenic signaling might be sufficient to bypass normal *cdkn2ab* growth arrest or oncogene-induced senescence and consequently additional alterations may not be required for progression to malignancy in the *Xiphophorus* system (Butler et al., 2007b). As a result, a downregulation of *cdkn2ab* expression might not be necessary for full neoplastic transformation, which could explain the elevated *cdkn2ab* levels in malignant melanomas found in this study.

Depending on its expression level, HERmrk is able to drive murine melanocytes into senescence or to keep them in a proliferative state. In this system, senescence induction is associated with oncogene-dependent ROS production. In contrast, the expression levels of the senescence mediators pRB and p53, and their upstream activators $p14^{ARF}$, $p16^{INK4a}$ and, $p15^{INK4b}$, although increased upon HERmrk activation, are not correlated with senescence (Leikam et al., 2008). Hence, it is conceivable that Xmrk expression may lead to a similar situation in melanomas of *Xiphophorus*. If, in the *in vivo* situation, Xmrk also shows such a dosage-dependent effect, the *Xiphophorus* melanomas might be phenotypically heterogeneous, with highly proliferative and differentiated or even senescent subpopulations of cells. To address this question, histological and immunohistochemical analyses of

Xiphophorus melanoma sections should be performed in future studies using markers for proliferation (Ki-67), senescence (senescence-associated β-galactosidase activity, senescence-associated heterochromatin foci (H3K9me3)), and melanocytic differentiation. Moreover, it would be reasonable to analyze whether these subpopulations differ in their gene expression patterns by performing in situ hybridizations and immunofluorescent stainings for genes like *xmrk* and *cdkn2ab*. However, as these melanomas are heavily melanized, it might be necessary to bleach the sections before the subsequent analyses or to use melanomas from albino mutants for these experiments.

It was shown that $p16^{INK4a}$ can be induced by MITF and that this is required for cell cycle arrest and terminal differentiation of melanocytes (Loercher, 2004). Notably, I found a massive overexpression of *mitfa* in malignant melanomas of *Xiphophorus* compared to normal healthy skin (Fig. S6). This is in line with previous data showing high *mitfa* expression in melanoma tissues and cell lines (Altschmied et al., 2002). In contrast, expression of the second *mitf* gene in *Xiphophorus*, *mitfb*, was only slightly increased in melanoma tissues (Fig. S7). In *Xiphophorus* and other teleost fish, the *mitfa* gene is crucial for the development of neural crest-derived melanophores, whereas the *mitfb* gene regulates the expression of melanin synthesis enzymes in the retinal pigment epithelium (Altschmied et al., 2002; Lister et al., 2001). Hence, an induction of *cdkn2ab* expression by Mitfa in melanocytes could be another possible mechanism leading to high *cdkn2ab* mRNA and protein expression levels in *Xiphophorus* melanoma cells. However, as there is evidence suggesting that Xmrk signaling induces Mitfa degradation (Delfgaauw et al., 2003), these data further indicate that *Xiphophorus* melanomas might be phenotypically heterogeneous with proliferative, dedifferentiated subpopulations highly expressing the *xmrk* oncogene and more differentiated subpopulations showing high *mitfa* and *cdkn2ab* expression levels. In human melanomas, MITF expression varies across tumor specimens, with higher MITF levels correlating with a more differentiated, less aggressive phenotype (Ibrahim and Haluska, 2009; Levy et al., 2006). However, MITF was found to be amplified in 10 - 20% of melanoma cases, where it might act as a dominant oncogene (Garraway et al., 2005).

As functional evidence supporting *cdkn2ab* as the critical constituent of the tumor suppressor locus *R* is scarce, in this study three additional candidate genes in close vicinity to *cdkn2ab* were tested for concordant expression in *Xiphophorus* melanomas. Of these genes, only *pdcd4a* showed the expression pattern expected for a potential tumor suppressor gene, namely a significant downregulation in melanoma tissues compared to pigment spots and normal skin.

As *PDCD4* is a known tumor suppressor gene in human, *pdcd4a* is a promising new candidate for the crucial *R* locus-encoded gene. In line with our data, *PDCD4* was also shown to be downregulated in malignant melanoma specimens (Guo, 2013) and in various other human tumors (reviewed in (Lankat-Buttgereit and Göke, 2009)). However, the mechanisms by which PDCD4 exerts its tumor-suppressive effect are only beginning to be understood. Hence, additional work is required to determine whether *pdcd4a* downregulation is causative for neoplastic transformation of melanocytes in *Xiphophorus* backcross hybrids, and consequently *pdcd4a* might be *R*, or if it is just a downstream consequence of *xmrk*-induced transformation of these cells. As a first approach to address this question, reporter gene assays should be performed in future studies to determine whether there are differences in the transcriptional activity between the *X. hellerii* and *X. maculatus pdcd4a* allele in melanoma cells, which would indicate that downregulation of *pdcd4a* in melanomas is not just a downstream effect. Moreover, it would be reasonable to search for sequence differences between the *X. hellerii* and *X. maculatus pdcd4a* alleles that might be responsible for these transcriptional differences.

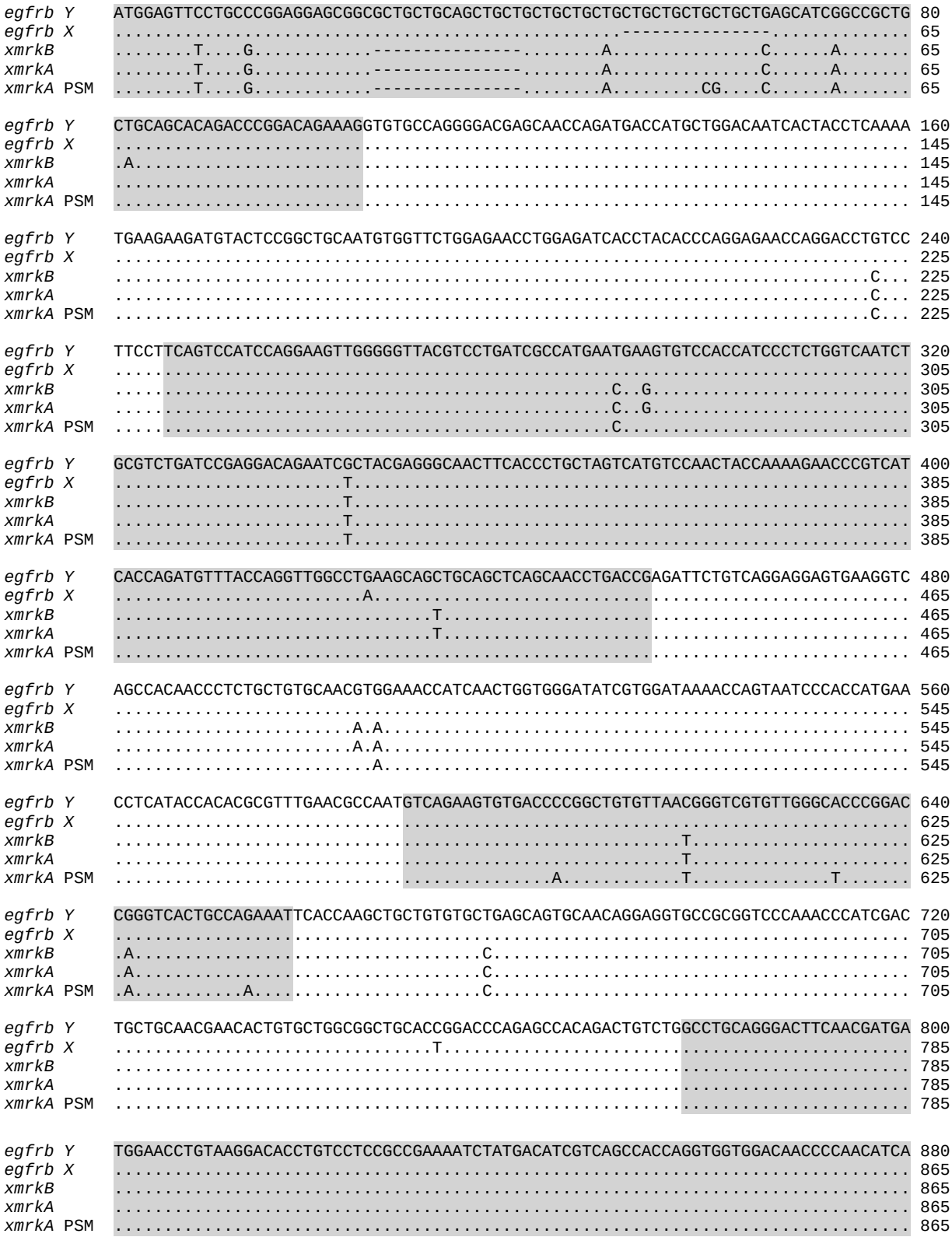
As a next approach to directly identify the *R* locus-encoded gene, a high density genetic map of *Xiphophorus* is currently being constructed by restriction site associated (RAD)-tag sequencing using a backcross mapping panel of 138 BC_1 animals typed for melanoma severity (John Postlethwait, Manfred Schartl, Ron Walter). As this map will give a detailed picture which genes in LG V are associated with the melanoma phenotype, this approach should provide several good candidate genes for the *R* locus that can subsequently be analyzed in more detail to define the molecular identity of the *R* locus-encoded gene and to understand its mode of action.

Perspectives

The data presented in this study should be the basis for future research as they strongly indicate that melanoma formation in *Xiphophorus* is regulated by a tight transcriptional control of the *xmrk* oncogene and that the *R* locus acts through this mechanism. Moreover, this study provides the first functional evidence that transcription of the *xmrk* oncogene is controlled by a melanoma cell specific promoter region. Hence, future studies aiming at understanding how melanoma formation in *Xiphophorus* is regulated should focus on analyzing the transcriptional regulation of the oncogene in more detail. The structural and functional characterization of the promoter regions of two *xmrk* alleles performed here furthermore provides a good starting point to identify the transcription factors and DNA elements involved in the tumor-inducing transcriptional activation of *xmrk* in melanocytes. However, to finally uncover how the *R* locus controls *xmrk*-induced melanoma formation, it will be necessary to identify the molecular nature of the *R* locus-encoded gene and to understand its mode of action.

Appendix

Figure S1:



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egfrb Y    AGTACACTTTCGGAGCCGCTTGTGTCAAGGAATGCCCTAGTAAGTACGTGGTGACAGAAGGCGCATGTGTTCGCTCTTGC 960
egfrb X    ...........................................C.................T.................. 945
xmrkB      ...........................................C..............G..................... 945
xmrkA      ...........................................C..............G..................... 945
xmrkA PSM  ...........................................C..............G..............T...... 945

egfrb Y    AGTGCAGGAATGCTTGAGGTAGATGAGAATGGCAAACGAAGCTGCAAGCCATGCGACGGAGTCTGTCCCAAAGTGTGTGA 1040
egfrb X    ................................................................................ 1025
xmrkB      ................................................................................ 1025
xmrkA      ................................................................................ 1025
xmrkA PSM  ................................................................................ 1025

egfrb Y    TGGAATTGGAATCGGGTCTCTGAGCAACACTATTGCTGTCAACTCAACCAACATCGGGTCCTTCAGCAACTGCACCAAGA 1120
egfrb X    ................................................................................ 1105
xmrkB      .......................................................A........................ 1105
xmrkA      .......................................................A........................ 1105
xmrkA PSM  .......................................................A........................ 1105

egfrb Y    TCAACGGCGACATCATCCTCAACAGGAACTCCTTTGAAGGGGATCCACATTACAAAATCGGGCCGATGGATCCTGAGCAT 1200
egfrb X    ................................................................................ 1185
xmrkB      ..............................................................A................. 1185
xmrkA      ..............................................................A................. 1185
xmrkA PSM  ..............................................................A................. 1185

egfrb Y    CTGTGGAACCTGACGACAGTGAAGGAAATCACTGGTTATCTGGTGATCATGTGGTGGCCTGAAAACATGACGTCTCTGTC 1280
egfrb X    ................................................................................ 1265
xmrkB      ................................................................................ 1265
xmrkA      ................................................................................ 1265
xmrkA PSM  ................................................................................ 1265

egfrb Y    GGTGTTCCAGAACCTGGAAATTATCAGAGGAAGAACTACGTTTTCCAGAGGGTTCAGCTTTGTGGTGGTTCAGGTGAGTC 1360
egfrb X    ................................................................................ 1345
xmrkB      ................................................................................ 1345
xmrkA      ....................................................C........................... 1345
xmrkA PSM  ............................................................................C... 1345

egfrb Y    ACCTGCAGTGGCTCGGTCTGCGCTCCCTGAAGGAGGTGAGCGCTGGGAATGTGATCCTGAAGAACATGCCGCAGCTGCGC 1440
egfrb X    ................................................................................ 1425
xmrkB      .T................................................................C..T.......... 1425
xmrkA      .T................................................................C..T.......... 1425
xmrkA PSM  ..................................................................C..T.......... 1425

egfrb Y    TACGCCAGCACCATCAACTGGAGGCACTTGTTCCGGTCTGAGGACCAGAGCATAGAGTATGACGCCAGGACTGAGAATCA 1520
egfrb X    .........................G...................................................... 1505
xmrkB      .......A.................G...................................................... 1505
xmrkA      .......A.................G...................................................... 1505
xmrkA PSM  .......A.................G...................................................... 1505

egfrb Y    AACCTGCAACAACGAGTGCTCAGAGGATGGGTGCTGGGGCCCGGGGCCCACAATGTGTGTCTCCTGTCTGCATGTGGACA 1600
egfrb X    ............TA................C................................................. 1585
xmrkB      ..............................C................................................. 1585
xmrkA      ..............................C................................................. 1585
xmrkA PSM  ..............................C................................................. 1585

egfrb Y    GAGGGGGGCGCTGTGTGGCATCCTGCAACCTGCTGCAGGGTGAACCCAGAGAGGCCCAGGTGAACGGCAGGTGTGTTCGG 1680
egfrb X    ..............................................................................A. 1665
xmrkB      ..............................................................G.T.............A. 1665
xmrkA      ..............................................................................A. 1665
xmrkA PSM  ..............................................................G.T.............A. 1665

egfrb Y    TGTCACCAGGAGTGTTTGGTGCAGACTGACAGCCTGACCTGCTACGGTCCGGGGCCAGCCAACTGCTCCAAGTGTGCACA 1760
egfrb X    ................................................................................ 1745
xmrkB      ........................................................................A....... 1745
xmrkA      ........................................................................A....... 1745
xmrkA PSM  ........................................................................A....... 1745

egfrb Y    CTTTCAAGATGGCCCCCAATGCATCCCTCGCTGCCCCCACGGCATGCTGGGAGACGGAGACATTCTGATCTGGAAATATG 1840
egfrb X    ................................................................................ 1825
xmrkB      .................................T..T........A................C................. 1825
xmrkA      .................................T..T........A................C................. 1825
xmrkA PSM  ....................................T........A................C................. 1825

egfrb Y    CGGATAAGATGGGCCAATGCCAGCCGTGTCATCAGAACTGCACCCAAGGGTGTTCTGGTCCTGGACTGTCTGGCTGTAGA 1920
egfrb X    ................................................................................ 1905
xmrkB      .A...........T..............................................................C... 1905
xmrkA      ............................................................................C... 1905
xmrkA PSM  .A..........................................................................C... 1905
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egfrb Y     GGCGATATCGTTTCCCACTCCTCTCTGGCAGTGGGGTTAGTCAGTGGACTCCTGATCACTGTAATTGTGGCGCTGCTCAT 2000
egfrb X     ..........................................................A..................... 1985
xmrkB       ............................................................................. 1985
xmrkA       C............................................................................. 1985
xmrkA PSM   ..........................................G.................................... 1985

egfrb Y     CGTGGTTCTACTGCGGCGCCGGCGAATCAAACGGAAGAGGACAATACGCCGCCTGCTCCAAGAGAAGGAGCTGGTGGAGC 2080
egfrb X     ..................................................................................... 2065
xmrkB       ............A................................................................... 2065
xmrkA       ............A.....................................T....................... 2065
xmrkA PSM   ............A.....................................T....................... 2065

egfrb Y     CGTTGACTCCGAGCGGTCAGGCTCCCAATCAGGCCTTCCTGAGAATCCTGAAGGAGACGGAATTCAAGAAGGACCGAGTT 2160
egfrb X     ..................................................................................... 2145
xmrkB       ..................................................................................... 2145
xmrkA       ............T........................................................................ 2145
xmrkA PSM   ..................................................................................... 2145

egfrb Y     CTGGGCTCCGGGGCATTTGGGACGGTCTACAAGGGTTTATGGAACCCTGACGGAGAAAACATCCGGATCCCAGTCGCTAT 2240
egfrb X     ......................................................................................  2225
xmrkB       ......................A...............................................................  2225
xmrkA       ......................................................................................  2225
xmrkA PSM   ......................................................................................  2225

egfrb Y     CAAAGTTCTGAGAGAGGCTACGTCACCAAAAGTCAACCAGGAAGTTCTGGACGAGGCGTACGTGATGGCAAGCGTGGACC 2320
egfrb X     ..................................................................G.......... 2305
xmrkB       ........................G.........................................G.......... 2305
xmrkA       ..............T.........G.........................................G.......... 2305
xmrkA PSM   ........................G.........................................G.......... 2305

egfrb Y     ATCCTCACGTCTGCCGCCTGCTGGGCATCTGCCTGACGTCGGCCGTGCAGCTGGTGACGCAGCTGATGCCGTACGGCTGC 2400
egfrb X     ..................................................................................... 2385
xmrkB       ......................T.............................................................. 2385
xmrkA       ..................................................................................... 2385
xmrkA PSM   ..................................................................................... 2385

egfrb Y     CTGCTGGACTACGTCCGGCAGCACCAGGAGCGGATCTGTGGCCAGTGGCTGCTGAACTGGTGTGTTCAGATCGCCAAGGG 2480
egfrb X     ..................................................................................... 2465
xmrkB       ............................A........................................................ 2465
xmrkA       ..................................................................................... 2465
xmrkA PSM   ............................A........................................................ 2465

egfrb Y     AATGAACTACCTGGAAGAGCGCCACCTGGTGCACCGCGACCTGGCAGCCAGGAACGTCCTGCTGAAAAACCCGAACCACG 2560
egfrb X     ..................................................................................... 2545
xmrkB       G................................................................................... 2545
xmrkA       G................................................................................... 2545
xmrkA PSM   G................................................................................... 2545

egfrb Y     TCAAGATCACAGACTTCGGTCTGTCCAAGCTGCTGACGGCTGACGAGAAGGAATACCAAGCCGACGGAGGAAAGGTTCCC 2640
egfrb X     ..................................................................................... 2625
xmrkB       ..................................................................................... 2625
xmrkA       ..................................................................................... 2625
xmrkA PSM   ..................................................................................... 2625

egfrb Y     ATTAAGTGGATGGCTTTGGAGTCGATCCTCCAGTGGACCTACACCCATCAGAGCGACGTGTGGAGCTACGGTGTGACGGT 2720
egfrb X     ..................................................................................... 2705
xmrkB       ..................................................................................... 2705
xmrkA       ..................................................................................... 2705
xmrkA PSM   ..................................................................................... 2705

egfrb Y     TTGGGAGTTGATGACGTTCGGATCCAAACCGTACGACGGCATCCCGGCCAAGGAGATCGCCTCGGTGCTGGAGAACGGGG 2800
egfrb X     ..................................................................................... 2785
xmrkB       ..............A..T.................................................................... 2785
xmrkA       ..............A............A.......................................................... 2785
xmrkA PSM   ..............A............A.......................................................... 2785

egfrb Y     AGCGGCTGCCGCAGCCTCCCATCTGCACCATCGAAGTCTACATGATCATCCTGAAGTGCTGGATGATCGACCCGTCCAGC 2880
egfrb X     ..................................................................................... 2865
xmrkB       ..................................................................................... 2865
xmrkA       ..................................................................................... 2865
xmrkA PSM   ..................................................................................... 2865

egfrb Y     AGACCCAGGTTCAGAGAGCTGGTGGGCGAGTTCTCCCAGATGGCCCGGGACCCGTCCAGGTACCTGGTCATACAGGGCAA 2960
egfrb X     ..................................................................................... 2945
xmrkB       ..................................................................................... 2945
xmrkA       ..................................................................................... 2945
xmrkA PSM   ..................................................................................... 2945
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egfrb Y     CCTGCCGAGTCCGTCTGATCGGAGGCTTTTCTCCCGCCTGCTGAGCTCTGATGACGACGTGGTCGACGCCGATGAATACC 3040
egfrb X     ................................................................................ 3025
xmrkB       ...........T.........................................................G.......... 3025
xmrkA       ...........T.................................................................... 3025
xmrkA PSM   ...........T.................................................................... 3025

egfrb Y     TGCTGCCGTACAAACGGATAAACCGCCAGGGCAGCGAGCCCTGCATCCCGCCGAATGGGCATCCAGTGAGAGAGAACAGC 3120
egfrb X     ................................................................................ 3105
xmrkB       .......A......T......................................--------------------------- 3078
xmrkA       .......A..................................................A..................... 3105
xmrkA PSM   .......A..............................................C...A..................... 3105

egfrb Y     ATGGCTCTCCGGTACATCTCCGACCCGACCCAGAACGCGCTGGAGAAAGACCTGGATGGTCACGAGTACGTTAACCAGCC 3200
egfrb X     ................................................................................ 3185
xmrkB       ------------------------------------------------------------------------------.. 3080
xmrkA       ..CA........A................................................................... 3185
xmrkA PSM   ..CA........A................................................................... 3185

egfrb Y     TGGGAGTGAAACCAGCAGCAGGCTGTCGGATATCTACAATCCCAACTACGAGGACCTGACGGACGGCTGGGGCCCGGTGT 3280
egfrb X     ................................................................................ 3265
xmrkB       ............................................G...............A................... 3160
xmrkA       ............................................................A..............C.... 3265
xmrkA PSM   ............................................................A................... 3265

egfrb Y     CGCTGTCCTCCCAGGAGGCGGAGACCAACTTCTCCAGACCGGAGTACCTGAACACCAACCAAAACTCGCTCCCCCTGGTG 3360
egfrb X     ................................................................................ 3345
xmrkB       ................................................................................ 3240
xmrkA       ................................G.......A....................................... 3345
xmrkA PSM   ........................................A....................................... 3345

egfrb Y     TCCAGTGGCAGTATGGACGACCCCGACTACCAGGCCGGCTACCAGGCTGCCTTCCTACCGCAGACTGGAGCGCTCACTGG 3440
egfrb X     ...........C.................................................................... 3425
xmrkB       ...........C.......................T..................................A......... 3320
xmrkA       ...........C.................................................................... 3425
xmrkA PSM   ...........C.................................................................... 3425

egfrb Y     GAACGGCATGTTTCTCCCTGCAGCAGAGAACCTGGAGTACCTGGGACTGGGAGGCGCACTGTACACTCCTGTCCGGTAG 3519
egfrb X     ............................................................................... 3504
xmrkB       ............C...............................A..A...........................C... 3399
xmrkA       ............C..................................A...........................C... 3504
xmrkA PSM   ............C..................................A...........................C... 3504
```

Fig. S1: Nucleotide alignment of coding sequences of different *xmrk* and *egfrb* alleles in *X. maculatus*. Light gray marks sequences of exons with odd numbers. The deletion in the *mdl*^{Sd}-*xmrk*^{B} allele is highlighted in turquois. Dots indicate conserved sites. Coding sequences of X- and Y-chromosomal *egfrb* alleles and of *mdl*^{Sr}-*xmrk*^{A} and *mdl*^{Sd}-*xmrk*^{B} are derived from genomic sequences of the *X. maculatus* BAC library (Froschauer et al., 2002). The sequence of the *mdl*$^{Sp\text{-}type}$-*xmrk*^{A} allele from PSM cells was obtained from NCBI (GenBank accession no. X16891.2).

Figure S2:

```
Egfrb Y    MEFLPGGAALLQLLLLLLLLLLSIGRCCSTDPDRKVCQGTSNQMTMLDNHYLKMKKMYSGCNVVLENLEITYTQENQDLS 80
Egfrb X    ............-----............................................................... 75
XmrkB      ....R.......-----.......S..S.................................................... 75
XmrkA      ....R.......-----.......S....................................................... 75
XmrkA PSM  ....R.......-----...V...S....................................................... 75

Egfrb Y    FLQSIQEVGGYVLIAMNEVSTIPLVNLRLIRGQNRYEGNFTLLVMSNYQKNPSSPDVYQVGLKQLQLSNLTEILSGGVKV 160
Egfrb X    ..................................L............................................. 155
XmrkB      ..................................L............................................. 155
XmrkA      ..................................L............................................. 155
XmrkA PSM  ..................................L............................................. 155

Egfrb Y    SHNPLLCNVETINWWDIVDKTSNPTMNLIPHAFERQCQKCDPGCVNGSCWAPGPGHCQKFTKLLCAEQCNRRCRGPKPID 240
Egfrb X    ................................................................................ 235
XmrkB      ........I....................................................................... 235
XmrkA      ........I....................................................................... 235
XmrkA PSM  .........................................H...................................... 235

Egfrb Y    CCNEHCAGGCTGPRATDCLACRDFNDDGTCKDTCPPPKIYDIVSHQVVDNPNIKYTFGAACVKECPSKYVVTEGACVRSC 320
Egfrb X    ...................................................................N............ 315
XmrkB      ...................................................................N............ 315
XmrkA      ...................................................................N............ 315
XmrkA PSM  ...................................................................N............ 315

Egfrb Y    SAGMLEVDENGKRSCKPCDGVCPKVCDGIGIGSLSNTIAVNSTNIGSFSNCTKINGDIILNRNSFEGDPHYKIGPMDPEH 400
Egfrb X    ................................................................................ 395
XmrkB      .............................................R............................T..... 395
XmrkA      .............................................R............................T..... 395
XmrkA PSM  .............................................R............................T..... 395

Egfrb Y    LWNLTTVKEITGYLVIMWWPENMTSLSVFQNLEIIRGRTTFSRGFSFVVVQVSHLQWLGLRSLKEVSAGNVILKNMPQLR 480
Egfrb X    ................................................................................ 475
XmrkB      ...........................................................................TL... 475
XmrkA      ............................................L..............................TL... 475
XmrkA PSM  ....................................................R......................TL... 475

Egfrb Y    YASTINWRHLFRSEDQSIEYDARTENQTCNNECSEDGCWGPGPTMCVSCLHVDRGGRCVASCNLLQGEPREAQVNGRCVR 560
Egfrb X    ........R......................K...............................................Q 555
XmrkB      ..N.....R.................................................................D....Q 555
XmrkA      ..N.....R......................................................................Q 555
XmrkA PSM  ..N.....R.................................................................D....Q 555

Egfrb Y    CHQECLVQTDSLTCYGPGPANCSKCAHFQDGPQCIPRCPHGMLGDGDILIWKYADKMGQCQPCHQNCTQGCSGPGLSGCR 640
Egfrb X    ................................................................................ 635
XmrkB      ........................S................I.....T................................ 635
XmrkA      ........................S................I.....T................................ 635
XmrkA PSM  ........................S................I.....T................................ 635

Egfrb Y    GDIVSHSSLAVGLVSGLLITVIVALLIVVLLRRRRIKRKRTIRRLLQEKELVEPLTPSGQAPNQAFLRILKETEFKKDRV 720
Egfrb X    ................................................................................ 715
XmrkB      ................................................................................ 715
XmrkA      R..........................................C.................................... 715
XmrkA PSM  ...........................................C.................................... 715

Egfrb Y    LGSGAFGTVYKGLWNPDGENIRIPVAIKVLREATSPKVNQEVLDEAYVMASVDHPHVCRLLGICLTSAVQLVTQLMPYGC 800
Egfrb X    ................................................................................ 795
XmrkB      ................................................................................ 795
XmrkA      ................................S............................................... 795
XmrkA PSM  ................................................................................ 795

Egfrb Y    LLDYVRQHQERICGQWLLNWCVQIAKGMNYLEERHLVHRDLAARNVLLKNPNHVKITDFGLSKLLTADEKEYQADGGKVP 880
Egfrb X    ................................................................................ 875
XmrkB      ................................................................................ 875
XmrkA      ................................................................................ 875
XmrkA PSM  ................................................................................ 875

Egfrb Y    IKWMALESILQWTYTHQSDVWSYGVTVWELMTFGSKPYDGIPAKEIASVLENGERLPQPPICTIEVYMIILKCWMIDPSS 960
Egfrb X    ................................................................................ 955
XmrkB      ................................................................................ 955
XmrkA      ................................................................................ 955
XmrkA PSM  ................................................................................ 955
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Egfrb Y     RPRFRELVGEFSQMARDPSRYLVIQGNLPSPSDRRLFSRLLSSDDDVVDADEYLLPYKRINRQGSEPCIPPNGHPVRENS 1040
Egfrb X     ................................................................................ 1035
XmrkB       ..............................L...........................W............--------- 1026
XmrkA       ..............................L................................................. 1035
XmrkA PSM   ..............................L........................................T........ 1035

Egfrb Y     MALRYISDPTQNALEKDLDGHEYVNQPGSETSSRLSDIYNPNYEDLTDGWGPVSLSSQEAETNFSRPEYLNTNQNSLPLV 1120
Egfrb X     ................................................................................ 1115
XmrkB       --------------------------...............S...................................... 1080
XmrkA       IT..N...........................................................A............... 1115
XmrkA PSM   IT..N........................................................................... 1115

Egfrb Y     SSGSMDDPDYQAGYQAAFLPQTGALTGNGMFLPAAENLEYLGLGGALYTPVR 1172
Egfrb X     .................................................... 1167
XmrkB       .......................E.................EQ......... 1132
XmrkA       ..........................................Q......... 1167
XmrkA PSM   ..........................................Q......... 1167
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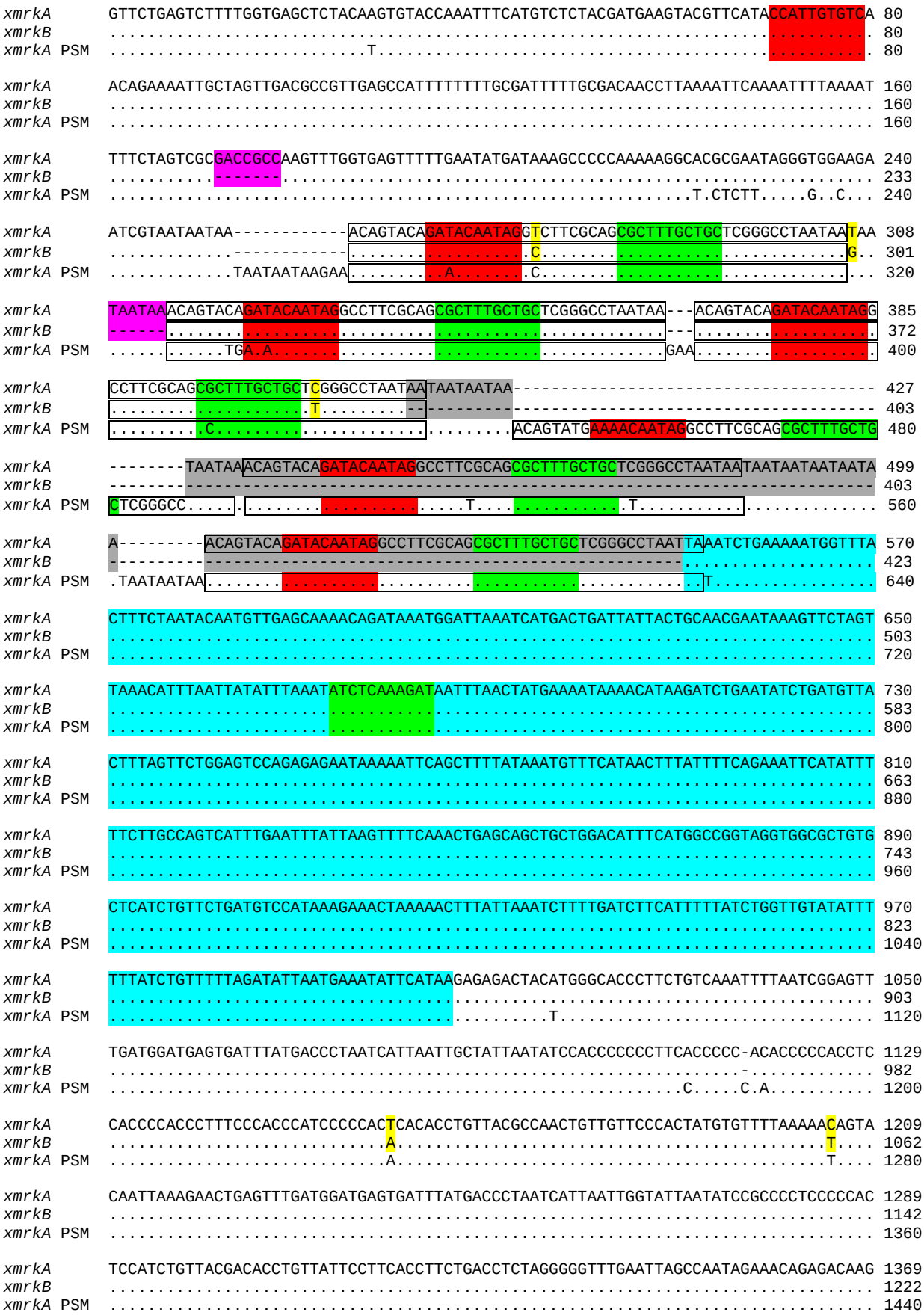
Fig. S2: Amino acid alignment of different Xmrk and Egfrb proteins in *X. maculatus*. Signal peptides are outlined in black. The kinase domain is highlighted in green and the transmembrane region is shown in red. Light and dark gray indicates extracellular domains 1-4. Amino acid exchanges between all mature Egfrb and Xmrk proteins are delineated in yellow. G336R and C555S, the two amino acid exchanges that interfere with the formation of intramolecular disulfide bridges, are shown in magenta (positions without signal peptide). Dots indicate conserved sites. The 35 amino acids missing from the cytoplasmic domain of the $Xmrk^{B}$ protein are highlighted in turquois.

Figure S3:


xmrkA GTTCTGAGTCTTTTGGTGAGCTCTACAAGTGTACCAAATTTCATGTCTCTACGATGAAGTACGTTCATACCATTGTGTCA 80
xmrkB . 80
xmrkA PSM .T. 80
xmrkA ACAGAAAATTGCTAGTTGACGCCGTTGAGCCATTTTTTTTGCGATTTTTGCGACAACCTTAAAATTCAAAATTTTAAAAT 160
xmrkB . 160
xmrkA PSM . 160
xmrkA TTTCTAGTCGCGACCGCCAAGTTTGGTGAGTTTTTGAATATGATAAAGCCCCCAAAAAGGCACGCGAATAGGGTGGAAGA 240
xmrkB . 233
xmrkA PSM .T.CTCTT.....G..C... 240
xmrkA ATCGTAATAATAA-----------ACAGTACAGATACAATAGGTCTTCGCAGCGCTTTGCTGCTCGGGCCTAATAATAA 308
xmrkB .----------. .C. .G.. 301
xmrkA PSM .....TAATAATAAGAA. ..A. .C. ... 320
xmrkA TAATAAACAGTACAGATACAATAGGCCTTCGCAGCGCTTTGCTGCTCGGGCCTAATAA---ACAGTACAGATACAATAGG 385
xmrkB ------. .---. . 372
xmrkA PSM ......TGA.A. .GAA. . 400
xmrkA CCTTCGCAGCGCTTTGCTGCTCGGGCCTAATAATAATAATAA---------- 427
xmrkB .T.--------- 403
xmrkA PSM .C. .ACAGTATGAAAACAATAGGCCTTCGCAGCGCTTTGCTG 480
xmrkA --------TAATAACACAGTACAGATACAATAGGCCTTCGCAGCGCTTTGCTGCTCGGGCCTAATAATAATAATAATAATA 499
xmrkB ---------- 403
xmrkA PSM CTCGGGCC.....T....T. 560
xmrkA A---------ACAGTACAGATACAATAGGCCTTCGCAGCGCTTTGCTGCTCGGGCCTAATTAAATCTGAAAAATGGTTTA 570
xmrkB ---------- 423
xmrkA PSM .TAATAATAA. .T. 640
xmrkA CTTTCTAATACAATGTTGAGCAAAACAGATAAATGGATTAAATCATGACTGATTATTACTGCAACGAATAAAGTTCTAGT 650
xmrkB . 503
xmrkA PSM . 720
xmrkA TAAACATTTAATTATATTTAAATATCTCAAAGATAATTTAACTATGAAAATAAAACATAAGATCTGAATATCTGATGTTA 730
xmrkB . 583
xmrkA PSM . 800
xmrkA CTTTAGTTCTGGAGTCCAGAGAGAATAAAAATTCAGCTTTTATAAATGTTTCATAACTTTATTTTCAGAAATTCATATTT 810
xmrkB . 663
xmrkA PSM . 880
xmrkA TTCTTGCCAGTCATTTGAATTTATTAAGTTTTCAAACTGAGCAGCTGCTGGACATTTCATGGCCGGTAGGTGGCGCTGTG 890
xmrkB . 743
xmrkA PSM . 960
xmrkA CTCATCTGTTCTGATGTCCATAAAGAAACTAAAAACTTTATTAAATCTTTTGATCTTCATTTTTATCTGGTTGTATATTT 970
xmrkB . 823
xmrkA PSM . 1040
xmrkA TTTATCTGTTTTTAGATATTAATGAAATATTCATAAGAGAGACTACATGGGCACCCTTCTGTCAAATTTTAATCGGAGTT 1050
xmrkB . 903
xmrkA PSM .T. 1120
xmrkA TGATGGATGAGTGATTTATGACCCTAATCATTAATTGCTATTAATATCCACCCCCCCTTCACCCCC-ACACCCCCACCTC 1129
xmrkB -. 982
xmrkA PSM .C.....C.A. 1200
xmrkA CACCCCACCCTTTCCCACCCATCCCCCACTCACACCTGTTACGCCAACTGTTGTTCCCACTATGTGTTTTAAAAACAGTA 1209
xmrkB .A. .T.... 1062
xmrkA PSM .A. .T.... 1280
xmrkA CAATTAAAGAACTGAGTTTGATGGATGAGTGATTTATGACCCTAATCATTAATTGGTATTAATATCCGCCCCTCCCCCAC 1289
xmrkB . 1142
xmrkA PSM . 1360
xmrkA TCCATCTGTTACGACACCTGTTATTCCTTCACCTTCTGACCTCTAGGGGGTTTGAATTAGCCAATAGAAACAGAGACAAG 1369
xmrkB . 1222
xmrkA PSM . 1440

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xmrkA     GGGTTCGGCCTTTAGTGTTTCTGCTATAAGTAATGTCGGAAAAAATCAAGCACTCTACTTGTAAGAGTTGTTCAAGAACT 1449
xmrkB     ....................................................................C........... 1302
xmrkA PSM ................................................................................ 1520

xmrkA     TTACAATGAATGGAACTAAACGAGTTCATCCTGCTGCTGAGAAAAATGGACCTACCTCCTCTGCATCAGCTGATACGGAT 1529
xmrkB     ................................................................................ 1382
xmrkA PSM ................................................................................ 1600

xmrkA     TCGAAGTTACCTAGCCCTAAGACATTCGCTAAGATGATGAAGTCAACACCGTTAACGAACAAATACCTGCTTGCTGAACA 1609
xmrkB     ..................................................................T............. 1462
xmrkA PSM ..................................................................T............. 1680

xmrkA     AGAAGAAGAACGTGCTGTTGTATATGAGATCCCAATCGCAGTGAAACCAGATTTGGGCCCAGTGTTGGATAAACCTGTAA 1689
xmrkB     ................................................................................ 1542
xmrkA PSM ....................................A........................................... 1760

xmrkA     GCTCACCTTCGTCGCCTGATGCATCTGCCTCAAAAACTGTGGATAAAAGAGAAATACCGACAGGCGGAACGTCTCTTCTA 1769
xmrkB     ..................................C....CT...............GT...................... 1622
xmrkA PSM ........................................................GT...................... 1840

xmrkA     TGCGAGACCCCGATATCCATCTATGACAACTCCAGGCTTAGAGCGGATGCCCCAAAGAAATCGAAATTTTATTTGTGCCG 1849
xmrkB     ........................................................................C....T.. 1702
xmrkA PSM ....................................A........................................... 1920

xmrkA     CATGCAGAGCCCGGTACTTGGATCCCTCAAGGCAGACTGGTCTTTAAAACCTCATGGTCTATGGGGGTCATGGGGTTATG 1929
xmrkB     TG.............................................................................. 1782
xmrkA PSM ................................................................................ 2000

xmrkA     TTTTCCGTTATGAGACCGGAGAGCTTTACCTGTACCAGCTGGACAAGGATGAGGAGCTTTCACCAATATCGGATACATGC 2009
xmrkB     ...............T......................................................AA........ 1862
xmrkA PSM ...............T................................................................ 2080

xmrkA     AGCCTCACCTATAATGACTGGGCAGTGCTAAGGCTTGCAATTCCTCGTGCCAATGAAGATATGG-AATTGGTCTTAGAGC 2088
xmrkB     ...................................C............................-............... 1941
xmrkA PSM ................................................................G............... 2160

xmrkA     GAAATACAAGAGAAGATGAGGCTGACCCTCGATCTGCAAAGAAAACCAGAAGACGGTGTTTCCCAACCGATACTGAAATT 2168
xmrkB     ................................................................................ 2021
xmrkA PSM ................................................................................ 2240

xmrkA     GGAAAGGGACAAATTCTTTTCAGCGCAGTGTGGGAATCGAAAACTACAGAATCTGGGCGCTGGTCCCTCTCAGAGGTCGA 2248
xmrkB     ..................................................................T.AA---------- 2091
xmrkA PSM ..........................G..................................................... 2320

xmrkA     CGCCCGCCCTGCGGCGGGCATGACGTCATGCATTTAAAAGACTTCTGTATAAAAACAAGACGCCATGAGAAGTGTGCACT 2328
xmrkB     -------------------------------------------------------------------------------- 2091
xmrkA PSM ................................................................................ 2400

xmrkA     TCATTTCTGTTGGACAGTAGAGACTTCAAACTTTGTAAAAGTAGTGTTTTTATATTGATTTTGTTTTATATTTACTTCCA 2408
xmrkB     -------------------------------------------------------------------------------- 2091
xmrkA PSM ................................................................................ 2480

xmrkA     AATAAAACCAGGAATATGATCGATCATTTTAGCCATAGCGTAGTGCGCATTTTACGCATGTTGAGTGCAATCAGTAGTTG 2488
xmrkB     -------------------------------------------------------------------------------- 2091
xmrkA PSM ................................................................................ 2560

xmrkA     TTTTATGACCAAAAAGTGAACGAATGCAACATGAAAGCCATTTTTCCGAGTTCCTCCAAGTGCACCACATTTCCGAAGCA 2568
xmrkB     -------------------------------------------------------------------------------- 2091
xmrkA PSM ................................................................................ 2640

xmrkA     ACC-AATCAGAAAGCATTTTCGGGCATTTTGACTTGAGATCGGAACAGCAGCGCGGGGAGAGAGTTTTGTGGCGGAAGAG 2647
xmrkB     -------------------------------------------------------------------------------- 2091
xmrkA PSM ...-............................................................................ 2719

xmrkA     AACGGTGTGCTGTTGAGATTTCGGATTTTACTAGTAGTGTTTTGTATTAGATTTTTTGGAAGTAGTGTTTTGTATTAGAT 2727
xmrkB     -------------------------------------------------------------------------------- 2091
xmrkA PSM .......C........................................................................ 2799

xmrkA     TTTGAGATCATACGTGTGTTAGTTAGTGTGCATTAGTGTGTGTAGACGGTTTTTAGTGTTTAGTGTGTGTCAAAATGAGT 2807
xmrkB     -------------------------------------------------------------------------------- 2091
xmrkA PSM ................................................................................ 2879

xmrkA     CGCTACATGACTGCGCAGCAAGCCCTGAAGTACATGTACGAATGTGAAGAGGACGAAGGCGCGCTGCCAGAAGGTTCGGA 2887
xmrkB     -------------------------------------------------------------------------------- 2091
xmrkA PSM .........C...................................................................... 2959
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xmrkA      CTCGGAGTTTGAGCTTGACGATGAGTTTTTTTTGTTTTTTTTTTCTTGTTTCATTTCTTTGTTTGGCATGGGATATGTAC 2967
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  .................................T.............................................. 3039

xmrkA      AGTAAATTATTATAATCGGAGTAGATGCACACTGAAACACATTATATGCTAATTTCCATCTCCAGCTTGGTGATCCCTCC 3047
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ................................................................................ 3119

xmrkA      ATTCTATTCAGGGAGGAGGAGGAGGAGGAGGAGGAGGAACATTTATTGGCGTACAATAAATACATGGGTGGGGTTGACCT 3127
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ..................................---........................................... 3196

xmrkA      GTCTGATCAACTCATCCAGTATTACTCTGCACAACGGAAAACTTATCGTTGGTACAAGACTGTGCTTATGCATTTGGTTG 3207
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ................................................................................ 3276

xmrkA      ACATTGCCACAGCAAATGCCTACATCCTACACCAAGAGTTGTGCAAAGCCAAAGGAGTGAAGCCCATGACACACAAAGAC 3287
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ................................................................................ 3356

xmrkA      TTCAATGTGGAGATGGCCAGTCAGCTTTGTAGTGTGGACATGGCAGGTGTTCCAAGGAGAAAGGCAGCTGAACACATTCC 3367
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ................................................................................ 3436

xmrkA      TGTGCCCATTAGTGCTTAGCAAAATGCCAGAAACGGCAGACTGCTGTGCAGACACTGCCTCCAGGTGAACAAAGTCAGGA 3447
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  .......G........C............................................................... 3516

xmrkA      AGGACACGCAATGGAAATGTGAGGGCTGTGATGTGCCATTGTGCCTTCTGATTGACAGAAACTGCTTTGCACAATGGCAC 3527
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ................................................................................ 3596

xmrkA      AAGTAATTTGTTTTGTCTTGTGGCACCCATTTTGCAAATATTGTAAATAGTTGTAAAAAAAA-CGCCTGGGAAATTACCC 3606
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ..............................................................A................. 3676

xmrkA      ACAATATGGTGAAAATATGACTAGTAATTTGTTTTTTACTAAGTTTGTACAAAAGTTCTTAAAATTTACCCAAAAACTTT 3686
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ................................................................................ 3756

xmrkA      TCATGTTAGGCTGTAAAAATGGTTTGTTCAACATTTTTGTGAATATTATAGTAAAAATATCAATATTTTTTCTACTCGGA 3766
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ................................................................................ 3836

xmrkA      TTTTGTTTCTGTGTGTGTTTTTTGCTCCAATGTGATAATAAATTTTGTCAAAATGAAACAAACACTGTAAAATCACAAAG 3846
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ................................................................................ 3916

xmrkA      TTTAGGATGTTCTGAAAAGAATGATACCAAACAAGGTAAGGTAAATAGTTTTTATGGTGAAAATATAAGGGTAAATTCAA 3926
xmrkB      -------------------------------------------------------------------------------- 2091
xmrkA PSM  ................................................................................ 3996

xmrkA      AATTAGACCAAAACGGCCATTTAGACCCAAGACTCGGGCGAGCAGACGGAGGGGCGAGAGCCTGAATTCCCCAGACCTGT 4006
xmrkB      -----------------------------------............................................. 2136
xmrkA PSM  ................................................................................ 4076

xmrkA      TTGTGTTGGAGGCTTTCAATGTGGACTGTGGTATATTCAGAACCATGCTGGCTCTCCCATTTGAGGCCTGGTCTGAGAAA 4086
xmrkB      ................................G............................................... 2216
xmrkA PSM  ................................................................................ 4156

xmrkA      ATGACGGAAGTCGGGGATCGGATTTCAGAACTGTTCCGAAAATCTCGCTGCTGGCTCGATATTTTGTCGGTGAAGAAAAC 4166
xmrkB      ................................................................................ 2296
xmrkA PSM  ................................................................................ 4236

xmrkA      TCTGACATTCCCTGTCCTGCCATCCTCTTCTGGCCCGACCCTCTTCTGAGGACCCGCCCCTCTACCTTATATAATGAGAG 4246
xmrkB      ................................................................................ 2376
xmrkA PSM  ................................................................................ 4316

xmrkA      CGCTGCGAGGACTTCTGACACCTTCGGCGCATGGAGCAGCAGTCCGACCTGCCGGACTCTAGTTTCTAACCGGACCGTCT 4326
xmrkB      ............................................T................................... 2456
xmrkA PSM  ................................................................................ 4396

xmrkA      TCATG 4331
xmrkB      ..... 2461
xmrkA PSM  ..... 4401
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Fig. S3: Sequence alignment of 5' flanking regions of different *mdl-xmrk* alleles in *X. maculatus*. A schematic drawing of this alignment is shown in Fig. 12. The start codon is outlined in black. The *piggyBac-like* DNA transposon that is integrated into the promoter region of the $mdl^{Sp\text{-}type}$-$xmrk^{A}$ (from PSM cells) and the mdl^{Sr}-$xmrk^{A}$ allele is shown in light gray. The 134 bp fragment present in the mdl^{Sr}-$xmrk^{A}$ but not in the mdl^{Sd}-$xmrk^{B}$ 5' region is highlighted in dark gray and the two micoindels are shown in magenta. Boxed areas mark copies of a repetitive sequence element. Sequence variations between mdl^{Sr}-$xmrk^{A}$ and mdl^{Sd}-$xmrk^{B}$ promoter sequences are delineated in yellow. Dots indicate conserved sites. Turquois marks regions showing a high nucleotide identity to the promoter region of the proto-oncogene *egfrb*. Putative binding sites for Lef1 and Sox9 transcription factors are shown in green and red, respectively. Putative transcription factor binding sites were determined using MatInspector of the Genomatix portal (http://www.genomatix.de/) (Cartharius et al., 2005).

Figure S4:

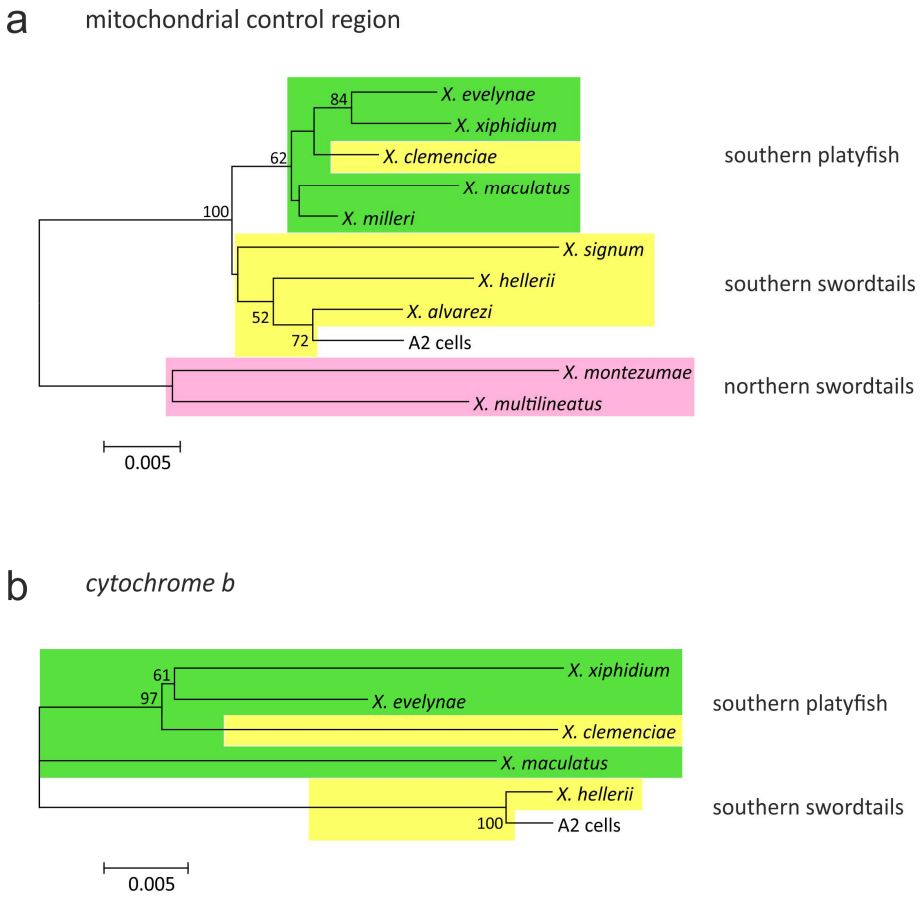

Fig. S4: Phylogenetic tree reconstruction to determine the *Xiphophorus* species the A2 cell line is derived from. Maximum-likelihood tree based on (a) the mitochondrial control region and (b) the *cytochrome b* gene of different *Xiphophorus* species and A2 cells. Numbers above the branches represent the corresponding bootstrap values. Southern platyfish species are highlighted in green, southern swordtail species in yellow and northern swordtail species in pink. In both phylogenetic trees, the A2 cells are nested among the southern swordtails. As *X. hellerii* was the only southern swordtail species present in the aquarium facility of the University of Giessen at the time this cell line was established, the A2 cells are probably derived from *X. hellerii*.

Figure S5:

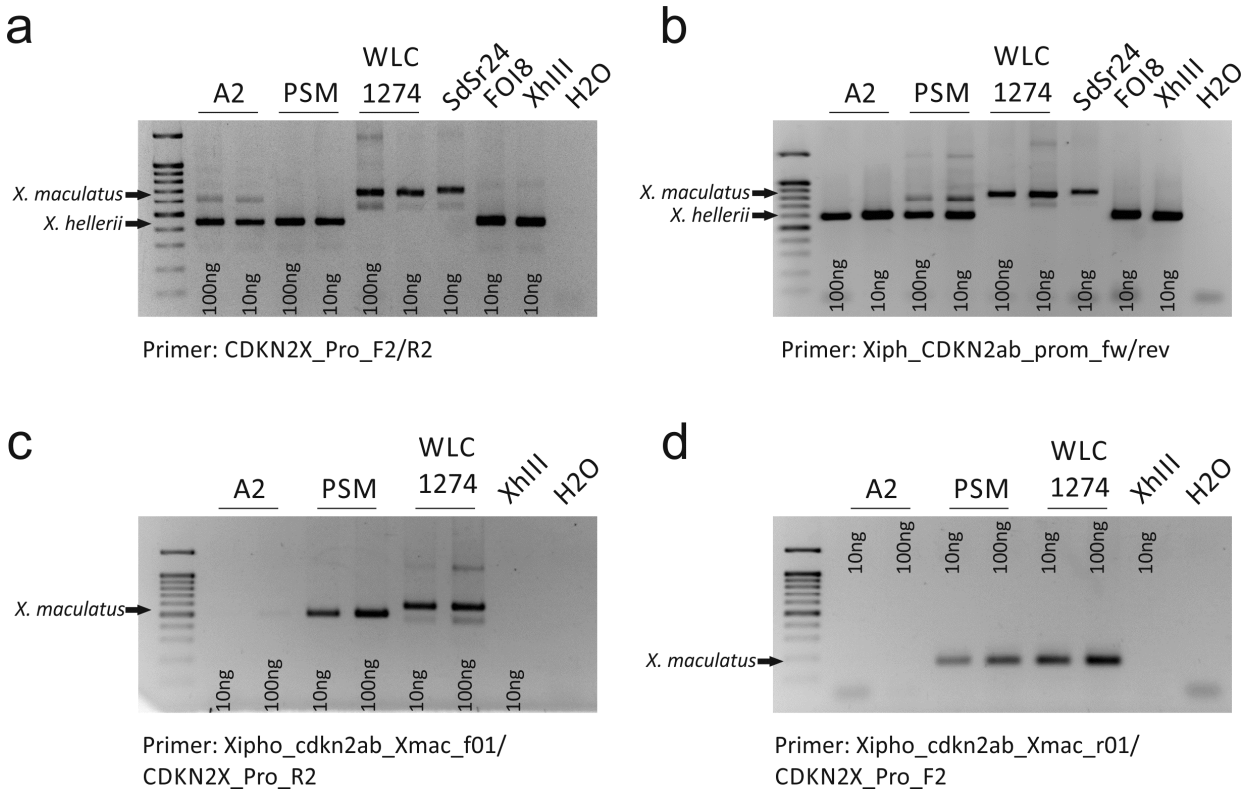

Fig. S5: Analysis of the *cdkn2ab* 5' region in A2 and PSM cells. For (c) and (d) primers specific for the promoter region of the *X. maculatus cdkn2ab* allele were used. These primers do not amplify the *X. hellerii cdkn2ab* allele. The primer pairs used for (a) and (b) amplify the 5' regions of both *cdkn2ab* alleles, but the size of the PCR product is different (indicated by arrows). As a control, PCR amplification was performed on gDNA from pooled tissues of *X. maculatus* (strain WLC 1274) and from *X. maculatus* (SdSr24) and *X. hellerii* (FOI8 and XhIII) derived cell lines. The data strongly indicate that A2 cells, which are probably derived from *X. hellerii* (Fig. S4), are homozygous for the *X. hellerii cdkn2ab* allele. This suggests that A2 cells are devoid of *R*. The melanoma cell line PSM, which was established from a F1 hybrid between *X. hellerii* and *X. maculatus*, is heterozygous, indicating that these cells contain one copy of the chromosomal region where the regulator locus *R* is located.

Figure S6:

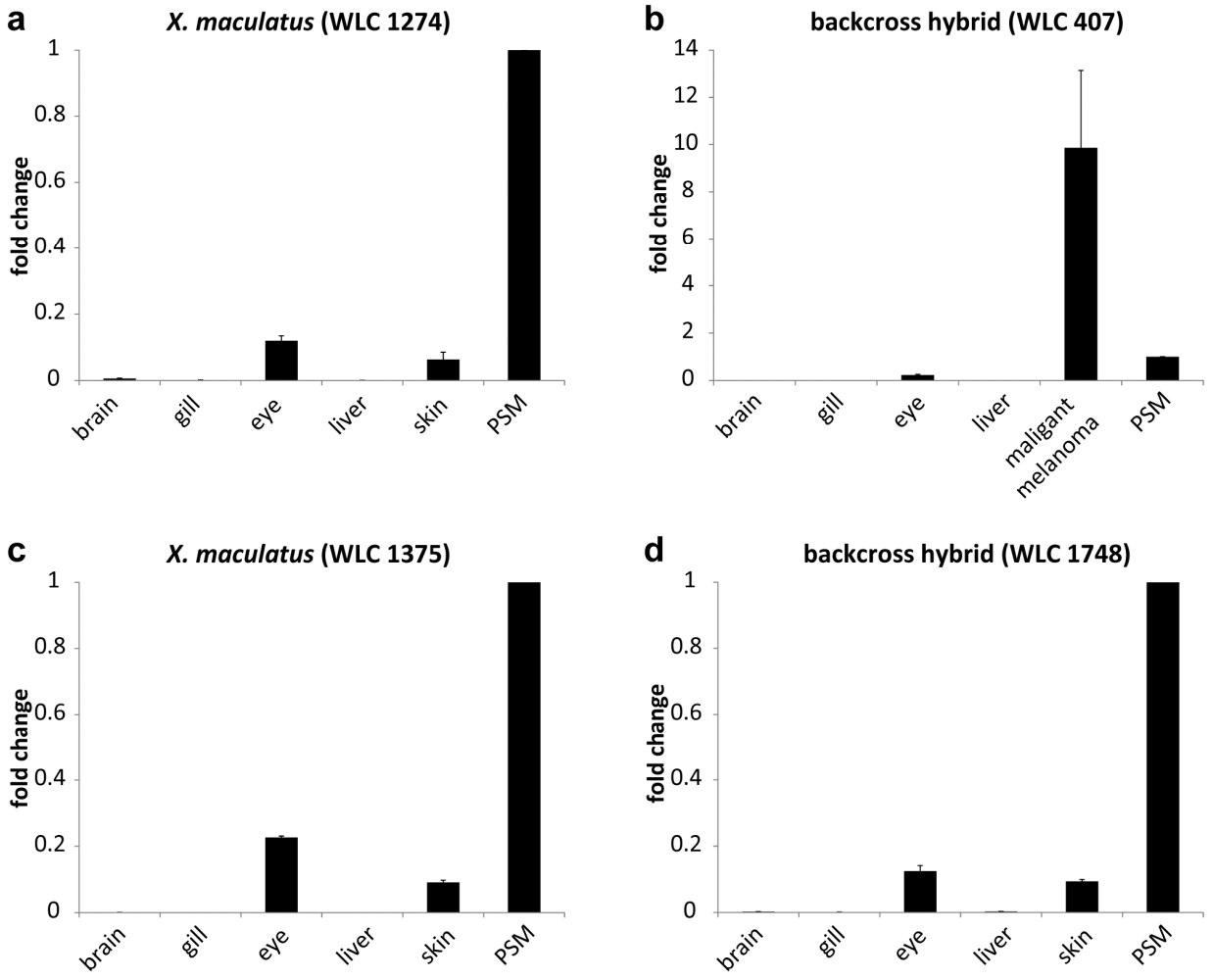

Fig. S6: Analysis of *mitfa* expression in parental and hybrid *Xiphophorus* genotypes. Quantitative real-time PCR analysis of *mitfa* expression levels in different tissues of (a) *X. maculatus* (WLC 1274) and (b) tumor-bearing backcross hybrids (WLC 407) carrying the tumorigenic mdl^{Sd}-$xmrk^{B}$ allele. *mitfa* mRNA expression patterns in (c) purebred platyfish (WLC 1375) and (d) tumor-free backcross hybrids (WLC 1748) carrying the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele. *mitfa* expression in the melanoma cell line PSM was set as reference. Data are presented as mean ± standard deviation of only two independent reverse-transcribed RNA samples.

Figure S7:

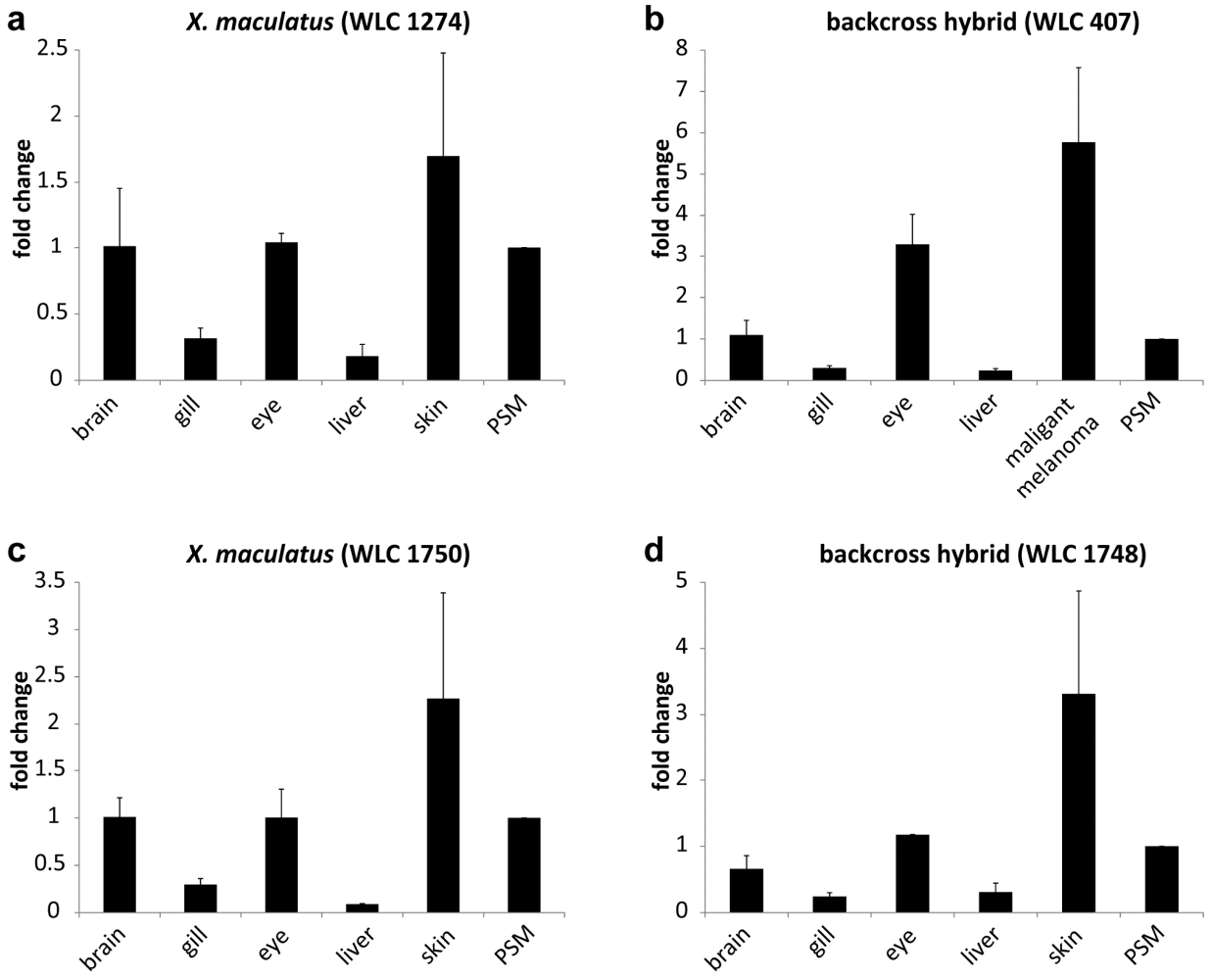

Fig. S7: Quantitative real-time PCR analysis of *mitfb* transcript levels in purebred and hybrid *Xiphophorus* genotypes. *mitfb* mRNA expression levels in tissues of (a) purebred platyfish (WLC 1274) and (b) tumor-bearing backcross hybrids (WLC 407) carrying the tumorigenic mdl^{Sd}-$xmrk^{B}$ allele. Transcript levels of *mitfb* in different tissues of (c) *X. maculatus* (WLC 1750) and (d) tumor-free backcross hybrids (WLC 1748) carrying the non-tumorigenic mdl^{Sr}-$xmrk^{A}$ allele. PSM cells were used as reference. Data are presented as mean ± standard deviation of only two independent reverse-transcribed RNA samples.

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