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Analysis of the putative tumor suppressor gene *cdkn2ab* in pigment cells and melanoma of *Xiphophorus* and medaka

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Summary

In humans the *CDKN2A* locus encodes two transcripts, INK4A and ARF. Inactivation of either one by mutations or epigenetic changes is a frequent signature of malignant melanoma and one of the most relevant entry points for melanomagenesis. To analyze whether *cdkn2ab*, the fish ortholog of *CDKN2A*, has a similar function as its human counterpart, we studied its action in fish models for human melanoma. Overexpression of *cdkn2ab* in a *Xiphophorus* melanoma cell line led to decreased proliferation and induction of a senescence-like phenotype, indicating a melanoma-suppressive function analogous to mammals. Coexpression of *Xiphophorus cdkn2ab* in medaka transgenic for the *mitfa:xmrk* melanoma inducing gene resulted in full suppression of melanoma development, whereas CRISPR/Cas9 knock-out of *cdkn2ab* resulted in strongly enhanced tumor growth. In summary, this provides the first functional evidence that *cdkn2ab* acts as a potent tumor suppressor gene in fish melanoma models.

Keywords

cell cycle regulation; senescence; *xmrk*; nevi; p16/INK4A

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INTRODUCTION

The cell cycle regulator *CDKN2A* is a tumor suppressor gene that is mutated in many human cancers (Zhao, Choi, Lee, Bode, & Dong, 2016). Particularly in melanoma, it is the second most frequently mutated gene being affected in 19% of all cases studied (http://cancer.sanger.ac.uk/cosmic). In familial melanoma hereditary mutations in *CDKN2A* are found in 40% of cancers. In a recent study of nevus-associated melanomas, a gradual development of melanoma lesions from nevi via dysplastic lesions to melanoma was proposed (Shain & Bastian, 2016). Analysis of 293 cancer-relevant genes in 37 primary melanomas and their adjacent melanoma precursors showed that early lesions were characterized by *BRAF* or *NRAS* mutations as well as *TERT* mutations, while inactivation of *CDKN2A* occurred later in invasive lesions.

From a large number of studies it is known that the *CDKN2A* gene plays a crucial role in the control of the G1/S-phase checkpoint. In humans and mouse two proteins, INK4A and ARF, are encoded by this gene. These two proteins are unrelated in sequence and are expressed from two different promoters, which give rise to two alternative transcripts. INK4A inhibits specifically the cyclin D dependent kinases 4 and 6 (Fahham, Ghahremani, Sardari, Vaziri, & Ostad, 2009; Kazennov et al., 2013) through binding to these proteins, thereby preventing them from forming productive complexes with cyclin D. In consequence, phosphorylation of the retinoblastoma protein RB that is necessary for cell cycle progression cannot occur (Duronio & Xiong, 2013). ARF inhibits the degradation of the cell cycle regulator p53 by forming a stable complex with the ubiquitin ligase MDM2 in the nucleus (Sperka, Wang, & Rudolph, 2012).

Small aquarium fish provide useful models for biomedical research. Many systems have been developed that perfectly mimic human diseases including cancer. Most importantly, they allow to study tumor development in the context of the intact organism. Hence, the fish models can help to obtain a better understanding of the mechanisms that drive malignant growth and to detect new disease markers and drugs (Schartl, 2014; White, Rose, & Zon, 2013). Fish of the genus *Xiphophorus* were amongst the first animals where cancer was studied and they represent a classical model for melanoma development (Patton, Mitchell, & Nairn, 2010; Schartl & Walter, 2016). In these fish, malignant skin cancers develop from naturally occurring large pigment spots. The spotting patterns are composed of a spectacular type of melanocytes, the so-called macromelanophore. These are giant pigment cells that can reach a diameter of up to 300 μm. Melanoma then develop from these macromelaonophore spots on the basis of an elaborate genetic interaction of a melanoma locus (*tu*), whose activity is downregulated by a tumor suppressor, designated *r* (sometimes also called *diff* or R_{Diff}) in purebred species (e.g. *Xiphophorus maculatus*). Because *tu* and *r* are located on different chromosomes, a crossing scheme can be set up to replace the tumor suppressor-containing chromosome pair by tumor suppressor free chromosomes of a closely related species that does not have the *tu/r* system (e.g. *Xiphophorus hellerii*). In the interspecific hybrids, in the absence of *r*, the oncogene can manifest its deleterious function and highly malignant melanoma develop. The *tu* locus encodes a dominantly acting oncogene, *xmrk*, which is a mutationally altered, constitutively activated version of the epidermal growth factor receptor (Meierjohann & Schartl, 2006; Schartl, 2008). The gene that represents the *r*

locus was not identified to date. A candidate has been proposed based on linkage studies, which placed the *Xiphophorus* ortholog of *cdkn2a* into the region on chromosome V to which *r* had been mapped before (Kazianis et al., 2004; Kazianis et al., 1999; Lu et al., 2017). Its candidacy was, however, called in question mainly due to a counterintuitive high expression in malignant melanoma (Butler, Trono, Beard, Fraijo, & Nairn, 2007).

As so far functional studies on Cdk inhibitors in fish are missing, we revisited this issue by interrogating if *cdkn2ab*, the fish ortholog of human and mouse *CDKN2A*, would exhibit a similar melanoma suppressor gene activity as in mammals. Using transgenic medaka we found that this gene indeed is a potent inhibitor of melanomagenesis. Conversely, CRISPR/Cas9 knock-out resulted in enhanced tumor development. Induction of a senescence-like state by *cdkn2ab* in transformed melanocytes was revealed as mechanism underlying this melanoma-suppressive function.

MATERIAL AND METHODS

Experimental animals/Fish strains:

Adult medaka fish (*Oryzias latipes*) were maintained under standard conditions (Kirchen & West, 1976) with an artificial photoperiod (10 h darkness, 14 h light) to induce reproductive activity. Clusters of fertilized eggs were collected 0.5–1 h after the onset of light and kept in rearing medium (0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2 \times 2H_2O$, 0.016% $MgSO_4 \times 7H_2O$ and 0.0001% methylene blue). Embryos were staged according to (Iwamatsu, 2004). Fish from the following strains were used: *Carbio*, closed colony bred strain carrying the *R'*, *variegated*, pigmentation locus; *HB32C*, inbred strain with wildtype pigmentation; *tg(mitfa:xmrk)*, transgenic line, genetic background *Carbio*, which carries the *xmrk* melanoma oncogene under control of the pigment cell specific *mitfa* promoter; fish of this strain develop various histotypes of melanoma and xanthophoroma/erythrophoroma (Schartl et al., 2010).

Fish of the genus *Xiphophorus* were maintained under standard conditions as described by (Kallman, 1975). For a detailed description of the genotypes used in this study, see Supplementary Materials and (Regneri & Schartl, 2012). Nomenclature of pigment cells and pigment cell tumors is according to (Schartl et al., 2016). For mRNA and protein expression analyses, melanocyte spots (composed of so-called macromelanophores) and benign pigment cell lesions were carefully excised from the dorsal and caudal fins to minimize contamination of the samples with normal tissue. Malignant melanoma were dissected from the dorsal and caudal fins and from the peduncle taking care not to include muscle or healthy skin.

All animals were kept and sampled in accordance with the applicable EU and national German legislation governing animal experimentation. In particular, all experimental protocols were approved through an authorization (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).

Production of stable transgenic *tg(mitfa:cdkn2ab)* medaka:

For generating stable transgenic lines the meganuclease protocol (Thermes et al., 2002) was used. For construction of the transgene vector see Supplementary Materials.

Disruption of *cdkn2ab* by CRISPR/Cas9:

For creating functional *cdkn2ab* knock-out medaka fish we designed guide RNAs for Cas9 directed against two target sites, which create a deletion of approximately 400 bp taking out exon 1 and part of intron 1 including the transcription start site of *cdkn2ab* (see Supplementary Materials).

Cell culture:

The PSM cell line was established from melanoma tissue of an adult F1 hybrid between a platyfish (*X. maculatus*) and an albino swordtail (*X. helleri*) (Wakamatsu, 1981). PSM cells highly overexpress the *xmrk* oncogene. The A2 cell line is derived from the southern swordtail species *X. hellerii* (Regneri, Volff, & Schartl, 2015) and thus is devoid of *xmrk*. The embryonic epithelial cell line SdSr24 is derived from *X. maculatus* (Altschmied et al., 2000). Cells were cultured as described ((Regneri et al., 2015).

Plasmids for cloning of expression constructs (see Supplementary Materials) were transiently transfected into PSM and A2 cells using FuGENE HD (Promega, Fitchburg, USA) and into SdSr24 cells using Xfect transfection reagent (Clontech Laboratories, Mountain View, USA). From d2 on, transfected cells were selected in the presence of 1 μg/ml puromycin. Unless noted otherwise, cells were seeded and transfected in duplicates and data are presented as mean ± standard deviation of these duplicates. For mRNA and protein expression analyses cells were harvested on d2, 5, 8 and 14 post transfection. To quantify the proportion of bi- and multinucleated cells, living cells were counterstained with 5 μg/ml Hoechst 33342 (Thermo Fisher Scientific, Waltham, USA) for 20–30 min on d1, 2, 5, 8 and 14 post transfection to visualize nuclei/DNA. The number of mono-, bi- and multinucleated GFP-positive cells in a region of interest (ROI) was counted using fluorescence microscopy.

Primary cultures from macromelanophores were established by dissecting two-dimensional black spots from tail fin clips of adult *tg(mitfa:xmrk)* medaka. Biopsies were washed for 30 s in 0.4% NaOCl, 2× 30s in EDTA solution, suspended in L15 medium (containing 15% FCS, 1 mM glutamin, 100U/ml penicillin, 100 μg streptomycin) and cut into small pieces. After centrifugation at 1000rcf for 1 min the pellet was resuspended in TE and incubated at 28 °C for 15 min. After centrifugation at 1000rcf for 5 min the pellet was resuspended in 2.5 ml of L15 medium and cultivated for 4–8 days on fish gelatin-coated wells of Ibidi μ slides (ibidi GmbH, Martinsried, Germany). Nuclei were stained with Syto24 (Sigma-Aldrich, St. Louis, USA) and plasma membranes were stained with CellMask Orange plasma membrane stain (Thermo Fisher Scientific, Waltham, USA). Cells were observed under an Eclipse Ti confocal microscope (Nikon, Tokio, Japan) and analyzed with software Volocity 5.4.1.

RNA expression analysis:

Total RNA was extracted from cell lines and *Xiphophorus* and medaka tissues using TRIzol Reagent (Thermo Fisher Scientific, Waltham, USA) according to the supplier's recommendation. For *Xiphophorus* and medaka pigment cell tumors, material of individual fish was used. For normal skin and fins of *Xiphophorus* and medaka, material of several fish (3–5 for normal skin, 10–11 for normal fins) was pooled. For RNA isolation from dorsal fin pigment spots of parental *Xiphophorus maculatus*, spots from 15–30 fish were carefully dissected and pooled. After DNase I treatment, reverse transcription was performed from total RNA using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA) and random hexamer primers according to the manufacturer's instructions. cDNA from 25–50 ng of total RNA was used for quantitative real-time PCR (qPCR) (for primer sequences, see Table S2) using SYBR Green reagent and amplification was monitored with i-Cycler (Bio-Rad, Hercules, USA) or Mastercycler ep realplex (Eppendorf, Hamburg, Germany). For quantification data were analyzed using the delta Ct method (Simpson, Feeney, Boyle, & Stitt, 2000) and normalized to expression levels of the housekeeping gene elongation factor 1 alpha 1 (*eef1α1)*. For spot check, negative control RNA (not reversely transcribed) was used in the PCR reaction. PCR values for each cDNA were determined from duplicates or triplicates and data are presented as mean ± standard deviation of independent (reversed-transcribed) RNA samples. Differences in mRNA expression levels were tested for significance using one-way analysis of variance (ANOVA) or Kruskal-Wallis test. Normally distributed data were analyzed using ANOVA. Data that were not normally distributed were analyzed using non-parametric Kruskal-Wallis test. (∗∗∗): $p<0.001$; (∗∗): $p<0.01$; (∗): $p<0.05$).

Protein analysis:

For Western blot analysis, pigment cell tumors of single fish as well as pooled healthy organs of two *X. hellerii* (WLC 1337) females were used. Western blot analysis were performed as described in (Regneri & Schartl, 2012). Primary antibodies used were: anti-Xmrk, 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, a rabbit polyclonal antibody directed against bacterially-expressed recombinant *Xiphophorus* Cdkn2ab (p13) (see Supplementary Materials). Custom antibodies anti-p21 (sc-469), anti-β-Actin (sc-47778), anti-Rb (sc-50) (Santa Cruz Biotechnology, Inc., Dallas, USA), anti-phospho-Rb (Ser780) and anti-Cleaved Caspase-3 (Asp175) (Cell Signaling Technology, Leiden, Netherlands) were used. As secondary antibodies, horseradish peroxidase-coupled antibodies directed against mouse (Thermo Fisher Scientific, Waltham, USA) or rabbit IgG (Bio-Rad, Hercules, USA) were used. The intensity of the bands of the Western Blots was quantified by ImageJ 1.51f software (https://imagej.nih.gov/ij/notes.html) and normalized to the β-actin loading control. The data represent two to three experiments and are expressed as the mean + SD (standard deviation). Differences in protein expression levels were tested for significance using Wilcoxon-Mann-Whitney U test.

Cdkn2 gene family analysis:

Cdkn2 genes were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/genome) and ENSEMBL (http://www.ensembl.org/index.html) vertebrate genomes by the BLAST/BLAT search tools implemented at those sites using the *Xiphophorus* and several annotated *cdkn2* genes from mammals and fish. Medaka *cdkn2ab* was not annotated in the genome but was retrieved from cDNA clones available through MBRP Medaka sequence collection (https://shigen.nig.ac.jp/medaka/). Orthology and paralogy relationships were determined by phylogenetic methods (Tamura et al., 2011) and according to synteny relationships using the Genomicus genome browser version 89.01 (http://www.genomicus.biologie.ens.fr/genomicus-89.01/cgi-bin/search.pl).

Data availability:

All primary data and reagents are available upon request.

RESULTS

Evolution of the *cdkn2* gene family:

Already when *cdkn2ab* was cloned from *Xiphophorus* in 1999 as the first non-mammalian representative of the *cdkn2* gene family (Kazianis et al., 1999), it became clear that fish have a different repertoire of these important cell cycle regulators (Kazianis et al., 2004). Using available whole genome sequence information and synteny databases we inferred the most likely evolutionary history of the *cdkn2* gene family in vertebrates (Figure 1; Table S1). Orthologs of the tetrapod *cdkn2c* and *cdkn2d* genes with high sequence conservation are present in *Xiphophorus* and other fish. Phylogenetic analyses of amino acid sequences of these paralogs indicated that both genes originated from a common ancestor most likely during the 1R or 2R whole genome duplication event at the base of vertebrates after the split of the lamprey lineage. A single *cdkn2ab* gene is found in the genomes of the coelacanth, actinopterygian fishes, amphibians, reptiles, birds and platypus. A *cdkn2a* and a *cdkn2b* homolog are present adjacent to each other in the opossum genome, but no ARF containing open reading frame is predicted from *cdkn2a*. Bona-fide ARF-like proteins are encoded in the *cdkn2a* genes of basal eutherian mammals, e.g. elephant and armadillo, indicating that the alternate reading frame evolved later after the local gene duplication at the base of the Eutheria. The situation in chicken is obscure. Besides cdkn2ab the ARF specific exon 1ß is found, which together with a duplicated exon 2 of *cdkn2ab* gives rise to a truncated ARF like transcript (S. H. Kim, Mitchell, Fujii, Llanos, & Peters, 2003). Like all other non-mammalian vertebrates medaka and *Xiphophorus* have the common precursor of the *cdkn2a* and *b* genes, termed *cdkn2ab* (Kazianis et al., 2004), with absence of an alternative ARF-related transcript from this gene.

Expression of *cdkn2ab* in pigment cell tumors:

Expression levels of *cdkn2ab* were first determined by quantitative real-time PCR in the *Xiphophorus* melanoma model from normal skin, micro-dissected spots from dorsal fins of the parental platyfish and melanoma of low, intermediate and high-grade malignancy from platyfish/swordtail backcross hybrids. All pigment lesions showed higher expression of

cdkn2ab than normal skin, however, these differences were not always significant (Figure 2A). The degree of malignancy of pigment lesions of *Xiphophorus* is characterized by different transcript amounts of the driver oncogene *xmrk* (Regneri & Schartl, 2012). Thus, *xmrk* expression levels were determined from the same tumors (Figure 2B) revealing higher *xmrk* expression with increasing aggressiveness of the melanomas. For further comparison *cdkn2ab* expression in the *Xiphophorus* melanoma cell line PSM, which has high *xmrk* expression comparable to malignant melanoma, and in the *Xiphophorus* fibroblast cell lines SdSr24 and A2 was determined (Figure 2C, D). A2 cells are derived from *Xiphophorus hellerii* and thus are devoid of *xmrk*, while expression of *cdkn2ab* in the *Xiphophorus maculatus* cell line SdSr24 was not detectable by qPCR analysis. In the highly proliferative PSM and A2 cells, expression of *cdkn2ab* was barely detectable, whereas the slower proliferating SdSr24 cells and biopsies from malignant melanoma showed similar *cdkn2ab* expression levels (Figure 2C). Western blot analyses revealed high expression of Cdkn2ab in some malignant melanoma while others had lower amounts comparable to benign pigment cell lesions (Figure 2E). PSM and A2 cells showed low protein expression in the range of gills or some other normal organs. The SdSr24 cell line had the highest protein levels, again comparable to malignant melanoma.

Inhibition of pigment cell tumor formation by overexpression of *cdkn2ab*:

Xiphophorus fish are livebearers and transgenic technologies are not available. As alternative a transgenic melanoma model in medaka can be used, where the *Xiphophorus xmrk* oncogene is expressed under control of the pigment cell specific *mitfa* promoter (Figure 3A). These fish, alike the platyfish/swordtail hybrids, develop spontaneously aggressive malignant pigment cell tumors that are pathologically and molecularly highly similar to *Xiphophorus* melanoma (Schartl et al., 2015; Schartl et al., 2010).

To investigate a tumor-suppressive effect of Cdkn2ab, stable transgenic lines of medaka were established that express a *cdkn2ab* transgene under the melanocyte and xanthophore/erythrophore specific *mitfa* promoter. In wildtype pigmented fish transgenic for *cdkn2ab* the additional expression levels of *cdkn2ab* contributed by the transgene led to an approximately 25% increase in the number of pigment cells (Figure S1A). No changes in the morphology of pigment cells were noted (data not shown).

However, there was a striking effect on neoplastically transformed pigment cells. After crossing to the *tg(mitfa:xmrk)* (Figure 3A) we observed a full suppression of melanoma and xanthophoroma/erythrophoroma (XE) formation (Figure 3B). Most interestingly, in the double transgenic fish not even benign hyperpigmentation or melanosis developed, which are generally seen as precursor lesions in the course of pigment cell tumor development in fish. This shows that overexpression of *cdkn2ab* suppresses initiation of melanomagenesis at a very early stage.

Enhanced tumor growth and malignancy in *cdkn2ab* knock-out fish:

To create a loss-of-function allele for *cdkn2ab* in medaka the CRISPR/Cas9 technology was employed. Two different guide RNAs were injected that were designed to produce a deletion of exon 1 (Figure S2). Homozygous knock-out fish did not exhibit a noticeable phenotype

different from wildtype fish except for a slight increase in pigment cell numbers (Figure S1B). Importantly, there was no increase of the low background tumor rate (<0.1%) of medaka laboratory strains.

When the deletion allele was crossed into the *mitfa:xmrk* line, a profound effect on melanoma development was noticed. The tumors started already during the first two weeks after hatching and adults developed very fast growing 3-dimensional exophytic or invasive nodular melanoma (Figure 3C, Figure S3). On the gene expression level the tumors from the *cdkn2ab* knock-out genetic background showed the expected reduction or absence of transcripts from the mutant gene (Figure S4). Despite the strong phenotypic effect no dramatic differences in the driver oncogene *xmrk* expression occurred. Also *mitfa*, a pigment cell specific transcription factor that is associated with melanoma development (Garraway et al., 2005; Wellbrock & Arozarena, 2015), and *tp53*, *rb and cdkn1a*, which are cell cycle regulators, whose expression is frequently affected in tumor development (Abbas & Dutta, 2009; Ozaki & Nakagawara, 2011; Sherr & McCormick, 2002; Zlotorynski, 2016) did not show significant differences between tumors from a *cdkn2ab* wildtype or mutant genetic background were seen (Figure S4).

Induction of a senescence-like phenotype in pigment cells by *cdkn2ab*:

To obtain insight into the molecular mechanism of the effect of *cdkn2ab* on pigment cell tumor development, the *cdkn2ab* gene was expressed in the *Xiphophorus hellerii/maculatus* hybrid melanoma cell line PSM and two cell lines established from embryonic fibroblasts of *X. hellerii* and *X. maculatus*. In the melanoma cell line overexpression of *cdkn2ab* led to a clear decrease in cell numbers (from d2 on) (Figure 4A). Western blot analysis of cleaved caspase 3, a marker for apoptosis (McIlwain, Berger, & Mak, 2013; Porter & Janicke, 1999) revealed no correlation with *cdkn2ab* overexpression. (Figure 4E, S5, S6). Interestingly, the decrease in cell number was accompanied by morphological changes, namely a “flattened” and enlarged cell shape and the appearance of large nuclei with prominent nucleoli (Figure 4D). Moreover, a strong increase in the proportion of bi- and multinucleated cells was observed (Figure 4B-D, S7). qPCR analyses revealed upon *cdkn2ab* overexpression no relevant changes in transcript levels of *mitfa*, *rb*, *tp53* and *cyclin E1* (*ccne1)*, which regulates the cell cycle progression from G1 to S phase (Geng et al., 2001). (Figure S4). Interestingly, we found upregulation of p21/Cdkn1a protein expression, which is a known feature of senescent cells (Chang et al., 2000; Georgakilas, Martin, & Bonner, 2017a), on d2 and d5 and downregulation on d14 (Figure 4E, S5). However, on the mRNA level an opposite regulation was seen (Figure S6). This discrepancy might be explained by the presence of a second *cdkn1a* gene in *Xiphophorus*. Moreover, analyzing transcript levels of the important regulator of mitotic entry to cytokinesis *aurkb* (H. J. Kim, Cho, Quan, & Kim, 2011) we detected a robust downregulation (Figure S6). On the protein level, a reduction in total Rb was found on d2 and d5, which, however, was not accompanied by a similar decrease in phosphorylation of Ser780. Over time, Rb protein level increased (d8 and d14) and this is in accordance with the Rb mRNA expression on d14 (Figure 4E, S5, S6). In the embryonic fibroblast cell lines A2 and SdSr24, *cdkn2ab* overexpression induced a similar change in cell morphology as observed in PSM cells (data not shown). However, no increase in the proportion of bi- and multinucleated cells was detected in the non-melanoma cell lines

(Figure S8). Moreover, despite a robust expression of *cdkn2ab* on the mRNA and protein level, no relevant expression changes in the analyzed genes were observed (Figure S6).

Multinucleated cells are a hallmark of human nevi (Leikam et al., 2015), which are made up of senescent cells. These are escapers from active oncogenic signaling, e.g. by mutant BRAF V600E, and express high levels of the *CDKN2A* gene product p16/INK4A (Michaloglou et al., 2005). To validate our *in-vitro* findings we next analyzed *xmrk* transformed melanocytes from *tg(mitfa:xmrk)* medaka. In this line melanoma formation in adult fish starts from small pigment spots that resemble the spots and benign pigment cell lesions of the *Xiphophorus* model. Spot containing fin biopsies were used for short-term primary cultures. It revealed that those spots contain very large pigment cells resembling the *Xiphophorus* macromelanophores. Remarkably, the *xmrk*-transformed medaka melanocytes showed a flattened morphology with multiple nuclei ranging from 2 to 5 per cell (Figure 5A, B) similar to the *cdkn2ab* transfected cells *in-vitro*.

Macromelanophores in *Xiphophorus* are identifiable by their large size (Figure 5C). Such macromelanophores are also clearly discernable in the periphery of highly malignant melanoma (Figure 5D), indicating that senescence may be an ongoing process even in the full-blown cancerous disease.

DISCUSSION

In this study we demonstrated that *cdkn2ab* impacts melanoma development in fish. While knock-out of *cdkn2ab* promoted tumor progression, expression of a *cdkn2ab* transgene in pigment cells of the *tg(mitfa:xmrk)* melanoma medaka line resulted in a full suppression of tumor formation. Overexpression of p16/INK4A in A375 human melanoma cells inhibited proliferation and migration *in-vitro* and led to decreased growth after xenotransplantation into nude mice (Bai, Yu, Long, Feng, & Wang, 2016). This is in agreement with our observation that *cdkn2ab* acts as a melanoma suppressor in fish. Of note, for inhibition of growth and migration of A375 cells the strong *CMV* promoter had to be used, while in our *in-vivo* system *cdkn2ab* transgene expression driven by the *mitfa* promoter at levels not much above the endogenous gene was sufficient. This may indicate that in the initial stage of melanoma development rather low physiological levels of *cdkn2ab* can prevent melanoma formation, while for the full blown malignant disease much higher level are necessary to inhibit progression of the cell cycle.

In line with a melanoma-suppressive function, CRISPR/Cas9 knock-out of *cdkn2ab* resulted in an enhancement of tumorigenesis in the *tg(mitfa:xmrk)* medaka. In the absence of *xmrk* we did not observe spontaneous tumor development in the *cdkn2ab* homozygous knock-outs. This is in contrast to the situation in humans, which have loss of both alleles of the *CDKN2A* locus. Here, a high susceptibility for melanoma (Hussussian et al., 1994) and to a lesser extent for pancreatic cancer (Zhen et al., 2015) has been reported.

In mouse and human cells p53 is activated by the ARF gene product of *CDKN2A*. We did not find evidence in any of the high quality fish genomes for such an alternative transcript. Senescence induction by *cdkn2ab* in *Xiphophorus* melanoma cells was accompanied by a

robust downregulation of the p53 target gene *cdkn1a*. The *CDKN1A* gene product p21 is a potent inhibitor of CDKs 1, 2, 4 and 6 (Bennett, 2016). Upregulation of p21 has been connected to the induction of many features of the senescence phenotype, including a RB dependent G1/S block as well as an arrest in the G2/M phase of the cell cycle (Abbas & Dutta, 2009; Georgakilas, Martin, & Bonner, 2017b). On the other hand, in confirmation of our findings, neither benign human nevi nor human neonatal melanocytes in culture express relevant levels of p53 or p21 (Gray-Schopfer et al., 2006), indicating an independent pathway for p16/INK4A induction of senescence.

Based on genetic linkage analyses the *cdkn2ab* gene was proposed as the critical component of the tumor suppressor locus *r* in the *Xiphophorus* melanoma model (Kazianis et al., 1999). Somehow counter-intuitively, *cdkn2ab* is nevertheless expressed at reasonable levels in malignant melanoma and no mutation that would interfere with a tumor-suppressive function was identified in those tumors (Butler et al., 2007). This called in question that *cdkn2ab* acts as a tumor suppressor gene. We find that Cdkn2ab protein expression levels are comparable in all normal tissues analyzed, independent of the proliferative state of the respective tissue, and lower than in malignant melanomas. On the other hand, the highly proliferative *Xiphophorus* cell lines PSM and A2 exhibit only low expression of *cdkn2ab* mRNA and protein, whereas the slow proliferating SdSr24 cell line expresses *cdkn2ab* on a higher level. *In-vitro* studies with mammalian cell lines revealed a clear inverse correlation between INK4A and/or ARF levels and proliferation (Bai et al., 2016) in agreement with the data from the fish cell lines. However, the expression levels of *cdkn2ab* determined from whole healthy organs or pigment lesion extracts are not reflecting the situation expected from the cell culture data, and thus do not provide a reliable read-out for the proliferative state of a normal tissue or tumor. This may be explained by the fact that *in-vitro* only a single uniformly proliferating cell type is analyzed, while in organs a mixture of cell types with different mitotic indices contribute to the amount of measurable *cdkn2ab* transcript and protein. Moreover, melanomas often contain stromal cells intermixed with the tumor cells. In isolating RNA from dissected melanoma and precursor lesions, these non-tumor stromal cells can skew the expression pattern, and thus may account for the discrepancy to the in-vitro data from the cell line studies. The admixture of normal and tumor cells may reduce the sensitivity of the expression analysis. Of note, the cell line SdSr24 had *cdkn2ab* expression comparable to biopsies of malignant melanoma from *Xiphophorus* backcross hybrids indicating that the *cdkn2ab* level in melanoma is compatible with a reasonable proliferation rate. It is certainly premature to conclude from the steady state expression level in whole organ extracts on the relevance of *cdkn2ab* for down-regulating the cell cycle and extrapolate on its role as a tumor suppressor in the *Xiphophorus* melanoma model.

In human nevi p16/INK4A, the gene product of *CDKN2A*, is upregulated in senescent cells in accordance with its cell cycle inhibiting function (Michaloglou et al., 2005). Our data point to a similar function of the ancestral gene in fish melanoma. Expression of *cdkn2ab* in pigment cells that have activated mitogenic signaling elicited by the oncogenic Xmrk receptor tyrosine kinase drives such cells into a senescence-like state, characterized by a flattened morphology and multiple nuclei. In addition, increased expression of p21 is a frequently seen characteristic of senescent cells (Althubiti et al., 2014; Stein, Drullinger, Soulard, & Dulic, 1999). We also noted a decrease in *aurkb* expression. It has been shown

that AURKB is downregulated during replicative senescence in human primary cells and the knock-down of this gene accelerates cellular senescence (H. J. Kim et al., 2011) accompanied by the appearance of multinuclear cells (Yang et al., 2005). However, unlike in human and mouse, SA-ß-gal staining cannot be used as marker for oncogene induced senescence in fish due to similar activity in non-senescent cells (unpublished data).

The benign pigment spots are composed of macromelanophores, which are multinucleated like human nevus-cells and show a considerable *cdkn2ab* expression. Consequently, we propose that these spots are the fish counterpart of human nevi. In this context it is worthwhile to note that even the most aggressive fish melanoma contain a considerable fraction of highly differentiated, heavily pigment cells and macromelanophores are apparent in periphery of the tumors. It is tempting to assume that these cells contribute considerably to the *cdkn2ab* expression levels measured from whole tumor extracts. Taken together, our results not only qualify *cdkn2ab* as a potent tumor suppressor gene in fish, they also reestablish this gene as a candidate for the *r* locus of the *Xiphophorus* melanoma system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SIGNIFICANCE

The *CDKN2A* gene is a frequently mutated tumor suppressor gene in human melanoma. It is upregulated in nevus cells lending support to the hypothesis that impairment of *CDKN2A* function results in senescence escape and thus is instrumental for transition to malignancy. In the *Xiphophorus* melanoma system the tumor suppressor gene *r* has been mapped to the genomic region that contains *cdkn2ab*, the fish homolog of *CDKN2A*. We show that *cdkn2ab* induces a senescence-like state of transformed fish melanocytes accompanied by a multinuclear phenotype and inhibits melanoma formation, while loss of this gene promotes melanomagenesis in a fish model system.

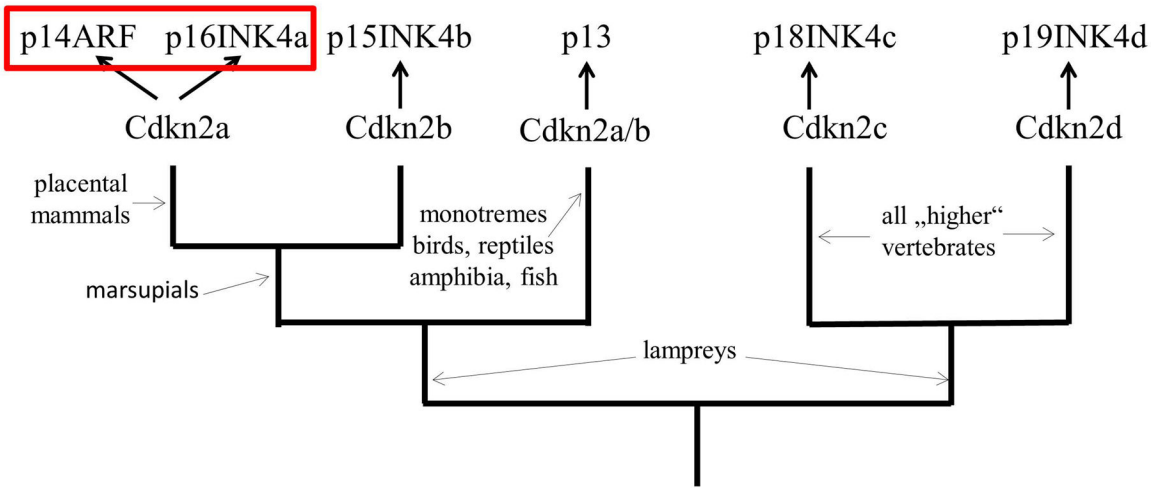

Figure 1:
Schematic representation of phylogenetic relationships of vertebrate *cdkn2* genes

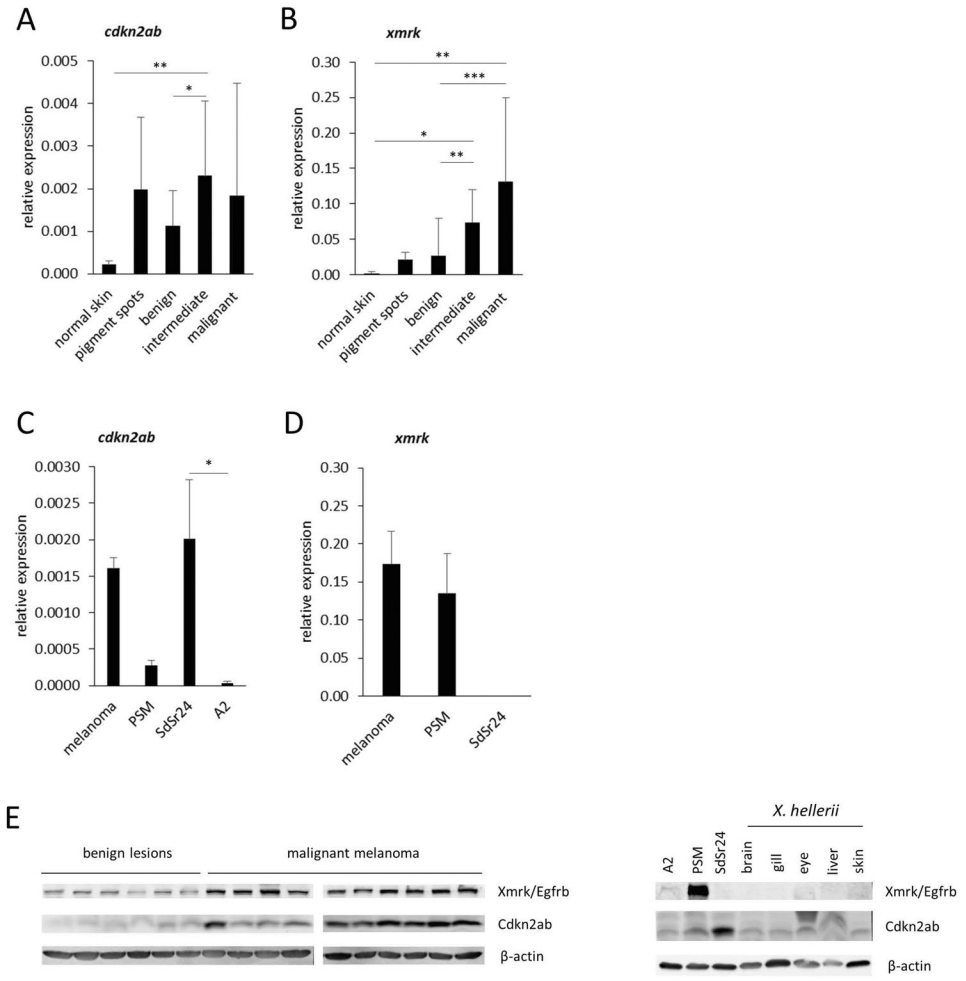

Figure 2: Expression of *cdkn2ab* and *xmrk* in *Xiphophorus* pigment cell lesions of different degrees of malignancy.

qPCR analysis of *cdkn2ab* (A, C) and *xmrk* (B, D) transcript levels in *Xiphophorus* tissues and cell lines. Expression levels were determined in normal skin and dorsal fin pigment spots of wildtype *X. maculatus* (*Jp163A*), in melanoma of low-, intermediate- and high-grade malignancy of *Xiphophorus* interspecific backcross hybrids, in the melanoma cell line PSM and in the embryonal fibroblast cell lines A2 and SdSr24. The number of independent samples (n) is: (A, B) normal skin: n=3, pigment spots: n=2 (pools of 15–20 fish), benign: n=17, intermediate: n=15, malignant: n=29; (C, D) melanoma: n=4, PSM: n=3, SdSr24: n=3, A2: n=3. (E) Western blot analysis of Xmrk and Cdkn2ab protein levels in benign lesions and malignant melanoma of *Xiphophorus* interspecific hybrids, in healthy tissues of *X. hellerii* and in *Xiphophorus* melanoma and embryonic fibroblast cell lines. The Xmrk antibody recognizes Xmrk as well as the proto-oncogene product Egfrb. β-actin was used as loading control. Statistical test used to determine significance are: (A, B,) Kruskal-Wallis. Unless otherwise stated, differences are not significant.

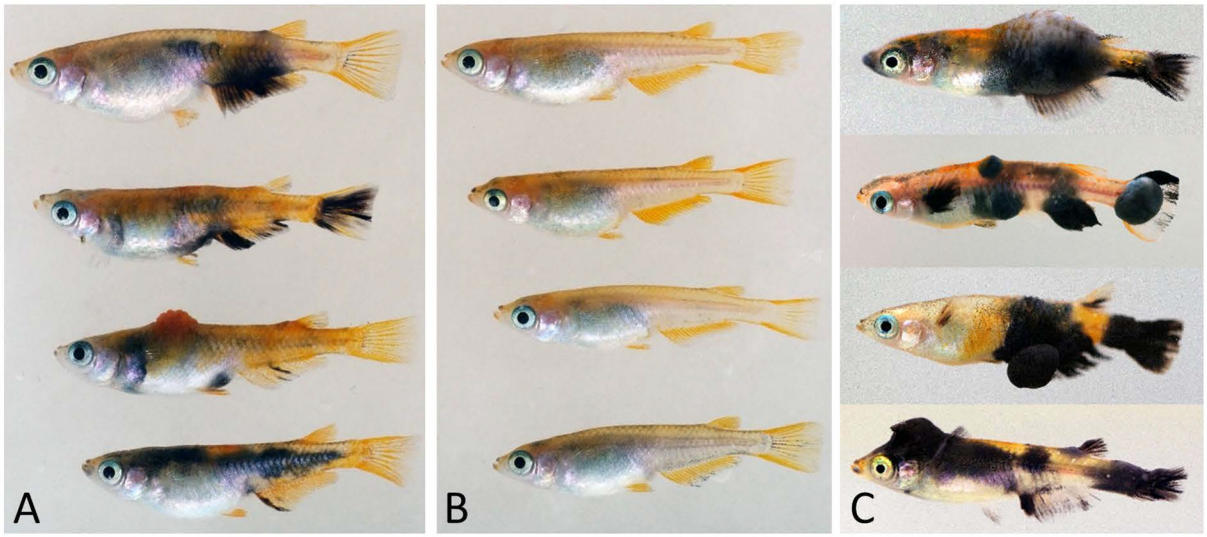

Figure 3: Effect of *cdkn2ab* on tumor formation in *tg(mitfa:xmrk)* medakas.

(A) Phenotype of *tg(mitfa:xmrk)* medakas with one copy of the transgene. (B) Suppression of melanoma and xanthoerythrophoroma development in double transgenic *tg(mitfa:xmrk)/tg(mitfa:cdkn2ab)* medakas in comparison to fish carrying only the *tg(mitfa:xmrk)* transgene (in A). Transgenic fish carry one copy of each transgene. (C) Development of fast growing focal melanoma in *tg(mitfa:xmrk)/cdkn2ab−/−* fish. The fish carry one copy of the transgene and are homozygous for the *cdkn2ab* knockout. Fish in A and B are 7–8 months old, in C from top 5–6 weeks, 5–6 months, 4–5 months, 2–3 months.

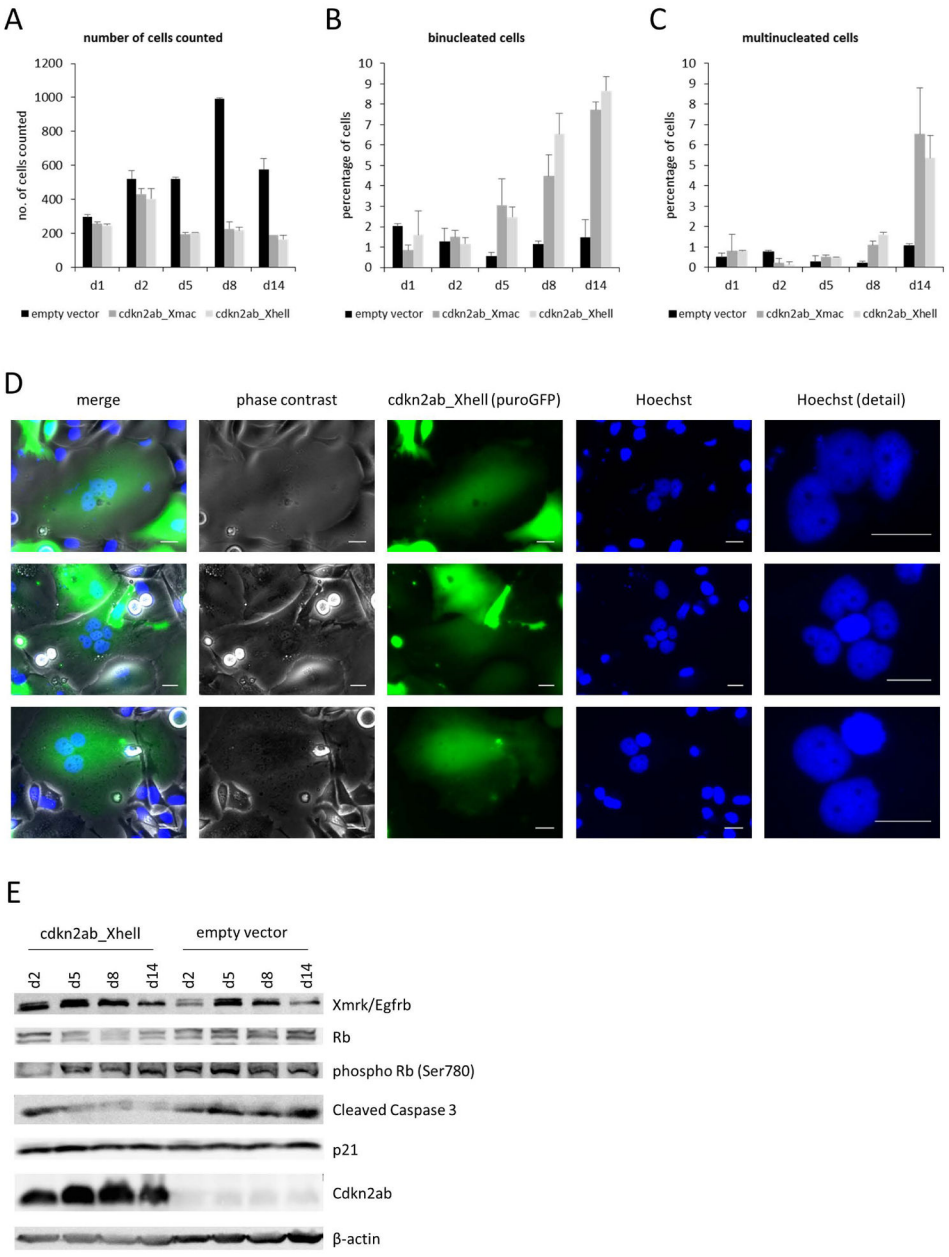

Figure 4: Ectopic expression of *cdkn2ab* leads to development of a senescent phenotype in *Xiphophorus* melanoma cells.

(A) Total number of GFP-positive (*cdkn2ab*-positive) cells per ROI (region of interest) on d1, 2, 5, 8, and 14 post transfection in the melanoma cell line PSM. Cells were transfected with the *cdkn2ab* expression vectors (pCS2+cdkn2ab/Xmac_pf or pCS2+cdkn2ab/Xhell_pf) or the empty vector control. Percentage of binucleated (B) and multinucleated cells (C) among the GFP-positive cells in the same ROIs as used for (A). (D) Representative pictures showing the development of a senescent phenotype in *cdkn2ab*-overexpressing *Xiphophorus* melanoma cells characterized by an enlarged and flattened cell morphology and the occurrence of multiple nuclei. Moreover, nuclei are enlarged and prominent nucleoli are visible. Cells shown here were transfected with the *cdkn2ab* expression vector containing the

X. hellerii cdkn2ab allele (pCS2+cdkn2ab/Xhell_pf) and pictures were taken on day 8 (upper row) or 14 (middle and lower row) post transfection. (E) Western blot analysis of PSM cells transfected with pCS2+cdkn2ab/Xhell_pf or empty vector control. Protein levels were detected on d2, 5, 8 and 14 post transfection. The Xmrk antibody recognizes Xmrk as well as the proto-oncogene product Egfrb. β-actin was used as loading control.

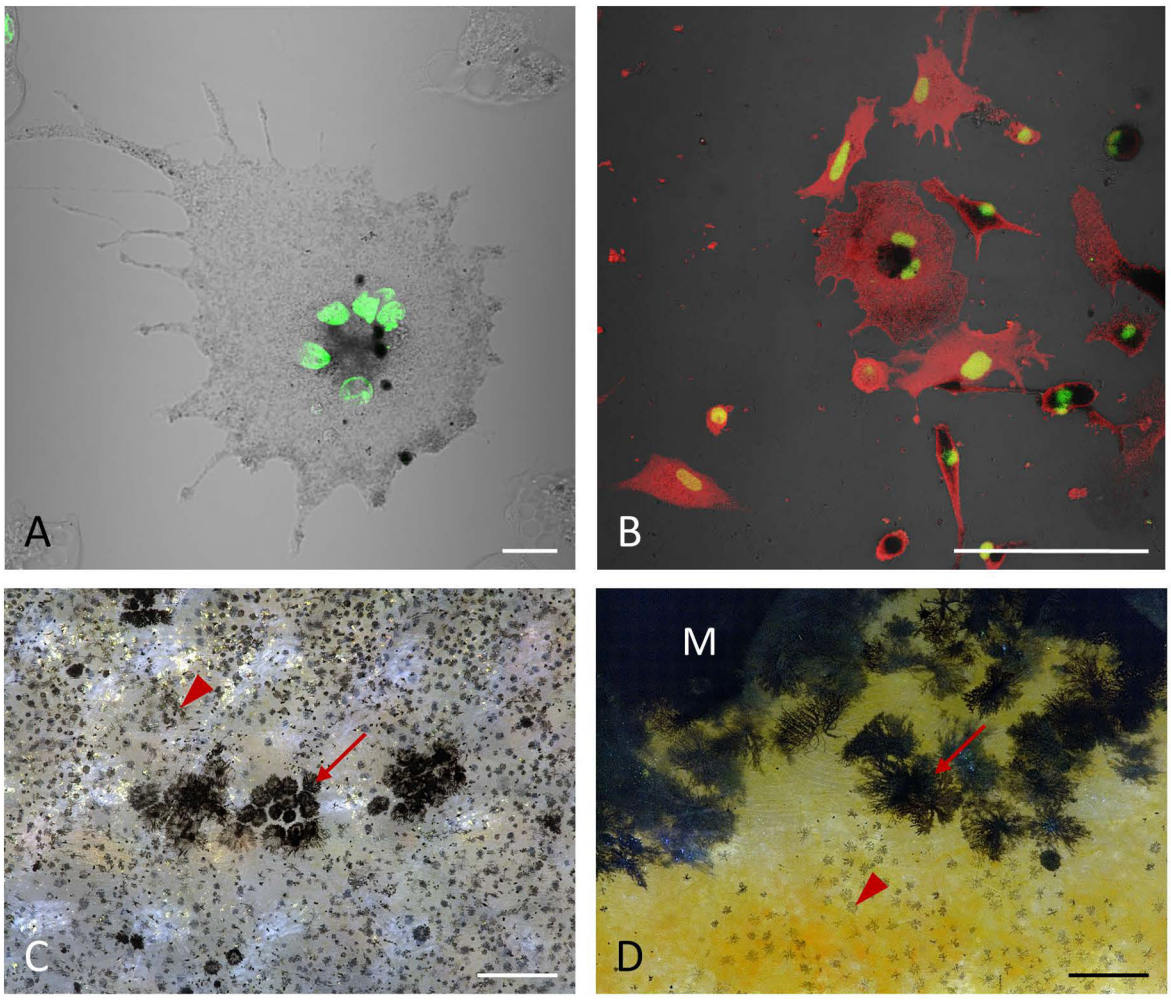

Figure 5. Macromelanophores in medaka and *Xiphophorus*.

(A, B) Confocal laser scanning microscopy images of primary cultures of fin biopsies of transgenic *tg(mitfa:xmrk)* medakas showing very large black pigment cells with multiple nuclei. Nuclei were stained with Syto24 and cell membranes with Cellmask. (A) Overlay of transmission light and Syto24 staining. (B) Overlay of transmission light, Syto24 and Cellmask staining. Macromelanophores in the skin of (C) wildtype platyfish (*Jp163B*) and (D) melanoma bearing *Xiphophorus* backcross hybrids. Macromelanophores (red arrow) and normal-sized melanocytes (red arrowhead). Macromelanophores in (B) are apparent at the margin of the melanoma (M). Pictures of integumental pigment cells of *Xiphophorus* were taken with a VH-Z20W zoom lens connected to a VHX-S90BE and VHX-2000D using the OP-87429 polarization illumination attachment (Keyence, Osaka, Japan). All pictures were taken under incident light conditions. Scale bars: (A) 20 μm, (B) 100 μm, (C, D) 500 μm.