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Genetics

A *CDKN2*-like polymorphism in *Xiphophorus* LG V is associated with UV-B-induced melanoma formation in platyfish–swordtail hybrids

(*CDKN2*/*Xiphophorus* hybrid melanoma/*DIFF* tumor suppressor gene/UV)

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ABSTRACT **The genetic basis of spontaneous melanoma formation in spotted dorsal (*Sd*) *Xiphophorus* platyfish–swordtail hybrids has been studied for decades, and is adequately explained by a two-gene inheritance model involving a sex-linked oncogene, *Xmrk*, and an autosomal tumor suppressor, *DIFF*. The *Xmrk* oncogene encodes a receptor tyrosine kinase related to *EGFR*; the nature of the *DIFF* tumor suppressor gene is unknown. We analyzed the genetic basis of UV-B-induced melanoma formation in closely related, spotted side platyfish–swordtail hybrids, which carry a different sex-linked pigment pattern locus, *Sp*. We UV-irradiated spotted side *Xiphophorus* platyfish–swordtail backcross hybrids to induce melanomas at frequencies 6-fold higher than occur spontaneously in unirradiated control animals. To identify genetic determinants of melanoma susceptibility in this UV-inducible *Xiphophorus* model, we genotyped individual animals from control and UV-irradiated experimental regimes using allozyme and DNA restriction fragment length polymorphisms and tested for joint segregation of genetic markers with pigmentation phenotype and UV-induced melanoma formation. Joint segregation results show linkage of a *CDKN2*-like DNA polymorphism with UV-B-induced melanoma formation in these hybrids. The *CDKN2*-like polymorphism maps to *Xiphophorus* linkage group V and exhibits recombination fractions with *ES1* and *MDH2* allozyme markers consistent with previous localization of the *DIFF* tumor suppressor locus. Our results indicate that the *CDKN2*-like sequence we have cloned and mapped is a candidate for the *DIFF* tumor suppressor gene.**

Genetic hybrids between species of the genus *Xiphophorus* (Teleostei: Poeciliidae) exhibit spontaneous melanoma formation in several different cross types and have been used for decades to investigate genetic factors contributing to melanoma formation (1). The most studied and best understood *Xiphophorus* hybrid melanoma is the spotted dorsal Gordon–Kosswig platyfish–swordtail model (2–5), represented by genetic hybrids derived from crossing F_1 hybrids between the platyfish *Xiphophorus maculatus* Jp 163 A and the swordtail *Xiphophorus helleri* back to *X. helleri.* Melanoma formation in this tumor model is genetically controlled by inheritance of a sex-linked receptor tyrosine kinase gene (*Xmrk*), associated with the spotted dorsal (*Sd*) pigment pattern locus, and segregation of an autosomal locus in *Xiphophorus* linkage group (LG) V, variously referred to in the literature as *DIFF*, R_{DIFF}, and *R* (3–6). The *DIFF* locus is believed to regulate macromelanophore pigment cell differentiation (4, 6), and behaves in the Gordon–Kosswig model as a classical tumor suppressor for which loss of species-specific alleles in pigmented backcross hybrids results in melanoma formation according to simple, Mendelian inheritance (1, 3–5).

Elegant experiments have shown that *Xmrk* is a duplicated gene, which has adventitiously acquired the promoter from another gene (7, 8). It has been postulated that expression of the oncogenic *Xmrk* gene duplicate is regulated by the *DIFF* autosomal locus (5, 8). Supporting this hypothesis are studies showing *Xmrk* overexpression (9), and differential expression (10) of the oncogenic *Xmrk* and its protooncogene copy [referred to as INV-*Xmrk* (11) or *Xmrk*-1 (10)], in spontaneous melanomas. Furthermore, the level of *Xmrk* overexpression correlates with the degree of malignancy of melanomas (7), and *Xmrk* overexpression in transgenic medaka (*Oryzias latipes*) is tumorigenic (12). These results strongly suggest that the regulation of *Xmrk* gene expression is a critical determinant of carcinogenesis in these tumor models. However, no candidate sequence for *DIFF* has been identified, and its putative role in regulating *Xmrk* expression has not been directly shown, but is inferred from genetic studies.

Genetic linkage analyses of melanoma in humans have indicated that alterations in the *CDKN2* gene, encoding the p16 protein, explains part of the clinical phenotype of familial atypical multiple-mole melanoma (FAMMM) syndrome (13, 14). *CDKN2* is also altered in many other primary tumors (15). Germ-line mutations in *CDKN2* have been detected in which p16 dysfunction results in the inability to bind cdk4 *in vitro*, and implicate *CDKN2* in development of heritable melanoma (16). Moreover, there is correlation of loss of expression of *CDKN2* with the invasive stage of melanoma progression (17). There is also evidence for UV induction of *CDKN2* mutations in human melanoma cell lines derived from patients without a family history of melanomas (18). Thus, *CDKN2* is a strong candidate for a human melanoma susceptibility gene.

UV-inducible *Xiphophorus* hybrid melanoma models have been developed to investigate the potential role of excessive sunlight exposure in melanoma formation (19, 20). Setlow *et al.* (19) used backcross hybrids from a spotted side (*Sp*) platyfish–swordtail hybrid model, closely related to the classical, spotted dorsal (*Sd*) Gordon–Kosswig spontaneous melanoma model, to demonstrate that UV irradiation was effective for melanoma induction, resulting in melanoma incidences of up to 40% in UV-B-irradiated backcross hybrids. However, the genetic basis of the UV-inducible, spotted side platyfish–swordtail hybrid model was not investigated in this initial study.

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Abbreviations: LG, linkage group; RFLP, restriction fragment length polymorphism; Lp, light pigmentation; Hp, heavy pigmentation.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U69273).

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We report results here that (*i*) establish that a probable homologue of a mammalian *CDKN2* is located in *Xiphophorus* LG V at or near the location of the *DIFF* tumor suppressor locus, and (*ii*) demonstrate that *CDKN2*-like genotypes are strongly correlated with susceptibility to UV-induced melanoma formation in the spotted side *Xiphophorus* hybrid model.

MATERIALS AND METHODS

Animal Strains and Genetic Hybridization. Parental and hybrid fish were derived from inbred genetic stocks maintained at the *Xiphophorus* Genetic Stock Center (Southwest Texas State University, San Marcos, TX). Stocks of platyfish strains *X. maculatus* Jp 163 A and Jp 163 B have been maintained by brother–sister inbreeding since establishment of *X. maculatus* Jp 163 from collections in the Rio Jamapa (Veracruz, Mexico), in 1939. These two platyfish strains are descended from the same *X. maculatus* female, and differ only in that different pigment pattern loci are carried on the X chromosomes, spotted dorsal (*Sd*) in the case of Jp 163 A, and spotted side (*Sp*) in the case of Jp 163 B (21). Stocks of the swordtail *X. helleri* Sarabia strain have been maintained in closed colony since establishment from collections in the Rio Sarabia (Oaxaca, Mexico), in 1963. Interspecific F_1 hybrids between *X. maculatus* Jp 163 B and *X. helleri* were produced by artificial insemination (22). Matings established between F_1 hybrids and *X. helleri* parental fish resulted in production of backcross hybrids, each of which exhibits heterozygosity for *X. maculatus* and *X. helleri* alleles, or homozygosity for *X. helleri* alleles, at one-half of genetic loci as determined by independent assortment of chromosomes at meiosis.

PCR Amplification and Cloning of a *CDKN2*-Like Sequence from *X. maculatus*. DNA was isolated from brain harvested from *X. maculatus* Jp 163 A according to a previously published protocol (23). Amplification primers were designed based on inspection of human and murine exon 2 *CDKN2A* (p16) and *CDKN2B* (p15) sequences (24–26). "Touchdown" PCR methodology, as described by Don *et al.* (27) and Roux (28), was used to amplify the *Xiphophorus CDKN2*-like sequence. Forward and reverse primers used for amplication were, respectively, P16F1: (GTCATGATGATGGGC) and P16R2: (GCGTGTCCAGGAAGC). The 130-bp amplification product was cloned into the pCRII plasmid by TA cloning (TA cloning kit, Invitrogen). Dideoxy sequencing (29) and Vent polymerase cycle sequencing (Circumvent kit, NEB, Beverly, MA) methods were used to determine the nucleotide sequence of the cloned insert.

UV-Irradiation of Backcross Hybrids. Backcross hybrids were irradiated in a UV-B exposure protocol based on the data of Setlow *et al.* (19). Irradiation conditions used cellulose acetate filtration of FS-20 sunlamps, with a cut-off of wavelengths below 290 nm, and mimic the solar spectrum. Five days after birth, individual broods of 6–24 animals were irradiated from above with three Westinghouse FS-20 sunlamps filtered through cellulose acetate film (Kodacel, Eastman-Kodak) in 2.5-gallon aquaria containing water to a depth of 5 cm. The dose rate was adjusted using a rheostat to modulate sunlamp intensity, resulting in a rate of 0.33 J/m^2 per sec, measured through the cellulose acetate film. Fluence was measured using a Model 1L 1400 A Radiometer/Photometer with a UVB-1 probe (International Light, Newburyport, MA). A total fluence of 1500 J/m^2 (incident to the surface of 5 cm of water) was delivered in equal doses of 300 J/m^2 per day for 5 days. Care was taken to keep irradiated fish completely dark for at least 16 hr after irradiation and in subdued light over the course of the total 5-day irradiation protocol. As they matured, *Sp*-inheriting fish were scored for macromelanophore spotting phenotype (light or heavy) and for presumptive tumors at 4 and 6 months, then sacrificed at 6 months for tissues to perform isozyme and DNA analyses to determine inheritance of genetic markers. Tumors were excised from the animals at the time of sacrifice and fixed in buffered 10% formalin.

Genetic Linkage Analysis. Dissection and tissue preparation for starch gel electrophoresis of proteins followed described methods (30). A listing of polymorphic proteins analyzed in this study, with conditions for electrophoresis and histochemical staining, may be found in Morizot and Schmidt (31). Tissues for DNA extraction (testis, spleen, kidney, gill) were dissected as rapidly as possible and flash frozen in a dry ice-ethanol bath, then stored at −80°C prior to DNA isolation. Genomic DNA for restriction fragment length polymorphism (RFLP) analysis was prepared from tissues of backcross hybrids using commercially available DNA extraction kits (either PureGene Kit, Gentra Systems or IsoQuick DNA Isolation Kit, MicroProbe, Garden Grove, CA). Purified DNA was dissolved in 0.1 × TE (1 mM Tris·HCl/0.1 mM EDTA, pH 7.5) and stored at −20°C until use. DNA samples were digested with appropriate restriction endonucleases, electrophoresed in 0.7% agarose gels, and blotted to Magna nylon membranes (Micron Separations) by capillary transfer. Conditions of hybridization and washing were essentially as described (32–34), except that in some cases random prime labeling (Deca-Prime II Kit, Ambion, Austin, TX) instead of nick translation was used to isotopically label DNA probes. DNA probes used to map *Xiphophorus* loci defined by RFLPs were excised as inserts from plasmids and purified from agarose gels prior to use in Southern hybridization. The origin of each DNA probe (except *ACTBL1* and *CDKN2*) and its use in genetic mapping in *Xiphophorus* genetic hybrids have been reported (32–35). The *ACTBL1* polymorphism was detected with a *Xiphophorus* β-actin cDNA (R.B.W., unpublished work).

DNA RFLP and isozyme nomenclature generally follows standardized human gene nomenclature (36); pigment pattern gene symbols are described in Morizot *et al.* (37). Allozyme and DNA RFLP phenotypes were scored as heterozygotes or homozygotes in accordance with codominant inheritance expectations. Segregation and linkage analyses of the resulting genotypic data were performed with the computer programs MAPMANAGER 2.6.5 (available from Kenneth F. Manly, Roswell Park Memorial Institute, Buffalo, NY) to generate recombination values, and MAPMAKER 3.0 (38) to calculate maximum likelihood gene map orders and map interval information.

Histological Evaluation of Melanomas. Tumors preserved in buffered formalin were imbedded in paraffin blocks, cut into 6-μm sections, and stained with hematoxylin/eosin. Samples were shipped to Avril Woodhead (Brookhaven National Laboratory, Upton, NY), who confirmed the characteristics of invasive melanomas using criteria established in the original description of the spotted side hybrid melanoma model (19). These included intense proliferation of dermal macromelanophores accompanied by inflammation at the margins of the tumors, and an ulcerated, swirled mass of spindle-shaped melanocytes in the interior of the malignant melanotic nodules, with invasion into surrounding tissues.

RESULTS

Joint Segregation Analysis of the *CDKN2*-Like Polymorphism in Backcross Hybrids. The nucleotide and computer-translated amino acid sequences for the PCR amplification product obtained using the *CDKN2* amplimers described in *Materials and Methods* are shown in Fig. 1. As shown by the comparisons of Fig. 1*B*, the *Xiphophorus* sequence in this region is conserved at the amino acid level with human *CDKN2A* (p16), *CDKN2B* (p15), and *CDKN2D* (p19) sequences (24–26, 39), and is only distantly related to human *ankyrin* (40) or to the *notch* homologue from goldfish (GenBank accession no. U09191); comparisons with murine *CDKN2* family members (not shown) also suggest that the

A.

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GTCATGATGATGGGCAGCTCGGAGGTGGCTCGGCTCTTACTGACGGCCGGAGCGG
ATCCAAACGTTACGGACAAAAGCACCGGCGCGACCCCGCTGCATGACGCGGCCCG
AACGGGCTTCCTGGACACGC
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B.

SSEVARLLLTAGADPNVTDKSTGAT-PLHDAART	*Xiphophorus CDKN2L1*
SARVAELLLLHGAEPNCADPATLTR-PVHDAARE	human *p15*
SARVAELLLLHGAEPNCADPATLTR-PVHDAARE	human *p16 (exon 2)*
NPEIARRLLLRGANPDLKDRTGFA--VIHDAARA	human *p18*
STAIALELLKQGASPNVQDTSGTS--PVHDAART	human *p19*
SANIISDLIYQGASLAAQTDRTGET-ALHLAARY	goldfish *notch*
HLEVVKFLLENGANQNVATEDGFTPLAVALQQGH	human *ankyrin*

FIG. 1. *Xiphophorus CDKN2*-like nucleotide sequence and computer-translated amino acid sequences of ankyrin-related genes. (*A*) *Xiphophorus* nucleotide sequence with amplimers underlined. (*B*) Computer-generated translations of nucleotide sequences from *Xiphophorus*, human, and goldfish ankyrin-related sequences, as indicated.

Xiphophorus CDKN2-like sequence corresponds to a *CDKN2*-related gene.

Fig. 2 is a representative Southern blot of *Pst*I-digested DNA from backcross hybrids of the *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) cross type. A distinct RFLP between *X. maculatus* (≈5-kb band) and *X. helleri* (≈1-kb band) *CDKN2*-hybridizing DNA is observable. This polymorphism was designated *CDKN2L1* based on homology with mammalian *CDKN2* sequences (Fig. 1). Table 1 shows the results of joint segregation analysis of *CDKN2L1* genotypes tested against 37 informative polymorphic markers available in this cross. These data clearly establish linkage of the *CDKN2L1* sequence with two allozyme (*ES1* and *MDH2*) and one RFLP (*ACTBL1*) markers in LG V. Multipoint linkage analysis indicates that location of *CDKN2L1* between *ES1* and *MDH2* is > 2500 times more likely than alternate locations: a gene order of *MDH2*–*CDKN2L1*–*ES1*–*ACTBL1* minimizes multiple crossovers in the subset of individuals informative for all LG V loci (totals of 28 single crossovers, 6 double crossovers, and 2 triple crossovers). Fig. 3 illustrates this gene order, and indicates recombination fractions observed between markers.

UV-Induced Melanoma Formation in Hybrids. The general phenotypes of parental and hybrid fish are shown in Fig. 4. The macromelanophore spotting of the spotted side pigmentation pattern in parental *X. maculatus* Jp 163 B animals (Fig. 4*B*) is overexpressed in F_1 hybrids (Fig. 4*C*), resulting in hyperplasia and a "marbled" pigmentation (19). Pigmented backcross hybrids inheriting the *Sp* pigment pattern locus constituted approximately one-half of the total backcross progeny. Pigmented backcross hybrids fell into two groups of roughly one-half heavily pigmented (Hp) fish (Fig. 4*E*) and one-half with a light pigmentation (Lp) pattern (Fig. 4*D*) resembling the F_1 hybrid pigmentation phenotype (Fig. 4*C*), although with somewhat more extensive hyperplasia. Determination of Lp or Hp phenotypes was not possible until the fish had matured to about 2 months of age; by 4 months of age, these pigmentation phenotypes were clearly discernible and distinct.

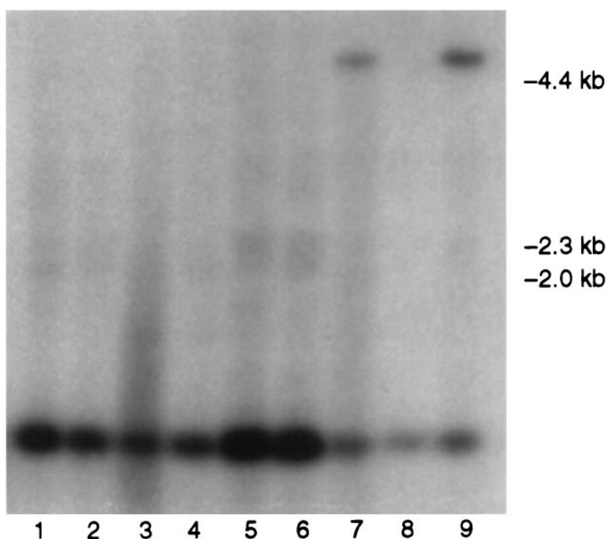

FIG. 2. Representative Southern blot of *CDKN2L1 Pst*I polymorphism in backcross hybrids. Lanes 1–6 and 8, homozygotes; lanes 7 and 9, heterozygotes.

Table 1. Joint segregation analysis of *CDKN2*-like polymorphism with 37 allozyme, RFLP, and pigment pattern loci informative in *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) backcross hybrids

Locus*	LG	No. of parentals	No. of recombinants	r†	χ^2
ACO1	XIV	57	45	0.44	1.4
ACTBL1	**V**	**65**	**19**	**0.23**	**25.2‡**
ADA	I	46	44	0.49	0
ATP	III	27	44	0.62	4.1
CKM	XI	42	44	0.51	0.1
EGFR	VI	16	15	0.48	0
ES1	**V**	**77**	**23**	**0.23**	**29.2‡**
ES2	II	58	53	0.48	0.2
ES3	II	41	32	0.44	1.1
ES5	II	18	18	0.50	0
ES7	III	9	10	0.50	0.1
FYN	XV	32	20	0.39	2.8
GALT1	VIII	50	42	0.47	0.7
GAPD1	III	40	60	0.60	2.0
GDA	XII	30	40	0.57	1.4
GLA	XV	52	47	0.48	0.3
G6PD	I	60	42	0.41	4.8
GPI1	IV	25	25	0.50	0
IDH1	IV	33	32	0.50	0
IDH2	VII	44	52	0.54	0.7
ITP	UA	25	19	0.43	0.8
LIG1	VI	40	48	0.49	0.8
MACR	XXIV	39	63	0.62	5.7
MDH2	**V**	**70**	**17**	**0.20**	**32.3‡**
MPI	II	48	51	0.52	0.1
PEPS	XII	45	37	0.45	0.8
PGAM1	XI	55	39	0.42	2.7
PGAM2	VIII	42	48	0.53	0.4
PGD	I	46	45	0.50	0
PGK	XI	50	41	0.45	0.9
PGM	IX	49	50	0.50	0
PK1	IV	24	26	0.52	0.1
PVALB2	X	38	46	0.55	0.8
SRC	I	27	24	0.47	0.2
TP53	XIV	28	21	0.43	1.0
UMPK	VI	47	52	0.53	0.3
YES	VI	47	45	0.49	0

*Gene symbols are defined in ref. 37, except *ACTBL1*, *Xiphophorus* β-actin like-1; *FYN*, *Xiphophorus FYN*; *LIG1*, *Xiphophorus* DNA ligase 1 (33); *SRC*, *Xiphophorus SRC*; *TP53*, *Xiphophorus* tumor protein p53 (34); *YES*, *Xiphophorus YES.*
†Recombination fraction.
‡Indicates χ^2 associated with $P < 0.001$.

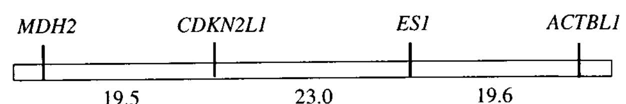

FIG. 3. Genetic linkage markers in *Xiphophorus* LG V. Numerical values refer to percent recombination between markers (see text).

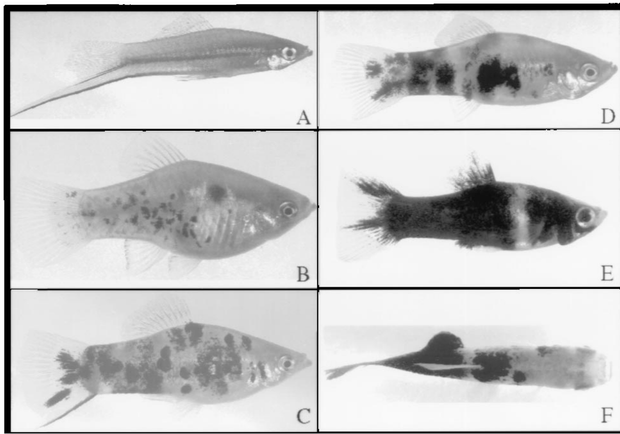

FIG. 4. Parental and F_1 hybrid fish from the *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) cross type. (*A*) *X. helleri*, Sarabia strain, male parent; (*B*) *X. maculatus* Jp 163 B, female parent; (*C*) F_1 hybrid; (*D*) light pigmentation (Lp) phenotype backcross hybrid; (*E*) heavy pigmentation (Hp) phenotype backcross hybrid; (*F*) tumor-bearing, UV-irradiated backcross hybrid. Animals shown in *D* and *E* are from the same brood, photographed at approximately 5 months of age.

Data presented in Table 2 show the frequencies of histologically confirmed melanomas in UV-B-irradiated and unirradiated control animals. An example of a fish with such a tumor is shown in Fig. 4*F*. The results in Table 2 show that the spontaneous incidence of melanomas at 4 and 6 months was very low, with no tumors detected in lightly pigmented hybrids, and only one histologically confirmed melanoma in heavily pigmented hybrids. In UV-irradiated backcross hybrids, lightly pigmented animals exhibited a low incidence of melanoma formation (5.4%), but the incidence of melanomas formed in heavily pigmented hybrids was 34.8%, or more than 6 times the incidence in unirradiated, heavily pigmented hybrids (5.5%). These results independently confirm the findings of Setlow *et al.* (19), demonstrating that *Sp* pigment pattern-derived platyfish–swordtail backcross hybrids are susceptible to UV-induced melanoma formation.

Linkage of *CDKN2L1* with UV-Induced Melanoma Formation. Table 3 presents joint segregation results for *CDKN2L1* genotypes with pigmentation phenotypes and UV-induced neoplasms in backcross hybrids. Analysis of the inheritance of Lp and Hp phenotypes represents a genetic test of *DIFF* regulation of melanocytic hyperplasia in backcross hybrids; the progressive loss of this regulation correlates with segregation of *X. maculatus DIFF* in *Sd*, Gordon–Kosswig backcross hybrids, and results in pigment pattern enhancement (1, 3–6). In *Sd* hybrids, inheritance of both Lp and Hp phenotypes is strongly associated with LG V genotypes, as is also spontaneous melanoma formation from melanoblasts of the spotted dorsal pigment pattern in Hp animals (1, 3–5). Surprisingly, the data in Table 3 show only a weak association of combined Lp and Hp phenotypes with *CDKN2L1* in *Sp* backcross hybrids, and no linkage at all with Lp phenotypes, although linkage of *CDKN2L1* with both UV-induced hyperplastic nodular lesions and the subset of histologically confirmed UV-induced melanomas is highly significant. Only the Hp phenotype is significantly associated with *CDKN2L1* genotypes; this LG V association is confirmed by linkage of Hp with *ES1* (42 parentals, 14 recombinants; $\chi^2 = 14.0$). Thus, although there is significant linkage of *CDKN2L1* genotypes with UV-induced tumor phenotypes, the LG V *DIFF* locus does not appear to solely regulate melanocytic hyperplasia in *Sp* hybrids. This result is in marked contrast to the extensively documented co-inheritance of pigment pattern phenotypes and spontaneous melanoma formation in *Sd* Gordon–Kosswig platyfish–swordtail hybrids (1–5).

Table 3. Joint segregation of *CDKN2L1* genotypes with pigmentation and tumor phenotypes in *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) backcross hybrids

Phenotype†	No. of parentals	No. of recombinants	r‡	χ^2
Lp	7	10	0.59	0.5
Hp	30	11	0.27	8.8**
Lp + Hp	37	21	0.36	6.1*
UV-HNL	28	3	0.10	20.2***
UV-HCM	17	2	0.11	11.8***

†Lp, light pigmentation phenotype; Hp, heavy pigmentation phenotype; UV-HNL, UV-induced hyperplastic nodular lesions; UV-HCM, UV-induced histologically confirmed melanomas.
‡Recombination fraction.
*, $P < 0.05$.
**, $P < 0.01$.
***, $P < 0.001$.

DISCUSSION

Melanoma incidence shows an alarming worldwide increase (41–43). Heredity, target cell susceptibility, and excessive sunlight exposure are all believed to be factors in cutaneous malignant melanoma (44, 45); however, a precise role for each of these factors in melanoma formation has not been established (45). *CDKN2A*, encoding the p16 gene product, is a strong candidate for a human melanoma susceptibility gene, based on pedigree analyses of families with hereditary melanomas (13, 14, 46), and genetic and biochemical studies of both hereditary and sporadic melanomas (16, 17). Mutations in *CDKN2A* consistent with UV induction have recently been reported in human melanoma cell lines (18), retrospectively suggesting a possible sunlight etiology. Directly testing a role for sunlight exposure in the etiology of melanoma, however, will require experiments in animal models exhibiting sunlight-inducible melanoma formation; the most useful of these models will be amenable to genetic analysis and offer correspondence between human and animal genes implicated in melanoma formation.

Xiphophorus genetic hybrids offer both spontaneous and sunlight-inducible melanoma models. Spontaneous melanoma formation in Gordon–Kosswig platyfish–swordtail hybrids has been studied for decades, and represents an experimental paradigm for hereditary tumor formation (1, 3–5). Recently, work by Setlow and colleagues has established the usefulness of other *Xiphophorus* hybrids for investigation of sunlight-inducible melanoma formation (19, 20). In this study, we subjected one of these sunlight-inducible *Xiphophorus* melanoma models, closely related to the classical Gordon–Kosswig

Table 2. Effects of UV-B irradiation on melanoma formation in *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) backcross hybrids

	Unirradiated				UV-B irradiated			
	Light phenotype		Heavy phenotype		Light phenotype		Heavy phenotype	
Scoring	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor
4 months	0	27	1	17	1	56	16	30
6 months	0	27	1	17	3	53	16	30

hybrid model, to linkage analysis for the inheritance of polymorphic DNA and protein markers with UV-induced melanoma formation. One of these markers is a *CDKN2*-like DNA sequence we recovered by PCR amplification from the *X. maculatus* genome. This *CDKN2*-related sequence maps to *Xiphophorus* LG V, in the close vicinity of the *DIFF* locus (Table 1, Fig. 3). Furthermore, there is significant linkage of *CDKN2L1* genotypes to UV-induced melanoma formation in *Sp* platyfish–swordtail backcross hybrids (Table 3). These results, and the evidence from a large number of studies implicating *CDKN2A* in human melanomas, establish a strong basis for considering this *CDKN2*-related sequence from *Xiphophorus* to be a candidate for the *DIFF* tumor suppressor gene.

The *Xiphophorus CDKN2*-related DNA sequence we have cloned and mapped exhibits an open reading frame with significant homology to a region in exon 2 of human *CDKN2A* coding for parts of the second and third of four ankyrin domain repeats (Fig. 1). Human sequences coding for homologous ankyrin domain regions in the p15 (*CDKN2B*) and p19 (*CDKN2D*) proteins likewise show extensive amino acid identity with the fish sequence (18 of 33 amino acids are identical compared with human p15 and p16; 18 of 32 compared with human p19). Human p18 possesses five ankyrin repeats and is somewhat less similar (16 of 32 amino acids identical) to the translated *Xiphophorus* sequence. Numerous possible alignments of non-*CDKN2*-encoded proteins with ankyrin domains are far less similar to the translated fish sequence than are sequences of *CDKN2* gene family members: in Fig. 1, the best alignments produced by CLUSTAL W (47) software yield only 10 of 33 identical amino acids for the goldfish *notch* gene (6 ankyrin repeats) and 9 of 34 identical amino acids for the prototypical human *ankyrin* gene (24 ankyrin repeats). Comparisons with mouse and rat sequences (data not shown) likewise support assignment of the fish DNA sequence to the *CDKN2* gene family, but establishment of orthology with a particular family member is not possible with the available sequence data. In future experiments, it will be very interesting to determine the size of the *CDKN2* gene family in *Xiphophorus,* and to establish orthology and gene map locations for comparison to mammalian *CDKN2* loci.

Results of the joint segregation analysis of *CDKN2L1* with the 37 other polymorphic genetic markers available in *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) backcross hybrids unequivocally establish its linkage with LG V markers *ES1, MDH2,* and *ACTBL1* (Table 1), and strongly support the gene order shown in Fig. 3. Precise localization of the *DIFF* tumor suppressor gene in *Xiphophorus* LG V has proven to be difficult, both in Gordon–Kosswig hybrids and in other *Xiphophorus* melanoma models, primarily because of variable recombination estimates with other LG V markers. Recombination estimates with *ES1* have ranged from ≈10-38% in various Gordon–Kosswig melanoma crosses, in a few cases being higher than estimates with *MDH2,* which often exceed 30% (48–50). While the reasons for such variability remain unclear, they may indicate interspecific differences in gene arrangement (4), or misclassification of intermediate pigment pattern intensity in some backcross individuals (48), or could reflect variable development of melanomas with different etiologies. Our localization of the *CDKN2L1* sequence in LG V is thus of considerable interest in assessment of the likelihood of its identity with *DIFF.* Our results from the *Sp* platyfish–swordtail UV-inducible melanoma model suggest that the *CDKN2L1* sequence resides near the predicted location of *DIFF* in LG V midway between *ES1* and *MDH2* (Fig. 3). Unfortunately, other LG V loci are uninformative in this cross. An additional marker, *GLYDH*, has been mapped between *ES1* and *MDH2* in LG V, again with variable recombination in different cross types ranging from 5–29% with *MDH2* (37, 51). Recently, we analyzed the inheritance of pigmentation phenotypes and tumor susceptibility in another inducible *Xiphophorus* melanoma model, in backcross hybrids of the cross type *Xiphophorus couchianus* × (*X. maculatus* Jp 163 B × *X. couchianus*), which is informative for *GLYDH* (52). Our preliminary linkage analyses (unpublished data) of this hybrid indicate a gene order of *GLYDH–CDKN2L1–ACTBL1*, confirming the localization of *CDKN2L1* in the *DIFF* region of LG V reported here.

The critical result in this study of significant association of *CDKN2L1* genotypes with UV melanoma induction (Table 3) strongly indicates that a *CDKN2*-related tumor susceptibility gene is a likely candidate for the *DIFF* tumor suppressor identified in the Gordon–Kosswig spontaneous hybrid melanoma model. However, results of Table 3 indicate that, although *CDKN2L1* genotypes are strongly associated with UV-induced tumor formation, there is only a weak association, or none at all, with inheritance of Lp and Hp pigmentation phenotypes in *Sp* platyfish–swordtail hybrids. In the Gordon–Kosswig model, it is impossible to separate genetic factors controlling pigmentation from those controlling melanoma susceptibility, because all heavily pigmented individuals develop melanomas with age, whereas almost no lightly pigmented individuals develop tumors during the usual time course of experiments (≤1 year). Several other *Xiphophorus* crosses yield heavy and light phenotypes in Mendelian proportions in backcrosses and variable frequencies of spontaneous melanomas in heavily pigmented individuals, independent of genotype at LG V markers (4, 51). The lack of association of Lp phenotypes with *CDKN2L1* and other LG V genetic markers is therefore not without precedent in these hybrid models.

In the *Sp* platyfish–swordtail hybrid model, loss of *X. maculatus* alleles in the LG V region containing *DIFF* does not ineluctably lead to formation of nodular lesions and melanomas in *Sp*-inheriting backcross hybrids, but does appear to be a necessary precondition for UV-induced tumorigenesis, which occurs only in heavily pigmented backcross hybrids at an appreciable frequency (Table 2). The strong correlation of *CDKN2L1* genotypes with tumor susceptibility (Table 3), coupled with its localization to the *DIFF* region of LG V, suggests that a *CDKN2* gene is a likely candidate for the *DIFF* tumor suppressor gene controlling development of malignant melanomas in *Xiphophorus* hybrid tumor models. The *CDKN2*-related sequence we have identified and linked to melanoma induction is behaving as would be predicted for a tumor susceptibility gene; its identification with *DIFF* relies on very strong genetic linkage and map order data with LG V markers (Table 1, Fig. 3), and the compelling association of *CDKN2L1* genotypes with susceptibility to UV-B-induced melanomas (Table 3). However, a two-gene inheritance model involving *DIFF* as a genetic trait determining pigment pattern phenotypes, as defined in the *Sd*, Gordon–Kosswig platyfish–swordtail hybrid, is not supported by our genetic linkage results for the *Sp* platyfish–swordtail hybrid. This finding suggests that proliferation and invasive tumor formation are separable in *Xiphophorus* melanoma models, and that other genes, in addition to *DIFF*, may regulate melanocyte proliferation; the identification of these genes will be an important focus of future studies.

In a recent study, Schartl *et al.* (53) investigated spontaneous melanoma formation in nonhybrid *Xiphophorus* species. In melanomas originating in certain purebred stocks, *Xmrk* overexpression was observed, consistent with a unifying mechanism for tumorigenesis in both hybrid and nonhybrid tumor models. However, the incidences of spontaneous melanoma formation in nonhybrid strains varies widely, from less than 1% to more than 25% (4, 23, 53), suggesting that simply overcoming suppression by *DIFF* is unlikely to be a common causal mechanism of melanoma formation. Our results, establishing linkage of a *CDKN2*-related sequence to the *DIFF* region in LG V, and to UV-B-induced melanoma formation in *Sp* platyfish–swordtail hybrids, support the role of *DIFF* as a

critical genetic determinant of tumor formation. In future experiments, it will be important to determine if the *CDKN2*-related sequence we have cloned and mapped is also genotypically associated with spontaneous and induced tumor formation in other *Xiphophorus* melanoma models (especially the Gordon–Kosswig model). Determination of the complete structure of the LG V *CDKN2* and its immediate genomic region in *X. maculatus* as well as other *Xiphophorus* species, and its gene expression characteristics in tissues and tumors from parental and hybrid animals, will lead to a more detailed and fundamental understanding of the genetic basis of these unique, heritable tumor models.

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