Comparison of Xiphophorus and Human Melanoma Transcriptomes Reveals Conserved Pathway Interactions

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Summary

To functionally assess the oncogene xmrk co-expressed genes, and compare the functional pathways of these genes to pathways represented in the human melanoma subgroups that were characterized by high and low pigmentation function, we performed gene expression profiling of Xiphophorus melanoma tumors, characterized xmrk co-expressed genes and identified the functional pathways they are associated with. The transcriptomic features and pathways related to the xmrk expression faithfully represent the genetic differences between non-proliferative differentiated and mitogenic dedifferentiated human melanoma. This property supports Xiphophorus melanoma as an appropriate disease model of human melanoma, enabling application of melanoma etiological discovery among vertebrates.

Comparative analysis of human and animal model melanomas can uncover conserved pathways and genetic changes that are relevant for the biology of cancer cells. Spontaneous melanoma in Xiphophorus interspecies backcross hybrid progeny may be informative in identifying genes and functional pathways that are similarly related to melanoma development in all vertebrates, including humans. To assess functional pathways involved in the Xiphophorus melanoma, we performed gene expression profiling of the melanomas produced in interspecies BC₁ and successive backcross generations (i.e., BC₅) of the cross: X. hellerii × [X. maculatus Jp 163 A × X. hellerii]. Using RNA-Seq we identified genes that are transcriptionally co-expressed with the

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driver oncogene, \textit{xmrk}. We determined functional pathways in the fish melanoma that are also present in human melanoma cohorts that may be related to dedifferentiation based on the expression levels of pigmentation genes. Shared pathways between human and \textit{Xiphophorus} melanomas are related to inflammation, cell migration, cell proliferation, pigmentation, cancer development and metastasis. Our results suggest \textit{xmrk} co-expressed genes are associated with dedifferentiation, and highlight these signaling pathways as playing important roles in melanomagenesis.

**Keywords**  
Melanoma; Xiphophorus; Bioinformatics; Signaling transduction; Xmrk; Gene expression; Transcriptome

**Introduction**  
While the incidence of most cancers is decreasing, skin cancer, including melanoma, continues to increase 3-7% per year, exhibiting a 6-fold increase in incidence over the past 40 years, with a 5-year survival rate of only 20% (Berger et al., 2012; Reed et al., 2012; Rigel, 2008; WE, 1982). Despite recent major advances in therapies, the prognosis for melanoma patients with advanced stage melanoma remains poor (Siegel, Naishadham, & Jemal, 2012). Approximately 50% of melanoma patients harbor a BRAF mutation (i.e., BRAF V600E), an oncogene driving the RAF/RAS/MEK signaling leading to proliferation and progression of melanoma cells (Davies et al., 2002; Gopal et al., 2010; Jakob et al., 2012; Long et al., 2011). Clinical data have shown that 50-60% of melanoma patients bearing BRAF V600E mutation respond to FDA approved BRAF inhibitor such as vemurafenib and dabrafenib (Chapman et al., 2011; Falchook et al., 2012; Flaherty et al., 2012; Flaherty et al., 2010; Hauschild et al., 2012; Lito et al., 2012; Long et al., 2012). Recently, immune checkpoint inhibitors targeting PD-1 or PD-L1 were approved by the FDA to treat advanced melanoma (e.g., Pembrolizumab). Approximately one-third of patients with advanced melanoma are responsive to PD-1 inhibitors. However, resistance to BRAF inhibitors is observed in a majority of the responsive patients. One mechanism accounting for the resistance is due to the aberrant up-regulation of EGFR and downstream PI3K/Akt signaling (Wang et al., 2015). Twenty-five percent of the immune checkpoint inhibitor responsive tumors also recur despite continued treatment. The mechanism of the resistance is still elusive although mutations in either JAK1 or JAK2 account for part of the resistance mechanism (Zaretsky et al., 2016). Therefore, mechanistic study of melanomagenesis, especially for the tumors that are not responsive to existing therapy, is needed in order to develop new effective treatments. Additionally, drug resistance observed in current melanoma therapy underscores the need for development of new therapeutic strategies that counteract therapy resistance and/or to target the disease through a different mechanism.

The \textit{Xiphophorus} melanoma model, also known as “Gordon-Kosswig-Anders” melanoma model, was originally introduced in the late 1920s, as one of the first animal models leading to genetic studies of cancer (Gordon, 1927; Häussler, 1928; Kosswig, 1928). This model
employs *X. maculatus* and *X. hellerii* interspecies hybrids to produce spontaneous, yet genetically controlled, melanoma. In the classical cross *X. maculatus* carries the spotted dorsal (*Sd*) macromelanophore pigmentation pattern while *X. hellerii* does not have such a pigmentation pattern (For reviews see: (Patton, Mathers, & Schartl, 2011; R. B. Walter & Kazianis, 2001). Neither the *Sd* locus, nor a functional equivalent of the *X. maculatus* R(*Diff*) locus, a hypothetical tumor suppressor mapped to linkage group 5, are present in *X. hellerii*. The X-chromosome and *Sd* linked oncogene, *xmrk*, is a mutant copy of the fish orthologue of the human EGFR and have been established as a melanoma driver oncogene in transgenic studies (Schartl et al., 2012; Schartl et al., 2015; Schartl et al., 2010). In contrast, the critical genetic component of the autosomal R(*Diff*) locus, which regulates *xmrk*, is still unknown. R(*Diff*) has been mapped to a 5.8 Mb region on linkage group 5, and is proposed to inhibit *xmrk* function in *X. maculatus* parental animals since they rarely develop melanoma tumors (Adam, Maueler, & Schartl, 1991; Kazianis et al., 1998; Kazianis et al., 1999; Lu et al., 2017). F1 interspecies hybrids between *X. maculatus* and *X. hellerii* (i.e., *Sd*-hellerii) exhibit enhanced dorsal fin pigmentation but do not develop melanoma, likely due to regulation by the remaining single copy of the *X. maculatus* R(*Diff*) locus. When F1 hybrids are backcrossed with *X. hellerii*, 25% of progeny that inherited the *xmrk* oncogene, but did not inherit the R(*Diff*) locus, develop spontaneous, lethal melanoma. Other model systems have been developed that take advantage of the *xmrk* driver oncogene, such as the *xmrk* transgenic medaka (Japanese rice fish, *Oryzias latipes*). In this transgenic model, *xmrk* is driven by pigment cell specific *mitf* promoter, resulting in early onset melanoma development with 100% penetrance (Schartl et al., 2012; Schartl et al., 2010).

The *xmrk* oncogene is capable of inducing transformation in melanocytes by maintaining sustained MAPK signaling. Similar to dedifferentiated melanocytes induced by other oncogenes (i.e., bFGF, myc, Ela, ras, or neu) dedifferentiated cells show enhanced proliferation, absence of dendrites, and a lack of melanin production (Dotto, Moellmann, Ghosh, Edwards, & Halaban, 1989; Wellbrock, Fischer, & Schartl, 1998; Wellbrock, Weisser, Geissinger, Troppmair, & Schartl, 2002; Wilson, Dooley, & Hart, 1989). The *xmrk* gene influences several EGFR regulated pathways that are consistent with published observations of mammalian melanomas that drive and maintain the dedifferentiated state (Ge, Fu, & Meadows, 2002; K. Smalley & Eisen, 2000; K. S. Smalley, 2003; K. S. Smalley & Eisen, 2002). These pathways include MAPK signaling, integrin signaling, PI3K signaling, STAT5 signaling, and repression of immune response (Delfgaauw et al., 2003; Geissinger, Weisser, Fischer, Schartl, & Wellbrock, 2002; Morcinek, Weisser, Geissinger, Schartl, & Wellbrock, 2002; Schartl et al., 2015; Wellbrock & Schartl, 1999, 2000; Wellbrock et al., 2002). Although some of the direct functions of *xmrk* driven pathways are well studied, a comprehensive understanding of genes and functional pathways that are associated with *xmrk*-induced dedifferentiation is lacking. As a continuation of previous studies, we used *xmrk* expression as a marker, and utilized contemporary RNA-Seq to perform global assessment of molecular genetic profiles in these *Xiphophorus* melanoma to hallmark genes that co-express or are reversely correlated with *xmrk*, to identify pathways that are associated with *xmrk* expression. We compare functional pathways associated with differentiated pigmentation related gene expression, a feature characterizing terminally differentiated pigment cells in human melanoma patients, to the functional pathways that are
associated with genes that are co-expressed with xmrk in Xiphophorus melanoma. This strategy allowed identification of gene clusters representing the dedifferentiated status of Xiphophorus melanoma, and may be related to the invasive capacity of the melanoma cells. The similarity in functional pathways between the Xiphophorus melanoma and human melanoma suggest that melanomagenesis in Xiphophorus is an informative genetic representation of human melanoma etiology.

Results

Identification of an xmrk co-expression signature in Xiphophorus melanoma

The backcross of F1 hybrid (Sd-hellerii) and X. hellerii lead to spontaneous melanoma in 25% of the BC1 and BC5 progeny (Fig. 1). These tumors expand from the dorsal fin and/or caudal fin and peduncle of the interspecies hybrid progeny. To profile global gene expression, we performed RNA-Seq and assessed gene expression of these melanomas (Supplement Fig. 1). High expression of the driver oncogene, xmrk, is capable of inducing spontaneous melanoma (Schartl et al., 2010; Wittbrodt et al., 1989). The expression of the xmrk in pigment cell both suppresses differentiation and induces a transformed dedifferentiated phenotype (Wellbrock et al., 2002). However, xmrk gene expression levels vary among melanomas by 9.6 fold in both BC1 and BC5 interspecies hybrid progeny (Fig. 2a). To identify genes that correlate in expression level with xmrk, we performed co-expression analysis between each gene and xmrk. In BC1 melanoma, there are 1279 genes showing a pattern of co-expression with xmrk, and 231 genes negatively correlated with the xmrk expression pattern. In BC5 melanoma, 2631 genes co-express with xmrk and 2589 genes negatively correlate with xmrk expression (Supplement Fig. 2). Six hundred and ten genes showed co-expression and 43 genes show negative correlation patterns with xmrk expression in both BC1 and BC5 melanoma (Fig. 2b, Supplement Table 2). Nine stem cell/melanoma cancer stem cell marker, cfl, prom1 (CD133), itga6, itga8, itgb1, tbx2, cdh7, cdh20 and zeb1 are co-expressed with xmrk in the Xiphophorus melanoma (Figure 2c,d).

This is in agreement with previous reports showing that xmrk is capable of dedifferentiating melanocytes in this model system (Delfgaauw et al., 2003; Wellbrock et al., 2002)

Differential gene expression between High- and Low-MITF-axis melanoma patient cohorts

Compared to terminal differentiated melanoma cells, invasive melanoma cells are characterized by loss of pigmentation related genes, amelanotic and dedifferentiated phenotypes (Delfgaauw et al., 2003; Dotto et al., 1989; Wellbrock et al., 2002; Wilson et al., 1989). To identify genes that are co-regulated with pigmentation related genes, we next assessed the gene expression differences between melanoma patient cohorts exhibiting differentiated pigmentation pathway activities. Terminally differentiated melanoma cells show higher expression of melanin biosynthesis genes than dedifferentiated melanoma cells, and this feature was used to identify genes that are related to the melanoma proliferation and invasion. The transcription factor MITF and its direct target genes TYR, TYRP1 and DCT, exert critical control of pigmentation and melanocyte development. We identified 93 human melanoma tumors that showed lower MITF, TYR, TYRP1 and DCT expression than 50% of all 472 SKCM samples (low-MITF-axis, dedifferentiated), and 59 melanoma tumors that showed higher level expression of these genes than 50% of all SKCM tumors (high-MITF-
A total of 491 genes showed differential expression between high-
MITF-axis-cohort and low-MITF-axis-cohort (252 genes highly expressed, and 239 genes
lowly expressed in high-MITF-axis-cohort; Fig. 3a; Supplement Table 3). Seventeen
pigmentation related genes, in addition to MITF, TYR, TYRP1 and DCT, also showed up-
regulation (Fig. 3b). Additionally, as expected, stem cell/melanoma stem cell markers
LIF, NFATC2, NGFR (CD271) showed lower expression in high-MITF-axis cohort.
Metabolism genes related to dedifferentiation (i.e., S100A4, MT1X, MT1A, NNM7, NT5E,
MT1G, AKR1C1, AKR1C2, GLDC) show lower expression, while metabolism genes
related to differentiation (i.e., PPARGC1A and GYG2) show higher expression in high-
MITF-axis cohort (Fig. 3b).

**Comparison of functional pathways between Xiphophorus melanoma and human melanoma**

As both the xmrk co-expressed genes, and the low-MITF-axis cohort show dedifferentiation
markers, we next attempted to identify functional pathways that are associated with the
observed gene expression signature that may represent dedifferentiation. The xmrk co-
expressed genes, and genes that show negative correlation to the xmrk expression pattern,
were analyzed using Gene Set Enrichment Analysis implemented within the Ingenuity
Pathway Analysis software to identify over-represented signaling pathways. Pathway
enrichment analysis of the genes co-expressed and negatively correlated with the xmrk
expression in the Xiphophorus melanoma are represented by 91 functional pathways (-
log\(_{10}\)(enrichment p-value) > 2; Supplement Fig. 4a, b; Supplement Table 4). Similarly,
pathway enrichment analysis was also performed on differentially expressed genes between
the two melanoma patient cohorts. These genes were clustered into 29 functional pathways
(-log\(_{10}\)(enrichment p-value) > 2; Supplement Fig. 4a, c; Supplement Table 4). Twelve
signaling pathways (Axonal Guidance Signaling, Colorectal Cancer Metastasis Signaling,
Epithelial Adherence Junction Signaling, Eumelanin Biosynthesis, IL8 Signaling, ILK
Signaling, Melanocyte Development and Pigmentation Signaling, Molecular Mechanism of
Cancer, Ovarian Cancer Signaling, Pancreatic Adenocarcinoma Signaling, Semaphorin
Signaling in Neurons, and Superpathway of Inositol Phosphate Compounds) are shared
between the Xiphophorus melanoma and human melanoma (Fig. 4a, b). Three of the 12
shared pathways (Colorectal Cancer Metastasis Signaling, IL-8 Signaling and Pancreatic
Adenocarcinoma Signaling) are repressed in the patient cohorts that highly express
pigmentation related genes and activated in the patient cohorts that lowly express
pigmentation related genes. The same 3 pathways are activated in the Xiphophorus
melanoma with highly expressed xmrk and its co-expressed genes (Supplement Table 4).

**Discussion**

The xmrk oncogene is a mutant copy of fish egfr gene encoding a Receptor Tyrosine Kinase
that forms constitutively homodimers and thereby becomes activated in a ligand-independent
manner. Although some downstream signaling pathways that are directly regulated by xmrk
in Xiphophorus melanoma have been well studied (i.e., MAPK signaling, STAT5 signaling,
P38K/Akt signaling; Meierjohann, Scharl, & Volff, 2004; Morcinek et al., 2002; Wellbrock,
Fischer, & Schartl, 1999; Wellbrock & Schartl, 2000; Wellbrock et al., 2002), other genes
that are co-expressed with xmrk and the pathways they associate with, have not been well defined. In this study, to test whether xmrk expression is associated with transcriptomic features of malignant melanoma and to further investigate the ability of Xiphophorus melanoma to model human melanoma, we identified genes and associated functional pathways involved in xmrk driven melanomagenesis in Xiphophorus and compared these to functional pathways associated with human melanoma. We analyzed Xiphophorus melanomas from both BC1 and BC5 hybrid fish, and observed more xmrk-correlating genes in BC5 (5220 genes in BC5) than in BC1 tumors (1510 genes in BC1). This is not due to different sequencing platforms or other technical differences. First, co-expression analyses were performed within tumors from BC1 and BC5 animals, respectively. Second, the gene expression values are normalized to library size (total read counts per sample). Thus, the different numbers of xmrk correlating genes is a result of differences in the genetic background between BC1 and BC5 hybrid fish. BC1 hybrids have 75% of the genome inherited from the recurrent parent (i.e., X. hellerii), while BC5 hybrids have over 98% of the genome derived from the recurrent parent. This difference in genome constitution leads to larger expression variation among BC1 individuals, and likely accounts for the lower number of xmrk correlating genes in BC1 than BC5 due to a greater degree of interspecies allele interactions from the 25% of the X. maculatus genome (non-recurrent parent) present in the BC1 genetic background. We included both BC1 and BC5 samples to capture the most conserved gene set associated with xmrk expression, regardless of the complexity of the genetic backgrounds.

Phenotype plasticity is an essential feature of melanoma. This feature is derived from neural crest progenitor cells that respond to morphogenetic cues from tissue microenvironments and give rise to respective lineages, including melanocytes (Simoes-Costa & Bronner, 2015; Takahashi, Sipp, & Enomoto, 2013). Studies have revealed that melanoma is organized and driven by a subpopulation of cancer cells that have the properties of dedifferentiated stem cells, such as disruption of dendricity, enhanced cell proliferation, and loss of pigmentation (Bracalente et al., 2016; Frank, Schatton, & Frank, 2010; Kalluri & Weinberg, 2009; Lee & Vasioukhin, 2008; Royer & Lu, 2011; Saez-Ayala et al., 2013; Schiaffino, 2010; Serafino et al., 2004). The xmrk oncogene can induce transformation of differentiated melanocytes (Delfgaauw et al., 2003; Wellbrock et al., 1998; Wellbrock et al., 2002). It has been shown to repress the MITF differentiation signal, implying MITF functional suppression partially accounts for the mechanism by which xmrk drives dedifferentiation (Delfgaauw et al., 2003). We found that xmrk expression varied in the Xiphophorus melanoma (Fig. 2a), even in those tumors that had been derived from successive backcrossing (i.e. BC5). This variation suggests that different levels of xmrk signaling activity may be associated with variable target gene regulation among melanomas in individual fish. The melanoma tumor mass is comprised of a large proportion of differentiated non-proliferating melanoma cells, and a small portion of dedifferentiated proliferating melanoma cells (i.e., malignant melanoma with high fraction of well differentiated, non-malignant pigment cells; Goodall et al., 2008). Considering xmrk is capable of transforming pigment cells to a dedifferentiated status, we hypothesized the level of xmrk expression may be an indicator of the relative size of the dedifferentiated melanoma cell population within the tumor mass. We used relatively high xmrk expression levels to hallmark a dedifferentiation state of Xiphophorus melanoma
cells. Melanoma stem cell makers and metastasis related genes *prom1* (CD133), *itga6, itgab, itgb1, tbx2, zeb1, cdh7, cdh20* and *cfl* (Fig. 2c; (Argaw-Denboba et al., 2017; Bosserhoff, Ellmann, & Kuphal, 2011; Bracalente et al., 2016; Madjd et al., 2016; Moore et al., 2004; Zhao et al., 2015; Zimmerer et al., 2016) were found to be co-expressed with *xmrk*. These observations show *xmrk* is associated with a cluster of genes that are capable of maintaining the cells in an undifferentiated state. Functional pathways that are known to associate with *xmrk* oncogenic effect are identified among the *xmrk* co-expressed genes, including EGF signaling, ERK/MAPK signaling, integrin signaling, PI3K/Akt signaling and PI3K related pathways, PTEN signaling, Melanocyte Development and Pigmentation Signaling (Supplement Fig. 4b; Supplement Table 4; Meierjohann et al., 2004; Schartl et al., 2015; Wellbrock et al., 1999; Wellbrock & Schartl, 2000; Wellbrock et al., 2002).

Since expression of MITF driven pigmentation related genes and melanogenesis hallmark the differentiated status of pigment cells, we used MITF and its target genes related to pigment synthesis *TYR, TYRP* and *DCT* to represent different transcriptomic features of disease subtypes in human melanoma (i.e., dedifferentiation and differentiation; Carreira et al., 2005; Carreira et al., 2006; Cheli et al., 2011; Cheli et al., 2012; Cheli, Ohanna, Ballotti, & Bertolotto, 2010; Garraway et al., 2005; Hoek & Goding, 2010; Loercher, Tank, Delston, & Harbour, 2005; Pinner et al., 2009). As expected, we identified two melanoma patient sample cohorts: a cohort that lowly expressed melanin synthesis genes (low-MITF-axis), and a cohort that highly expressed melanin synthesis genes (high-MITF-axis). Along with the lower expression of pigmentation related genes, the low-MITF-axis cohort shows higher expression of stem cell, neural-crest progenitor cell and melanoma cells dedifferentiation markers *LIF, NGFR* (CD271), and *NFATC2* (Fig. 3b; Bernhardt et al., 2017; Boiko et al., 2010; Landsberg et al., 2012; Martello & Smith, 2014; Perotti et al., 2016; Riesenber et al., 2015). Their higher expression suggested that melanomas in low-MITF-axis cohort have a higher percentage of dedifferentiated melanoma cells. Low-MITF-axis cohort also highly expresses *S100A4*, a metastasis-promoting microenvironment factor (Berge et al., 2011; Schmidt-Hansen et al., 2004), as well as several dedifferentiation related metabolism genes *MT1X, MT1A, MT2A, NNMT, NT5E, MT1G, AKR1C1, AKR1C2, GLDC*. Additionally, Low-MITF-axis cohort lowly expresses differentiation related metabolism genes *PPARGC1* and *GYG2* (Bettum et al., 2015). These observations suggest the low-MITF-axis cohort represents dedifferentiated melanoma tumors that are characterized by stem cell-like transcriptional features, while the high-MITF-axis cohort is associated with differentiated non-invasive melanoma (Fig. 3b). Genes co-regulated with *MITF* and its target genes in human melanoma are mainly related to expected pigmentation, inflammation, cell migration and proliferation, cancer development and metastasis, and stem cell (Supplement Fig. 4c; Supplement Table 4). The presence of these signaling pathways is consistent with the dedifferentiation status of low-MITF-axis melanoma cohort, suggesting the low expression of *MITF* and its target genes are indicative of the dedifferentiation expression signature and the phenotype of a subtype of melanoma cells.

To test whether dedifferentiation of *Xiphophorus* melanoma and human melanoma involved similar signaling pathways, we next compared the functional pathways associated with genes that were co-expressed with *xmrk* in *Xiphophorus* melanoma to pathways associated with genes that were differentially expressed between the high- and low-MITF-cohort. We
found 12 functional pathways that are shared between Xiphophorus melanoma and human melanoma (Fig. 4a, b). These pathways involved in inflammation (IL-8 signaling), cell migration (Axonal guidance signaling, Epithelial adherence junction signaling, ILK signaling, Semaphorin signaling, Superpathway of Inositol phosphate compounds), pigmentation (Eumelanin biosynthesis, Melanocyte development and pigmentation signaling), proliferation, cancer development and metastasis (Colorectal cancer metastasis signaling, Ovarian cancer signaling, Pancreatic adenocarcinoma signaling, Molecular mechanism of cancer). To summarize the genetic signature comparisons between Xiphophorus and human melanoma correspond to very similar groups of functional pathways and suggest that all vertebrate melanomas may share disease specific genetic signatures reflecting common developmental mechanisms (Fig. 5). Additionally, IL8 signaling and two signaling pathways related to cancer metastasis (Colorectal cancer metastasis signaling, Pancreatic adenocarcinoma signaling) are activated by xmrk co-expressed genes. The same pathways are also activated in melanoma cohorts that lowly expressed pigmentation related genes (Supplement Table 4). This consistency in functional changes indicates the high expressing xmrk Xiphophorus melanoma share transcriptomic features, and molecular functions of highly proliferative, dedifferentiated human melanoma. These results further substantiate the Xiphophorus melanoma model as representing melanoma cell plasticity at the genetic level, and its potential utility as a model to delineate the genetic etiology of select states in melanoma progression.

In conclusion, the transcriptomic features and tumorigenic pathways related to the xmrk expression faithfully represent the genetic differences between non-proliferative differentiated and mitogenic dedifferentiated human melanoma. This property supports Xiphophorus melanoma as an appropriate disease model of human melanoma, enabling application of melanoma etiological discovery among vertebrates. Additionally, delineating the mechanism of xmrk-driven melanomagenesis and identifying compounds that are able to repressing the xmrk-initiated transcriptional changes that may be applicable to human melanoma treatment.

Materials and Methods
Animal model

A total of 16 first generation backcross (BC1) animals used in this study were supplied by the Xiphophorus Genetic Stock Center (Fig. 1. For contact information see: http://www.xiphophorus.txstate.edu/). Specifically, a X. maculatus Jp 163 A female was artificially inseminated with sperm from a male X. hellerii (Sarabia) to produce F1 interspecies hybrids. F1 interspecies hybrid males were then backcrossed to X. hellerii females to generate the BC1 hybrid progeny. About 25% of the BC1 progeny developed melanoma tumors. At dissection, fish were anesthetized in an ice bath and upon loss of gill movement sacrificed by cranial resection. Organs were either dissected directly into TRI-Reagent (Sigma Inc. St. Louis) placed in a dry ice-ethanol bath if the RNA was isolated at the time of dissection, or dissected into RNAlater (Ambion Inc.) and kept at -80°C for later use. All BC1 fish were maintained and samples taken in accordance with protocol approved by IACUC (IACUC2015107711).
A total of 13 fifth generation melanoma tumor-bearing backcross hybrids (BC5) were produced in an independent series of successive crosses, utilizing F1 hybrids originating from a reciprocal cross in the Biocenter fish facilities (University of Würzburg, Würzburg, Germany). These BC5 progeny were produced from X. maculatus Jp 163 A males mated to X. hellerii (Rio Lancetilla) females. The F1 hybrid females, which developed benign pigment cell precursor lesions, were then successively backcrossed to X. hellerii males to produce BC5. All BC5 fish used in this study were from laboratory stocks maintained in the governmentally certified animal facilities of the Biocenter. All BC5 fish were maintained and samples taken as described above in accordance with the applicable EU and national German legislation governing animal experimentation. Fish were sacrificed by overanesthetization with MS222. All animal experimentation was done under authorization (AZ 568/300-1870/13) of the Veterinary Office of the District Government of Lower Franconia, Germany, in accordance with the German Animal Protection Law (TierSchG).

**RNA isolation and RNA sequencing**

RNA from a total of 16 melanoma tumors and skin dissected from BC1 interspecies hybrid progeny, as well as 13 BC5 melanoma tumors was isolated as previously detailed (Lu et al., 2015; D. J. Walter et al., 2014) using TRI-Reagent (Sigma Inc., St. Louis, MO, USA). Briefly, samples were homogenized in TRI-reagent followed by addition of 200 μl/ml chloroform and the samples vigorously shaken and subjected to centrifugation at 12,000 g for 15 min at 4°C. Total RNA was further purified using RNeasy mini RNA isolation kit (Qiagen, Valencia, CA, USA). Residual DNA was eliminated by incubating RNA samples with DNase for DNA digestion at 25°C for 15 min. Total RNA concentration was determined using a Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY, USA). RNA quality was verified on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) to confirm that RIN scores were above 8.0 prior to sequencing.

RNA sequencing of BC1 fish was performed upon libraries constructed using the Illumina TruSeq library preparation system (Illumina, Inc., San Diego, CA, USA). RNA libraries were sequenced as 125 bp paired-end fragments using Illumina Hi-Seq 2000 system (Illumina, Inc., San Diego, CA, USA). RNA libraries of BC5 fish were sequenced as 100 bp pair-end fragments using the Illumina Hi-Seq 4000 system (Illumina, Inc., San Diego, CA, USA) by the Beijing Genomics Institute (BGI, Hong Kong, China). Sequencing adaptors were removed from raw sequencing reads. The processed reads were subsequently trimmed and filtered based on quality scores by using a custom filtration algorithm that removed low-scoring sections of each read and preserved the longest remaining fragment (Garcia et al., 2012). For RNA-Seq statistics, see Supplement Table 1.

**Gene expression profiling and co-expression analysis in Xiphophorus melanoma model**

To fully represent gene expression profiles of the Xiphophorus melanomas, a concatenated reference transcriptome was constructed by combining the Ensembl X. maculatus transcriptome (ftp.ensembl.org/pub/release-80/fasta/xiphophorus_maculatus/cdna/Xiphophorus_maculatus.Xipmac4.4.2.cdna.all.fa.gz), X. hellerii transcriptome and xmrk sequence (GenBank: X16891.2; Schartl et al., 2013; Shen et al., 2016; Wittbrodt et al., 1989). The trimmed and filtered short sequencing reads were aligned to the custom
transcriptome using Bowtie2 (Langmead & Salzberg, 2012). Custom Perl scripts were developed to count short sequencing reads with either a perfect alignment to one transcript or a perfect secondary alignment to include all short reads mapped to both X. maculatus and X. hellerii alleles of a given gene (Lu et al., 2015; Shen et al., 2013). Sequencing read counts of each gene were normalized to the corresponding library size. BC₁ and BC₅ melanomas were ranked on their xmrk expression, respectively. A gene expression correlation coefficient was calculated for each coding gene using Spearman Ranking Correlation Analysis. A gene with correlation coefficient ≥0.5 or ≤-0.5 was classified as xmrk co-expressed gene or xmrk negative correlated gene. Only genes that showed co-expression or negative correlation with xmrk in both BC₁ and BC₅ melanoma are further analyzed. The workflow of sample collection and data processing is given in Supplement Fig. 1.

**Differential gene expression analysis in human melanoma**

A total of 473 gene expression profiles from human skin cutaneous melanoma (SKCM) were retrieved from The Cancer Genome Atlas (TCGA, tcga-data.nci.nih.gov) SKCM dataset through TCGA data portal. A custom perl script was used to combine the dataset and append a patient-specific sample name to corresponding expression profiles. To separate tumor samples with high pigmentation pathway gene expression and low pigmentation pathway gene expression, tumor samples were ranked on expression levels of MITF, TYR, TYRP1 and DCT. Tumor samples with these 4 genes expressed in lower than 50% of all samples were classified as low pigmentation pathway activity samples (low-MITF-axis-cohort). Tumor samples with these 4 genes expressed in higher than 50% of all samples were classified as high pigmentation pathway activity samples (high-MITF-axis-cohort). The Low-MITF-axis-cohort consists of 93 tumor samples, and the High-MITF-axis-cohort of 59 tumor samples. Differential gene expression analyses were performed between high and low pigmentation pathway activity samples using edgeR (Log₂FC ≥1 or Log₂FC ≤-1, FDR ≤0.05; (Robinson, McCarthy, & Smyth, 2010). To identify the most diagnostic differentially expressed genes in human SKCM dataset, a receiver operating characteristic (ROC) curve was plotted for each differentially expressed gene using R/Bioconductor package pROC. Only differentially expressed genes with ROC Area Under Curve (AUC) ≥ 0.8 were kept for further analysis.

**Gene set enrichment analysis**

Gene Ontology (GO) analysis was conducted using R package “GOstats” (Falcon & Gentleman, 2007). All genes with designated GO term in the GO database (GO.db) were used as background genes, and an enrichment p-value of 0.001 was used to determine statistically significant enrichment. Pathway analysis of xmrk co-regulated genes in the Xiphophorus melanoma, and differentially expressed genes in human melanoma were conducted by implementing Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, California). Pathway enrichment was determined by a p-value < 0.01 (or -log₁₀ p-value>2). Signaling pathways that share genes are connected to form functional network using R package “igraph”. Node size represents number of genes belonging to certain pathways. Width of edges represents number of shared genes of connected pathways. Functional networks were formed using a force-directed layout algorithm.
**Quantitative real time PCR**

*Xiphophorus* melanoma derived gene expression was compared to paired normal skin for identification of differential gene expression (Log₂FC ≥ 1 or Log₂FC ≤ -1, FDR ≤ 0.05, Log₂CPM ≥ 1). A total of 2044 genes differentially expressed (1057 genes down-regulated, 987 genes up-regulated) between BC₁ tumors and paired normal skin tissue. Ten genes were chosen to be validated using QRT-PCR. QRT-PCR was performed by SYBR Green-based detection with an Applied Biosystems 7500Fast system (Applied Bioscience, Carlsbad, CA, USA). Each reaction was subjected to 40 cycles each at 95 °C for 20 s, 95 °C for 15 s, and 60 °C for 30 s. The 18S gene was selected for normalization of all samples. The mean CT values from triplicate runs were used to calculate relative expression levels between tumors and paired skin samples.

**Data Availability**

All sequencing files are submitted to Gene Expression Omnibus (GEO).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Significance

Our observation that the transcriptomic feature of *Xiphophorus* melanoma represents dedifferentiated, proliferative human melanoma suggests that *Xiphophorus* melanoma model is an appropriate model system of human melanoma, enabling application of melanoma etiological discovery among vertebrates. Additionally, delineating the mechanism of *xmrk*-driven melanomagenesis and identifying compounds that are able to repressing the *xmrk*-initiated transcriptional changes may be applicable to human melanoma treatment.
Figure 1. “Gordon-Kossig-Anders” melanoma model

The F₁ interspecies hybrid was produced by crossing *X. maculatus* Jp 163 A (*xmrk *+/+, *R(Diff) */+) to *X. hellerii* (*xmrk */-, *R(Diff) */-). The F₁ hybrid shows enhanced dorsal fin pigmentation but does not develop invasive melanoma due to regulation by the remaining copy of the *R(Diff)* locus. When an F₁ hybrid is backcrossed with *X. hellerii*, 25% of the hybrid progeny that inherited the *xmrk* oncogene but did not acquire the *R(Diff)* locus, develop melanoma tumors.
(a) 

**xmrk expression**

Normalized Expression (CPM)

0 500 1000 1500 2000

BC₁  BC₅
Figure 2. Co-expression of genes with xmrk in Xiphophorus melanoma tumors
(a) The xmrk expression varies by up to 9.6 fold in BC\textsubscript{1} and BC\textsubscript{5}. (b) 610 genes co-expressed with xmrk, of which 43 genes negatively correlate with xmrk expression in both BC\textsubscript{1} and BC\textsubscript{5} Xiphophorus melanoma. (c) Nine stem cell markers co-expressed with xmrk in BC\textsubscript{1} and BC\textsubscript{5} Xiphophorus melanoma. Heatmap represents gene expression in each melanoma tumor of one backcross individual. Melanoma samples are ordered according to xmrk expression level. (d) Correlation coefficients of stem cell markers. Spearman correlation coefficient between two stem cell makers, and between each stem cell marker and xmrk are presented in a heatmap. The dendrogram represents clustering of the coefficients.
Figure 3. Differential gene expression in human melanoma
(a) To identify human melanoma patient samples with high MITF and MITF target genes, samples were categorized based on MITF, TYR, TYRP1 and DCT expression. Samples with each individual gene expressed higher than 50% of all patient samples were classified as high MITF axis cohort while samples with each MITF target gene expressed lower than 50% of all patient samples were classified as low MITF axis cohort. Differential gene expression between these two cohorts of melanoma patients showed 491 genes to be differentially expressed.
expressed (\(|\log2FC| \geq 1, \text{FDR} < 0.05, \text{AUC} \geq 0.8\)). (b) In addition to MITF, TYR, TYRP1 and DCT, 17 other pigmentation related genes were also higher expressed in patients that over-expressed MITF and its target genes. Thirteen dedifferentiation related genes showed lower expression, and 2 differentiation related genes showed higher expression in high MITF axis cohort.
Figure 4. Comparison of Signaling pathways enriched in *Xiphophorus* and human melanoma
(a) Genes enriched in the 12-shared functional pathways in *Xiphophorus* melanoma. (b) Genes enriched in the 12-shared functional pathways in human melanoma.
Figure 5. Model for the relation melanoma cell dedifferentiation with *xmrk* expression