Xiphophorus Interspecies Hybrids as Genetic Models of Induced Neoplasia

Ronald B. Walter and Steven Kazianis

Abstract

Fishes of the genus *Xiphophorus* (platyfishes and swordtails) are small, internally fertilizing, livebearing, and derived from freshwater habitats in Mexico, Guatemala, Belize, and Honduras. Scientists have used these fishes in cancer research studies for more than 70 yr. The genus is presently composed of 22 species that are quite divergent in their external morphology. Most cancer studies using *Xiphophorus* use hybrids, which can be easily produced by artificial insemination. Phenotypic traits, such as macromelanophore pigment patterns, are often drastically altered as a result of lack of gene regulation within hybrid fishes. These fish can develop large exophytic melanomas as a result of upregulated expression of these pigment patterns. Because backcross hybrid fish are susceptible to the development of melanoma and other neoplasms, they can be subjected to potentially deleterious chemical and physical agents. It is thus possible to use gene mapping and cloning methodologies to identify and characterize oncogenes and tumor suppressors implicated in spontaneous or induced neoplasia. This article reviews the history of cancer research using *Xiphophorus* and recent developments regarding DNA repair capabilities, mapping, and cloning of candidate genes involved in neoplastic phenotypes. The particular genetic complexity of melanoma in these fishes is analyzed and reviewed.

Key Words: *CDKN2*; melanoma; MNU; platyfish; swordtail; UV; *Xmrk*

Introduction

One of the oldest and best defined groups of established inbred strains consists of internally fertilized and livebearing platyfishes and swordtails of the genus *Xiphophorus* (Teleostei: Poeciliidae). These fishes are small (typically <50 mm standard length) and are derived from freshwater habitats in Mexico, Guatemala, Belize, and Honduras. Use of these fishes as a research model to study the genetic components of carcinogenesis has a history encompassing more than 70 yr. In the 1920s and early 1930s, the American biologist Myron Gordon and German scientists G. Haussler and C. Kosswig independently discovered that interspecies hybrids between strains of the southern platyfish *Xiphophorus maculatus* and the green swordtail *Xiphophorus helleri* develop melanomas spontaneously (Gordon 1931; Haussler 1928; Kosswig 1927, 1928). These neoplasms develop exclusively after interspecific crossing and originate from the extreme phenotypic enhancement of melanistic "macromelanophore" (Gordon 1927) pigment patterns derived from the southern platyfish. Pigment patterns in *Xiphophorus* are typically polymorphic and are composed of terminally differentiated micro- or macromelanophores and their precursors (melanoblasts and melanocytes) (Gordon 1927, 1959; Kallman and Atz 1966). Melanomas, which arise from phenotypic overexpression of macromelanophore pigment patterns and interspecific crossing, typically exhibit a disproportionate number of melanocytes that actively proliferate without sufficient regulation (Anders 1991; Gordon 1959; Schartl 1995; Vielkind 1976; Vielkind et al. 1989).

Although humans do not possess a cell type equivalent to *Xiphophorus* macromelanophores, human melanomas are similarly composed of improperly regulated melanocytes (Kraehn et al. 1995; Sauter and Herlyn 1998; Welch and Goldberg 1997). Additionally, in both human and *Xiphophorus*, melanocytes of these distantly related vertebrates are derived from the embryonic neural crest (Gordon 1959; Humm and Young 1956). Melanomas from fish and mammals also exhibit similar histopathological characteristics (Ishikawa et al. 1975; Sobel et al. 1975; Vielkind and Vielkind 1970; Vielkind et al. 1971; Yanar et al. 1996). Tumor similarity is underscored by the observation that fish melanoma cells proliferate in a manner virtually identical to those from humans after transplantion into thymus-aplastic nude mice (Schartl and Peter 1988). Fish melanoma cells are able to undergo serial passage, whereas xenografts from other nonmammalian vertebrates (reptiles and amphibians) generally exhibit rapid degeneration (Reed and Manning 1978). Melanomas from diverse vertebrates reveal a similar expression of extracellular gangliosides (Felding-Habermann et al. 1988). These studies clearly document the similarity between melanoma tumors from *Xiphophorus* and those from humans.

Herein, we review the results of research that has used the *Xiphophorus* genetic system as an experimental model to study melanoma formation and tumorigenesis in general. Although the *Xiphophorus* system is well suited to investi-

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gate the genetics of carcinogenesis, the attributes and variability between *Xiphophorus* species make this system equally valuable to investigate multifactorial genetic inheritance of any complex trait (e.g., behavior and optic or auditory sensing). Thus, we first outline below the availability of strains and describe the *Xiphophorus* Genetic Stock Center.

Resources for *Xiphophorus* Research

Dr. Gordon realized that precise identification of genes responsible for development of cancer would require genetically inbred platyfish and swordtails. Therefore in 1939, he established the *Xiphophorus* Genetic Stock Center and maintained it until his death in 1959. For the ensuing 30 yr, the Stock Center was maintained and expanded by Dr. Klaus Kallman at the New York Aquarium. Upon Dr. Kallman's retirement in 1993, the *Xiphophorus* Genetic Stock Center was transferred to Southwest Texas State University in San Marcos, Texas. Several of the original strains of platyfish and swordtails developed by Dr. Gordon in the 1930s are still available today. These strains comprise the products, in some cases, of more than 94 generations of brother-to-sister matings. This animal resource has enabled scientists to use defined genetic lines across the globe and has greatly facilitated genetic understanding of both the genus and the expansion in number and variety of interspecies tumor models. The Stock Center maintains more than 60 pedigreed *Xiphophorus* lines that represent 21 of the 22 species. Models of both spontaneous and induced carcinogenesis for several tumor varieties can be produced by select backcross matings between pairs of the 22 described *Xiphophorus* species. Examples of a few of these appear in Table 1. Information regarding *Xiphophorus* and availability of fish can be obtained online by accessing <www.xiphophorus.org> and using the links provided.

Xiphophorus are also actively used in other fields of study including evolution (Meyer 1997; Meyer et al. 1994), behavioral ecology (Beaugrand and Goulet 2000; Hoefler and Morris 1999; Rosenthal and Evans 1998; Trainor and Basolo, 2000), toxicology (De Wolf et al. 1993), parasitology (Dove 2000; Schmahl et al. 1996), and immunology (McConnell et al. 1998). Research within these fields also may focus on the identification of genetic factors associated with complex phenotypes. Therefore a well-developed *Xiphophorus* gene map, with supporting methodologies that enable quick and reliable isolation of genes, is a primary research interest within the Stock Center and collaborating research laboratories. Current gene map development is focused on the use of isozyme, restriction fragment length polymorphism, arbitrarily primed-polymerase chain reaction/randomly amplified polymorphic DNA , and microsatellite methodologies to increase marker saturation. Construction of the first complete (24 linkage groups for 24 pairs of acro- or telocentric chromosomes) genetic linkage map is currently under way (Kazianis et al. 1996, 1998b; Morizot et al. 1998a and unpublished).

Gordon-Kosswig Melanoma Model

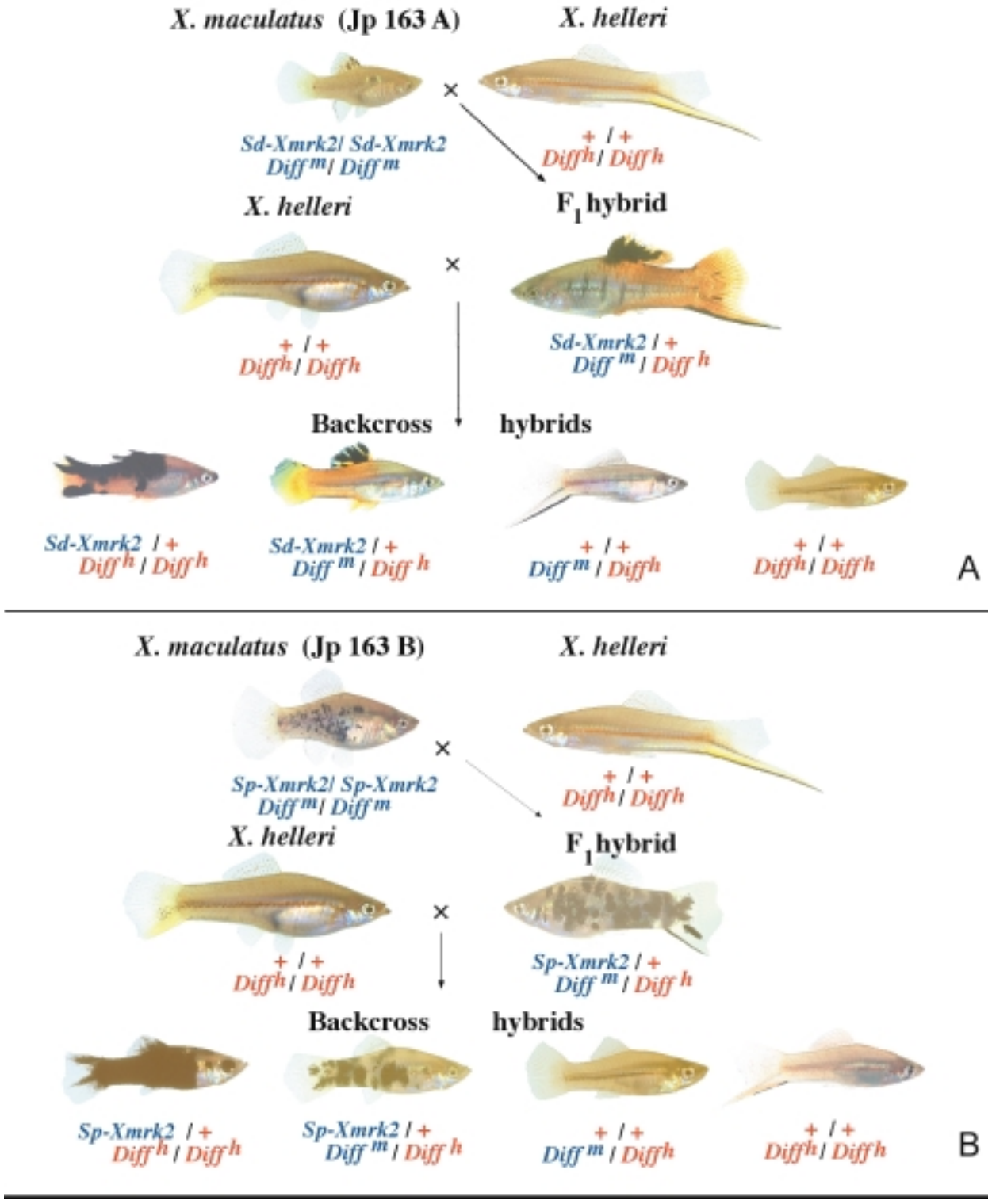
Among the 22 recognized *Xiphophorus* species (Meyer et al. 1994; Rauchenberger et al. 1990), a very large number of interspecific hybrid crosses have been produced that result in phenotypic overexpression of melanistic pigment patterns derived from one of the progenitor species (Anders et al. 1973a; Gordon 1931; Kallman and Atz 1966; Kazianis et al. 1998b; Weis and Schartl 1998; Zander 1969). Examples of these crosses are presented in Table 1. However, the majority of contributions toward the understanding of *Xiphophorus* melanomas is derived from one specific hybrid cross (Anders 1967). This cross (Plate 1A) pairs the southern platyfish, *X. maculatus,* and the green swordtail, *X. helleri*. These two species, which very rarely develop neoplastic lesions, are sympatric over part of their range. However, they can be hybridized through artificial insemination (Clark 1950). The

(text continues on page 309)

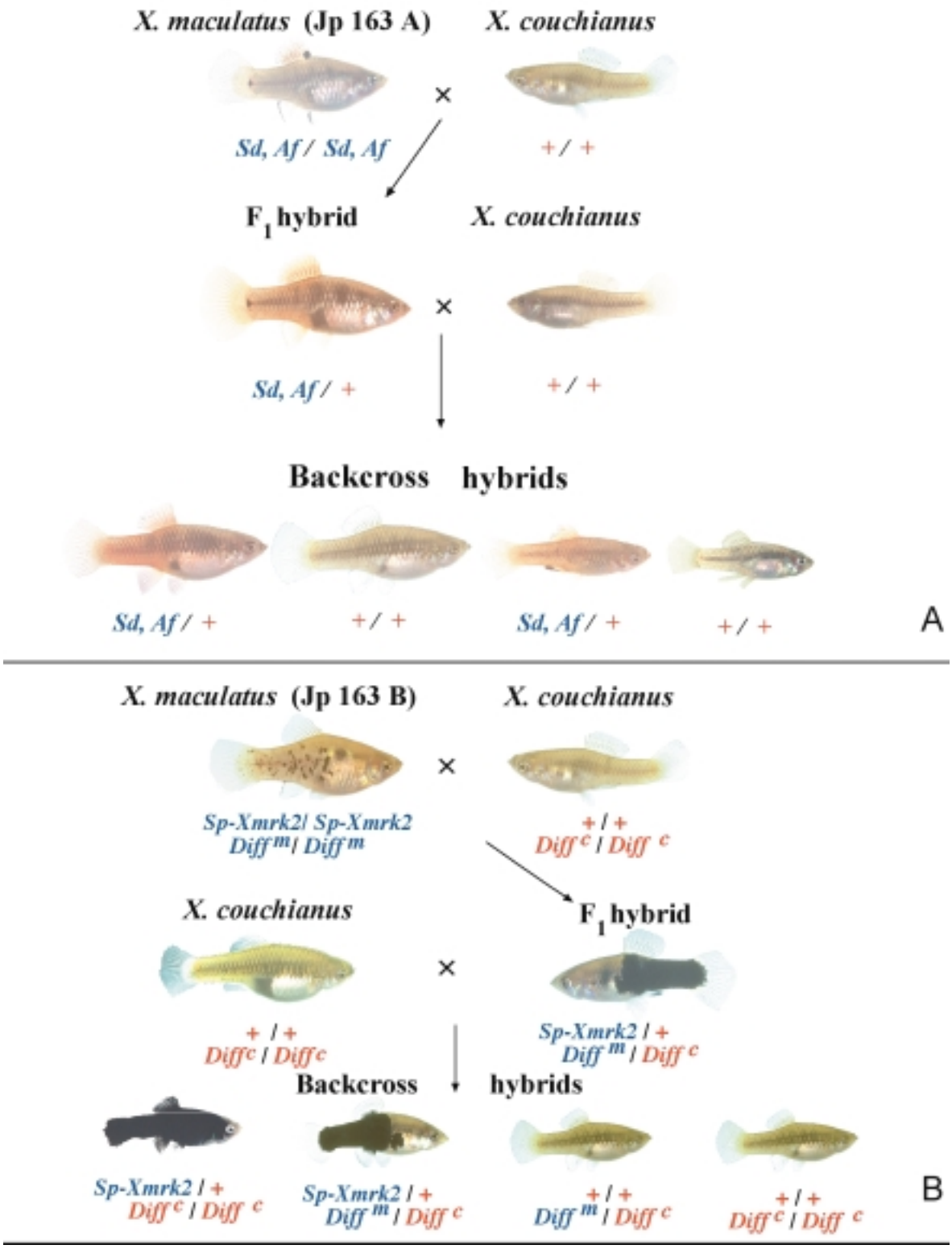
Table 1 Selected *Xiphophorus* hybrid crosses and their utility

Crossing scheme	Tumor type(s)
X. helleri × (*X. maculatus* Jp 163 A × *X. helleri*)	Melanoma
X. helleri × (*X. maculatus* Jp 163 B × *X. helleri*)	Melanoma, renal adenocarcinoma[a]
X. couchianus × (*X. maculatus* Jp 163 A × *X. couchianus*)	Pigment pattern melanosis,[a] retinoblastoma,[a] fibrosarcoma,[a] and schwannoma[a]
X. couchianus × (*X. maculatus* Jp 163 B × *X. couchianus*)	Melanoma, fibrosarcoma[a]
X. andersi × (*X. maculatus* Jp 163 A × *X. andersi*)	Melanoma[b]
X. andersi × (*X. maculatus* Jp 163 B × *X. andersi*)	Melanoma[b]
X. helleri × (*X. helleri* × *X. variatus*)	Melanoma[c]

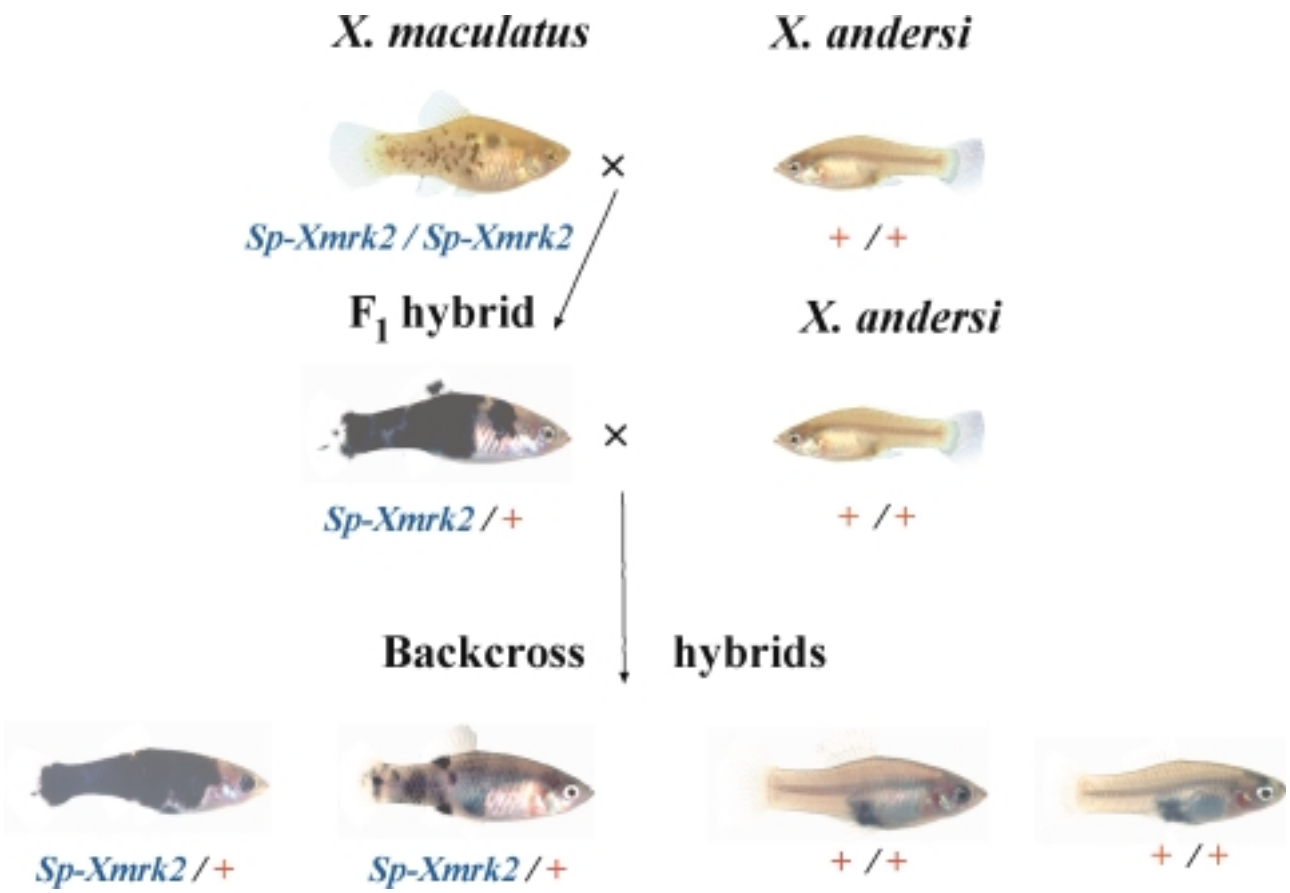
[a]Induced exclusively by *N*-methyl-*N*-nitrosourea (Kazianis et al. 2001a,b).
[b]Melanoma development not associated with linkage group (LG) V (Vielkind et al. 1989 and unpublished).
[c]Pigment pattern enhancement associated with LG III (see text).



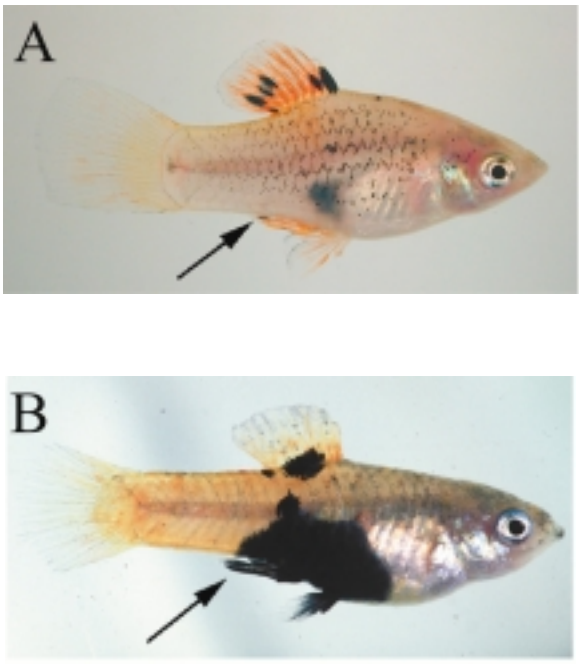
Walter Plate 1 (A) Gordon-Kosswig cross following the inheritance of two genomic regions: linkage group (LG) XXIV (*Sd*, *Xmrk-2*) and LG V (*Diff*). Loci derived from *X. maculatus* Jp 163 A strain, carrying *Sd*, are blue; and the corresponding loci from the swordtail are red. Parentheses indicate specific strains. The BC_1 hybrid (bottom left) exhibits invasive melanoma causing necrosis of the dorsal fin region, which occurs spontaneously in 25% of BC_1 hybrids simply due to the interspecies cross. (B) *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) cross used in ultraviolet light and *N*-methyl-*N*-nitrosourea tumor induction. The *X. maculatus* Jp 163 B strain carries *Sp*, which consists of macromelanophore spots on the flanks of the animal. This pattern becomes enhanced upon crossing with *X. helleri* but does not lead to melanoma at appreciable incidences (7%) unless BC_1 hybrid fish are treated with tumor induction protocols soon after birth (see text). Note the heavily pigmented (bottom left) and lightly pigmented (bottom, second from left) phenotypes segregating in the BC_1 hybrids. This pigmentation is enhanced melanosis, but not a melanoma.



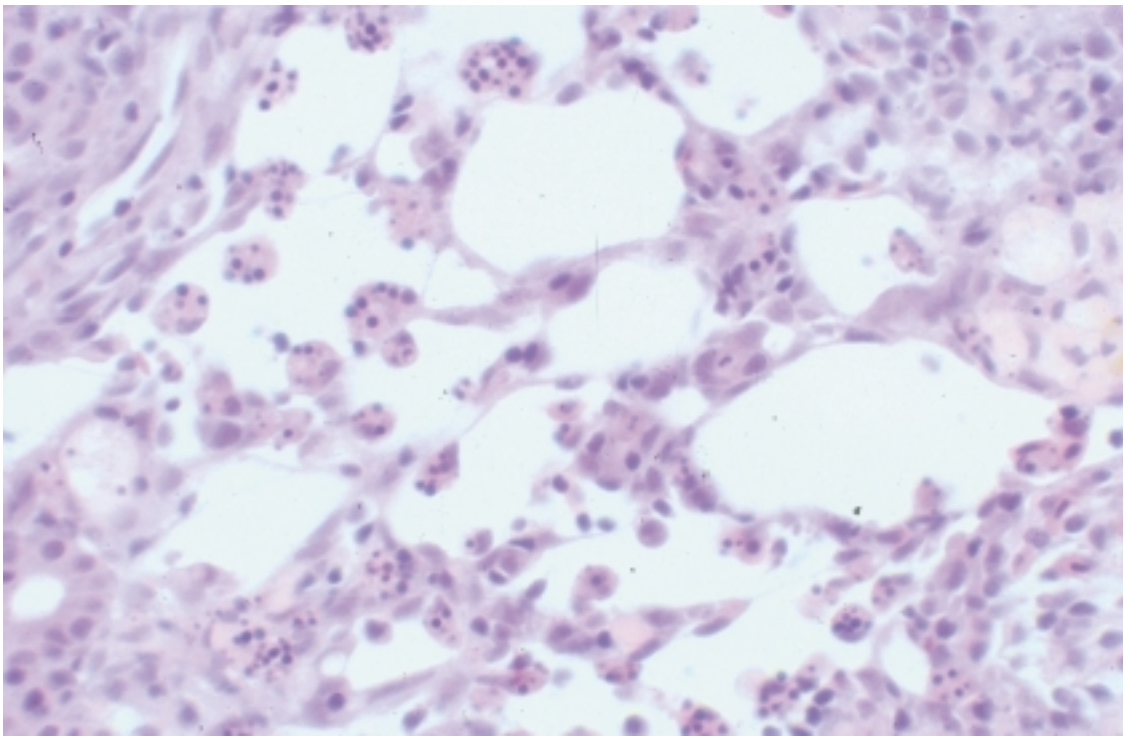
Walter Plate 2 *X. couchianus* × (*X. maculatus* × *X. couchianus*) crosses used in tumor induction experiments. (A) When *X. maculatus* Jp 163 A, harboring *Sd* is crossed with *X. couchianus*, the pigment pattern becomes largely suppressed (note, e.g., the relative absence of Sd in the dorsal fin [bottom left] animal). In contrast, the dorsal red (Dr) erythrophore pattern becomes enhanced in the same BC_1 hybrids (note orange color of 50% of the BC_1 hybrids). The loci carried by the *X. maculatus* parent are indicated in blue, and +/+ in red indicates the corresponding *X. couchianus* alleles. (B) Same cross as panel A but using the *X. maculatus* Jp 163 B carrying the Sp pigment pattern. Loci indicated are as described in Figure 1, with the exception that a "c" superscript indicates the *X. couchianus* allele. In this case, the Sp macromelanophore pigment pattern becomes severely enhanced in *Sp*-inheriting BC_1 hybrids, resembling the melanotic enhancement observed for Sd in the Gordon-Kosswig cross or Sp in the cross with *X. helleri* (see Plate 1). Despite enhanced melanosis, BC_1 hybrids from this cross do not develop tumors at appreciable incidences unless treated shortly after birth with a tumor induction protocol (see text).



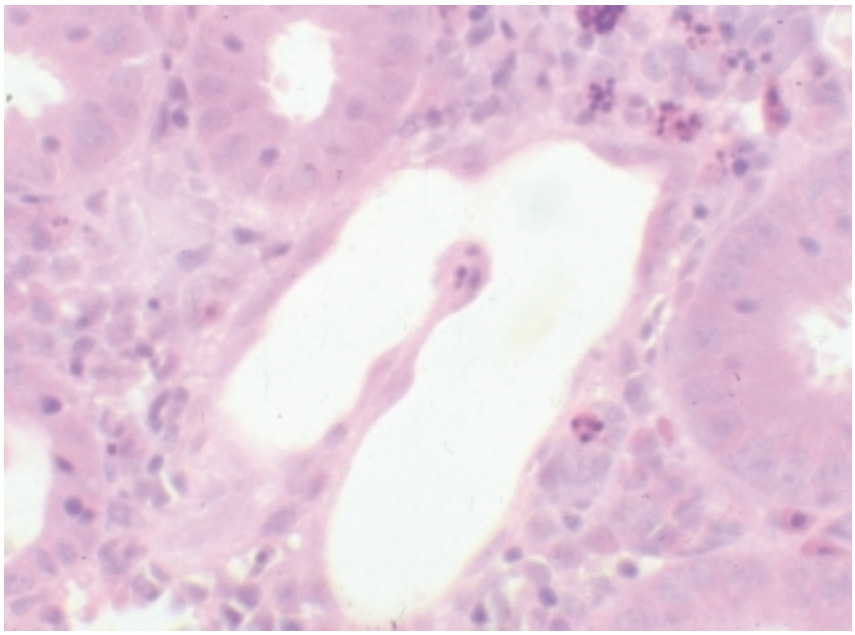
Walter Plate 3 Model cross, *X. andersi* × (*X. maculatus* Jp 163 B × *X. andersi*). The BC_1 hybrids exhibit severe enhancement of the Sp pigment pattern, which is very similar to the enhancement observed for the heavily pigmented hybrids shown in the *X. couchianus* backcross (Plate 2B). Attempts at UVB induction of melanoma in this cross have not been successful (see text). However, *N*-methyl-*N*-nitrosourea-induced tumorigenesis in this hybrid model occurs at very high incidences, and genetic analyses do not indicate association of *CDKN2X* inheritance with melanoma development.



Walter Plate 4 Example of *Af* melanosis. (A) Af pattern (arrow, left) is normally difficult to discern in normal male *X. maculatus* Jp 163 A fish. (B) After treatment with *N*-methyl-*N*-nitrosourea, the melanocytes comprising the Af pattern often become enhanced and grow up the gonopodium and into the ventral visceral cavity region.

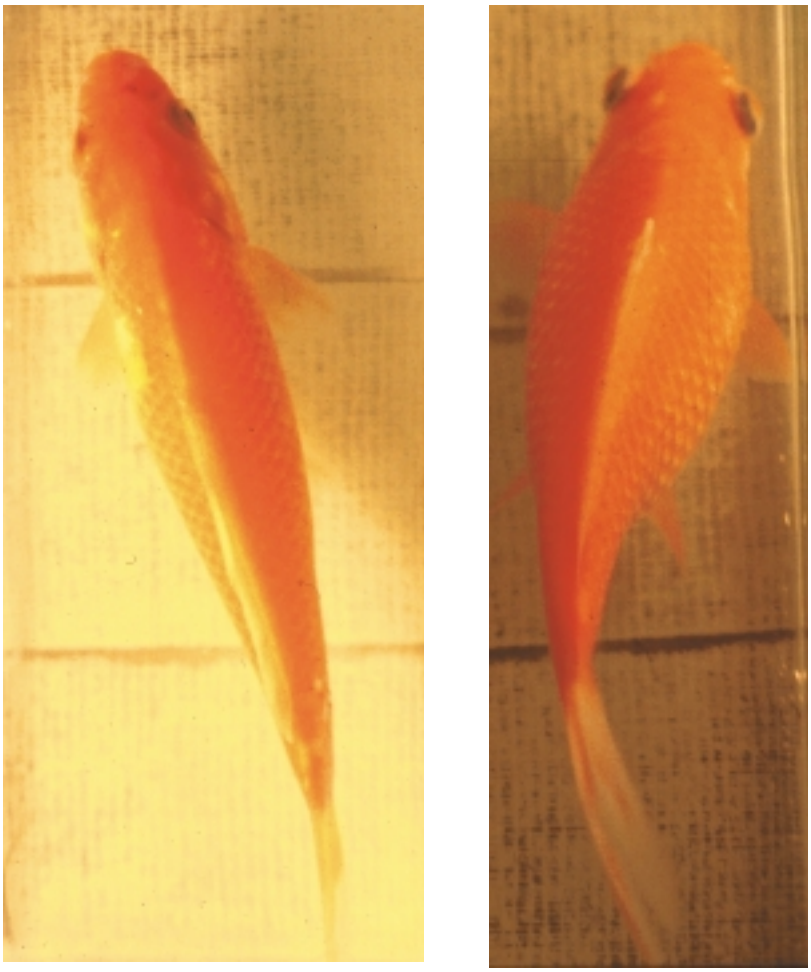


A



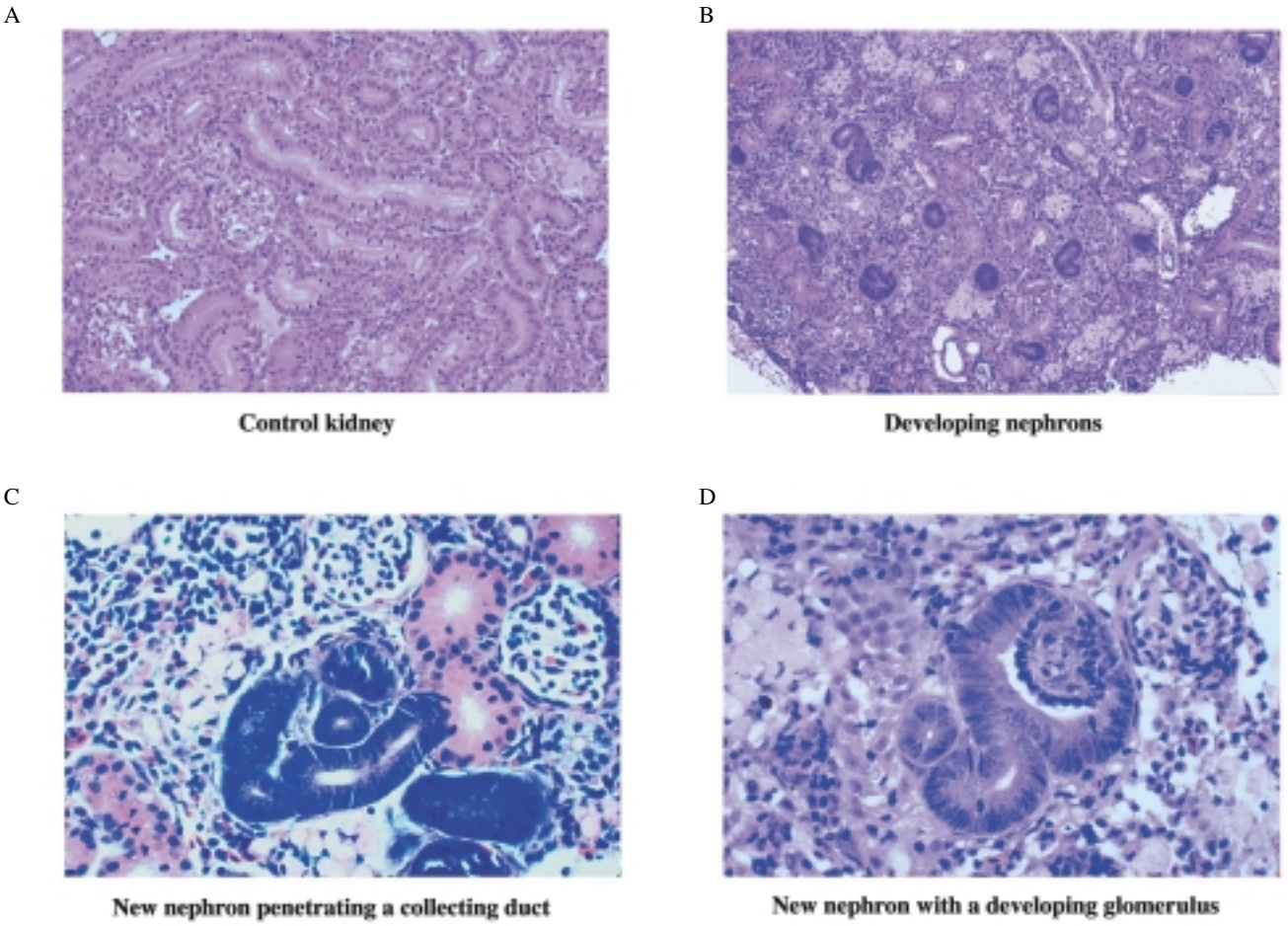
B

Reimschuessel Plate 1 Regeneration of existing goldfish nephron after peritoneal injection of a nephrotoxic dose (50 mg/kg) of gentamicin. (A) Renal tubular necrosis 3 days after injection. (B) Basophilic flattened epithelium in a regenerating tubule 1 wk after injection.



A B

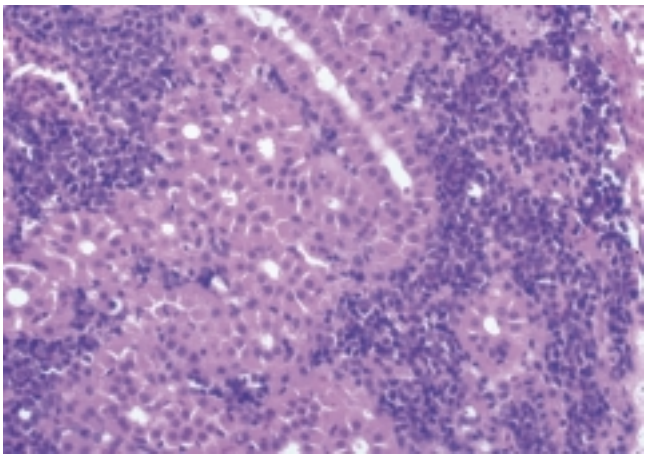
Reimschuessel Plate 2 (A) Control goldfish. (B) Goldfish in renal failure, with ascites and exophthalmia.



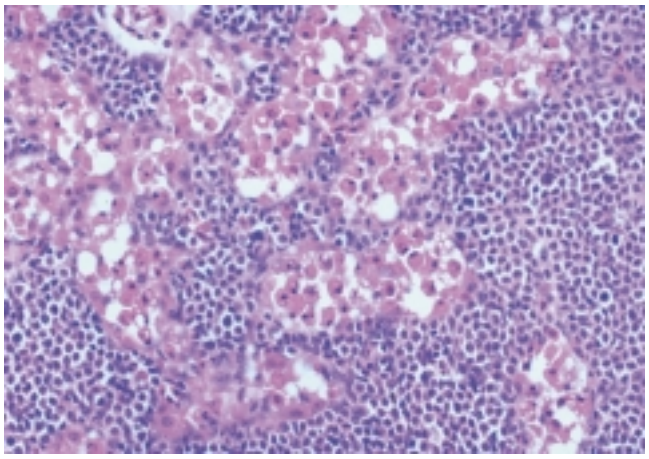
Reimschuessel Plate 3 Nephron neogenesis in the goldfish kidney 3 to 4 wk after gentamicin-induced tubular necrosis (50 mg of gentamicin/kg body weight).

A

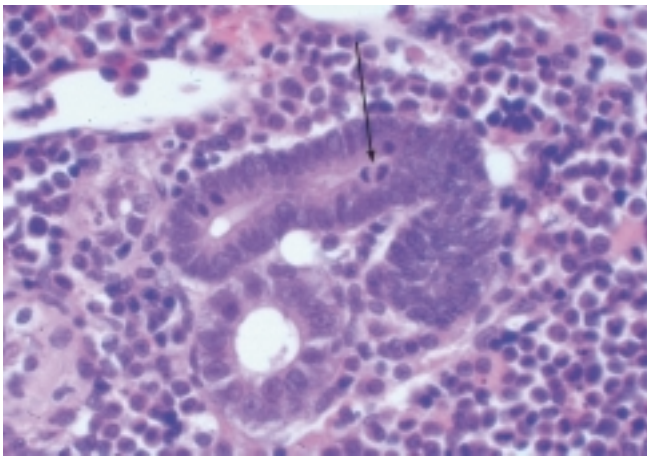
B



C



D



Reimschuessel Plate 4 Nephron neogenesis in the aglomerular toadfish after administration of 3.5 mg of genamicin/kg body weight. (A) Toadfish. (B) Control toadfish kidney. (C) Necrosis of proximal tubular epithelium 3 days after injection. (D) Developing nephron 9 wk after injection.

platyfish-specific macromelanophore pigment locus known as *spotted dorsal* (*Sd*[1]) is located on the subtelomeric region of the X chromosome of *X. maculatus* (Ahuja et al. 1979; Nanda et al. 2000). *Sd* is phenotypically enhanced in first filial generation (F_1)[1] hybrids. Within the first generation backcross (BC_1)[1] to *X. helleri*, a Mendelian 1:1 segregation is reported between individuals with and without the *Sd* locus. In addition, there is a 1:1 distribution of fish revealing moderate enhancement of *Sd*, thus resembling the F_1 hybrid progenitors and individuals that exhibit a greatly enhanced phenotypic expression of this pigment pattern. Within this latter class of fish, exophytic nodular melanomas form, which eventually leads to necrosis of the dorsal fin and surrounding tissues. Dr. Fritz Anders (1967) attributed these phenomena predominantly to two distinct genes: the *Sd* locus, which resides on the X chromosome of *X. maculatus,* and an autosomal locus, which he termed *repression gene 1* (RG_1)[1]. He speculated that the *Sd* locus had the potential to be improperly regulated within a hybrid context, and this gene hypothetically could be regulated by the RG_1 gene. In modern terminology, these loci equate to those of an oncogene (*Sd*) and tumor suppressor (RG_1). The hypothetical tumor suppressor locus was later renamed *Mel Sev* (Morizot and Siciliano 1983; Siciliano et al. 1976) or the more common and current designation, *Diff* (Vielkind 1976). Genetic mapping studies subsequently localized *Diff* to an autosome represented by *Xiphophorus* linkage group (LG[1]) V (Ahuja et al. 1980; Fornzler et al. 1991; Morizot and Siciliano 1983).

Histopathological studies of melanoma cells from Gordon-Kosswig tumor-bearing BC_1 hybrid fish revealed a marked decline in the differentiated state of melanin-containing cells compared with the lightly pigmented siblings (Ahuja et al. 1980; Siciliano et al. 1976; Vielkind 1976). Although the normal macromelanophore spots within *X. maculatus* are composed mainly of macromelanophores, melanotic tissues within hybrids reveal greater numbers of actively proliferating melanocytes, and this effect is manifested particularly within melanomas (Ahuja et al. 1980; Vielkind 1976).

The genetic model developed for Mendelian segregation of phenotypes in the Gordon-Kosswig model is believed to require two events. The first prerequisite is overexpression of a copy of a melanoma-determining gene (variously termed, *Tu*, *erb-B*a*, *ONC-Xmrk*, and *Xmrk-2*; Ahuja and Anders 1976; Wittbrodt et al. 1989; Woolcock et al. 1994; Zechel et al. 1989), which is tightly linked to the *Sd* pigment pattern locus on the X chromosome. For clarity herein, we use the term *Xiphophorus melanoma receptor tyrosine kinase-2* (*Xmrk-2*)[1] to indicate this oncogene. In addition to overexpression of *Xmrk-2*, the second prerequisite of tumorigenesis is loss of the *X. maculatus Diff* tumor suppressor locus, which normally regulates growth of macromelanophores and melanocyte precursor cell populations in parental *X. maculatus* fish. Hypothetically, either *X. helleri* does not have *Diff* or it fails to regulate macromelanophores and their precursors properly. Thus, F_1 hybrids hypothetically harbor one functional copy of *Diff*, as do 50% of BC_1 hybrid animals. The F_1 hybrid fish would hypothetically exhibit moderate phenotypic enhancement of *Sd*. Among the BC_1 hybrids, 50% of the *Sd*-bearing individuals would not inherit *Diff* from the platyfish and thus would reveal melanosis and melanoma.

Xmrk-2 Oncogene and *Xiphophorus* Melanoma

Considerable work has focused on isolation and molecular characterization of candidate genes having functions ascribed to the two antagonistic melanoma regulators based on the "two-hit" Gordon-Kosswig model. Studies conducted over the past several years have resulted in the isolation and characterization of the *Xmrk-2* oncogene and have demonstrated its association with melanoma in the Gordon-Kosswig hybrid cross and several other *Xiphophorus* tumor models. The *Xmrk-2* locus, and a related proto-oncogene *Xmrk-1*, were cloned using reverse genetic approaches (Wittbrodt et al. 1989; Zechel et al. 1989), and they have been mapped within 0.6 cM of each other on the *X. maculatus* X chromosome (Gutbrod and Schartl 1999; Schartl 1990). Both loci are tightly linked to the *Sd* pigment pattern locus and code for transmembrane receptor tyrosine kinases, which are similar in structure to the human epidermal growth factor receptor (HER-1)[1] (Wittbrodt et al. 1989). Each of the *Xmrk* genes encompasses ~23 kb of genomic sequence and possesses exon/intron structures nearly identical to higher vertebrate receptor tyrosine kinases (Gutbrod and Schartl 1999). *Xmrk-1* and *Xmrk-2* can be distinguished by either restriction fragment length polymorphisms or polymerase chain reaction amplification using selected primers within genomic areas of divergence (Weis and Schartl 1998; Woolcock et al. 1994). The *Xmrk-1* gene, which is considered the "normal" or "proto-oncogenic" gene copy, produces a 5.8-kb transcript, whereas the *Xmrk-2* "oncogenic" copy produces a shorter, 4.7-kb transcript (Adam et al. 1991). The *Xmrk-1* transcript is expressed at low levels in all tissues tested (using Northern blot hybridizations; Dimitrijevic et al. 1998; Wittbrodt et al. 1989) and is developmentally regulated in embryos inasmuch as the level of RNA expression is high in the early stages (0-4; also found in unfertilized eggs), declines during cleavage and neurula stages, and is expressed once again during organogenesis (Wittbrodt et al. 1989). In contrast, the *Xmrk-2* 4.7-kb transcript is virtually undetectable in normal tissues using Northern blot methodologies and is distinctly overexpressed in melanomas derived from F_1 and BC_1 hybrids within the Gordon-Kosswig cross. These results

[1]Abbreviations used in this article: *Af*, *anal fin spot*; BC_1, first generation backcross hybrids; BER, base excision repair; *CDKN2*, *cyclin-dependent kinase inhibitor-2* (with *CDKN2X* specifically coding for a *CDKN2* gene of *Xiphophorus*); CPD, cyclobutane pyrimidine dimer; F_1, first filial generation; HER-1, human epidermal growth factor receptor 1; LG, linkage group; MNU, *N*-methyl-*N*-nitrosourea; O6MGMT, O^6 methylguanine-DNA-methyltransferase; (6-4)PD, 6-4 ultraviolet light photoproduct; PI3-kinase, phosphotidylinositol-3 kinase; RG_1, repression gene 1; *Sp*, *spotted dorsal*; UV, ultraviolet light; *Xmrk-1* and *-2*, *Xiphophorus melanoma receptor tyrosine kinase-1* and *-2*.

have been corroborated by additional studies using reverse transcription polymerase chain reactions and Western blotting (Dimitrijevic et al. 1998; Wellbrock et al. 1998a; Woolcock et al. 1994).

Xmrk-2 encodes a 160-kDa protein. The predicted secondary structure consists of a cytoplasmic tyrosine kinase subunit connected by two cysteine-rich hydrophobic transmembrane sequences to an extracellular ligand-binding domain (Wittbrodt et al. 1989). Analysis of several *X. maculatus* mutant fish lines (arising spontaneously or through x-ray irradiation) revealed insertions or deletions within the *Xmrk-2* oncogene, and BC_1 hybrids that had been produced by crossing these mutants with *X. helleri* failed to show melanotic hyperplasia/tumorigenesis (Schartl et al. 1999; Wittbrodt et al. 1989). When Winkler and colleagues (1989) placed the *Xmrk-2* coding region under strong constitutive promoter regulation and used it to microinject medaka (*Oryzias latipes*) embryos, several kinds of tumors developed very early, in some cases resulting in embryonic lethality (Winkler et al. 1994).

At the intracellular level, gene fusion experiments between the Xmrk-2 intracellular domain and the extracellular ligand binding domain of the human HER-1 protein have shown that Xmrk-2 is able to bind or phosphorylate a number of intracellular targets, including the ubiquitous general receptor tyrosine kinase substrate phospholipase C-gamma and cytoplasmic kinases src, fyn, yes, Shc, and the adaptor protein GRB2 (Wellbrock et al. 1995, 1998a, 1999). Constitutive expression of *Xmrk-2* also leads to activation of a transcriptional regulator STAT5 and subsequent upregulation of downstream target loci (Wellbrock et al. 1998b). In addition, Wellbrock and Schartl (1999) have shown that phosphotidylinositol-3 kinase (PI3 kinase[1]) can associate with the intracellular portion of Xmrk-2 and become activated via this interaction. PI3 kinase is known to be responsive to growth factors and to play an intermediate role in cell cycle progression. Whether the PI3 kinase alone, or one or more Xmrk-2-associated factors are the true mediators of downstream signal transduction contributing to the Gordon-Kosswig melanoma phenotype is still under investigation. Clearly the recent emphasis on biochemical studies to identify intracellular proteins targeted by the Xmrk-2 kinase are well under way and should illuminate our understanding of this interesting model in the near future.

In an effort to identify potentially important differences between *Xmrk-1* and *Xmrk-2*, researchers have determined the cDNA and genomic sequences of both genes (Adam et al. 1991; Gutbrod and Schartl 1999). Of note are several genomic sequence differences between them, including two deletions (1344 and 581 bp) found exclusively in *Xmrk-2*. The first deletion is an in-frame deletion located only in one *Xmrk-2* allele, and the second deletion is in the last exon of two studied alleles, downstream from polyadenylation consensus sequences (Adam et al. 1991). These changes are not considered to be associated significantly with the tumorigenicity of *Xmrk-2* (Adam et al. 1991; Gomez et al. 2000). However, analysis of the coding region at the 5′ end of these genes has recently revealed two key codon differences between them (Dimitrijevic et al. 1998; Gomez et al. 2000). These differences are within the extracellular domain (C578S and G359R), resulting in ligand-independent dimerization and activation of the Xmrk-2 protein (Dimitrijevic et al. 1998; Gomez et al. 2000). Furthermore, the ability of these differences to lead to tumor development has been tested by in vivo experiments that involve microinjection of medaka embryos and result in the different tumorigenic potency of *Xmrk-1* and *Xmrk-2* genes (Winkler et al., 1994).

Although these differences may affect Xmrk (-1 and -2) protein function, they do not account for the large transcriptional differences observed between them. Extensive characterization of the promoter regions of both genes revealed significant sequence differences in regulatory elements (Adam et al. 1993; Altschmied et al. 1995, 1997; Fornzler et al. 1996; Woolcock et al. 1994). The *Xmrk-1* promoter contains regulatory elements consistent with its ubiquitous and low expression pattern, and it likely provides "housekeeping" function. In contrast, the *Xmrk-2* promoter region contains structural elements inconsistent with "housekeeping" genes and completely dissimilar to that of *Xmrk-1*. The promoter sequence of *Xmrk-2* exhibits distinct DNA sequence similarity to promoters derived from a sex-linked locus referred to as *Donor* (or "*D*") locus. This gene is found in multiple copies throughout the genome of *Xiphophorus* and related taxa (Fornzler et al. 1996; Nanda et al. 1996). Each of the *Donor* loci may code for two distinct polypeptides. One of these genes exhibits a high level of homology to a zinc finger protein of the krüppel type (Schuh et al. 1986), and the other is an unknown gene with great similarity to a putative gene sequence from *Caenorhabditis elegans* (F54H12.3; Fornzler et al. 1996). Researchers have proposed that the divergent promoters of the two *Xmrk* genes have arisen from an ancient gene duplication, presumably involving a nonhomologous recombination event (Adam et al. 1993; Fornzler et al. 1996).

Detailed study of the *Xmrk-2* promoter resulted in the identification of a critical GC-box element, which sequesters proteins with structural similarity to mammalian Sp1 transcription factors (Baudler et al. 1997). Also, the *Xmrk-2* promoter region contains a 5′ CpG island. Cytosines within this genomic region are methylated in tissues derived from nonhybrid fish, but they are unmethylated in melanotic tissues derived from hybrids and in a melanoma-derived cell line (Altschmied et al. 1997). Thus, such differential methylation might contribute to *Xmrk-2* expression and the development of melanomas (Altschmied et al. 1997).

In summary, it is evident that *Xmrk-2* differs from the *Xmrk-1* proto-oncogene in two significant ways: (1) through a mutation in the extracellular protein domain, which leads to a potent, constitutive tyrosine kinase receptor activity that exhibits ligand-independent activation, and (2) via constitutive oncogenic RNA/protein overexpression in melanotic tissues derived from hybrid fish. However, because the parental *X. maculatus* Jp 163 A stock very rarely develops melanoma, these phenomena alone clearly do not indepen-

dently lead to spontaneous melanoma within Gordon-Kosswig BC_1 hybrids. Melanoma formation is also mediated by the *Diff* locus, located in *Xiphophorus* LG V. Consequently, much recent effort has focused on gene mapping and candidate gene isolation of the hypothetical *Diff* tumor suppressor gene.

Diff Tumor Suppressor

Evidence that *Diff* could be a prominent locus involved in melanoma formation within the Gordon-Kosswig cross was assembled from linkage analyses to polymorphic isozyme markers analyzed in BC_1 hybrids (Ahuja et al. 1980; Fornzler et al. 1991; Morizot and Siciliano 1983; Siciliano et al. 1976). Until recently, no reasonable candidates for *Diff* could be proposed. Researchers have mapped known vertebrate tumor suppressor (*TP53*; Kazianis et al. 1998a; Nairn et al. 1996a), oncogene (*JUNA*, *JUNB*, *ERBB*, *SRC*, *Xyes*, and *Xfyn;* Hannig et al. 1991; Morizot et al. 1998a and unpublished), and DNA repair genes (*ERCC2/XPD*, and *LIG 1*; Della Coletta et al. 1995, Walter et al. 1993) orthologs in *Xiphophorus* to other linkage groups and have failed to evidence genetic association with melanoma formation in BC_1 hybrids. More recent development of modern gene mapping methodologies has enabled the production of a finer scale map and consequently finer localization of the *Diff* tumor suppressor gene on LG V (Kazianis et al. 1998b, 1996; Morizot et al. 1998b).

Recently, scientists cloned a fish homolog of the *cyclin-dependent kinase inhibitor-2* (*CDKN2*, also known as *INK4*) gene family from the *Xiphophorus* genome (Kazianis et al. 1999; Nairn et al. 1996b). This gene, designated *CDNK2X* (see below), was subsequently mapped to LG V and has become the primary candidate for *Diff*. The CDKN2 family of proteins is implicated in the regulation of the G1 checkpoint phase of the cell cycle and modulate the kinase activity of CDK4 and CDK6, which in turn regulate the phosphorylation/inactivation of the *Rb* tumor suppressor. Inactive *Rb* leads to the release of several proteins, including members of the E2F transcription factor family, which directly regulate the expression of genes involved in S phase (Chin et al. 1998; Drexler 1998; Ruas and Peters 1998). The coding sequence of *CDKN2X* reveals significant homology to but is equally distant from mammalian *CDKN2A* (*P16*) and *CDKN2B* (*P15*) tumor suppressor genes (Kazianis et al. 1999). Because primary structure alignments with all four known mammalian gene family members made assignment of orthology impossible, the gene was designated *CDKN2X* (wherein the "*X*" after the gene designation denotes the *Xiphophorus* form; Kazianis et al. 1999). The derived *CDKN2X* polypeptide codes for a 13-kDa protein, a size that has been confirmed by Western blotting (M. Schartl, University of Würzburg, personal communication, 2000). All known *CDKN2* gene family members share a protein structure composed of four to five ankyrin repeats (Chin et al. 1998; Drexler 1998; Luh et al. 1997; Ruas and Peters 1998; Venkataramani et al. 1998), and the fish polypeptide $p13^{CDKN2X}$ possesses four of these, similar to mammalian $p15^{CDKN2B}$ (Kazianis et al. 1999). *CDKN2X* has been sequenced from both *X. maculatus* and *X. helleri*. The two species exhibit only two conservative amino acid differences, neither of which would be expected to alter function (Kazianis et al. 1999). In both species, as in the human *CDKN2B* locus, there is a single intron separating two exons (Jiang et al. 1995).

The map position of the *CDKN2X* gene correlates with localization of *Diff* in *Xiphophorus* LG V (Kazianis et al. 1998b). Analysis of melanoma-bearing BC_1 hybrids revealed that the vast majority (136/165) of these fish did not inherit the *X. maculatus CDKN2X* allele (Kazianis et al. 1998b). A similar relation was evident between phenotypic severity of pigment pattern enhancement and *CDKN2X* allelotype in that heavily pigmented BC_1 were usually homozygotes for the *X. helleri* alleles whereas lightly pigmented fish retained one *X. maculatus CDKN2X* allele (see Plate 1A and Table 2). Additionally, fine mapping of the *CDKN2X* locus with other flanking LG V markers, and analysis with pigmentation degree as a quantitative trait locus, indicated a peak of association between pigmentation and LG V markers that centered on the *CDKN2X* locus (likelihood ratio of >10; Kazianis et al. 1998b). Based on these cumulative results in addition to the documented involvement of mammalian *CDKN2* gene family homolog *P16* in human melanoma (Chin et al. 1998; Drexler 1998; Ruas and Peters 1998), *CDKN2X* is a candidate for the historical *Diff* tumor suppressor gene proposed in the Gordon-Kosswig classical melanoma model (Kazianis et al. 1998b, 1999, 2000).

Table 2 Degree of pigmentation and ultraviolet light B-induced melanoma development versus inheritance of *CDKN2X* in first generation backcross (BC_1) hybrid fish[a]

Parental	Recombinant types	Recombination (%)	LOD[b,c]
	Pigmentation[d]		
55	22	28.6	3.2
	Melanoma[e]		
50	14	21.9	4.7

[a]Using the *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) cross (Kazianis et al. 1998b).
[b]LOD, log of odds.
[c]LOD score >3.0 is considered significant evidence of linkage/association.
[d]Parental animals representing heavily pigmented phenotypes, which proved to be homozygous for *CDKN2X*, and lightly pigmented phenotypes, which were heterozygous. Recombinants were either heavily pigmented and heterozygous or lightly pigmented and homozygous.
[e]Parental animals were tumor bearing and homozygous; recombinants were tumor bearing and heterozygous for *CDKN2X*.

To assess potential involvement of *CDKN2X* in fish melanomagenesis, scientists have determined the genomic structure and expressional characteristics in parental fish (*X. maculatus* and *X. helleri*) used in the Gordon-Kosswig cross. One of the *CDKN2X* promoter region's two CpG islands is located immediately upstream of the start of transcription and continuing into exon 1, and the other is within the 5′ end of exon 2. This organization is evolutionarily conserved in other *CDKN2* gene family members (reviewed by Kazianis et al. 2000). After promoter hypermethylation leading to tumor suppressor gene silencing had been documented for mammalian *CDKN2A* and *CDKN2B* (Herman et al. 1995, 1996; Malumbres et al. 1999), researchers sought to assess the methylation status of the *CDKN2X* locus. Both of the CpG islands proved to be unmethylated at virtually every CpG dinucleotide site (Kazianis et al. 2000 and unpublished). In stark contrast, our analysis of 10 other *Xiphophorus* genes, including DNA repair genes (uracil-N-glycosylase, *ERCC2/XPD*, and DNA ligase 1), tumor suppressors and oncogenes (*TP53*, *JUNA*, and *Xmrk-1*), and general function genes (β-actin, *JUNB*, 5-methylcytosine methyltransferase, *S15-rig,* and *Xmrk-1*), indicates that they are methylated at every available CpG site in all tissue sources examined, despite the presence of CpG islands equal to those in *CDKN2X* with regard to GC content and length (Walter and Li unpublished). However, analyses of *CDKN2X* methylation in many different tissues from F_1, BC_1 hybrids, and parental controls failed to reveal significant differences in *CDKN2X* promoter region methylation. Therefore, it is unlikely that methylation plays a prominent role in *CDKN2X* gene silencing and melanoma tumor suppression (Kazianis et al. 2000).

The *CDKN2X* promoter region exhibits distinct differences between *X. helleri* and *X. maculatus*, including a 20-bp region that is absent in *X. maculatus* and, conversely, an expanded GT-repeat sequence (80 GT-repeats) present in the southern platyfish but much reduced (11 GT-repeats) in the green swordtail (Kazianis et al. 1999). In several BC_1 hybrid tissues, the *X. maculatus CDKN2X* allele is expressed at higher levels than the *X. helleri* allele, and this effect is manifested particularly in melanotic tissues (Kazianis et al. 1998b). Reduced expression of the *X. helleri* allele in melanotic tissues would be a predicted result if *CDKN2X* were functioning as a tumor suppressor gene. Thus, the experimentally established differential *CDKN2X* expression patterning is consistent with its putative function as *Diff*. In addition, reduced *CDKN2A* (*P16*) expression in humans, which occurs via several distinct mechanisms, has been implicated in melanoma (Chin et al. 1998; Drexler 1998; Ruas and Peters 1998). However, detailed examination revealed that RNA expression of *CDKN2X* was more robust in numerous *Xiphophorus* melanomas such as those located on skin and fin than in control tissues (Kazianis et al. 1998b, 2000). These results have led to an alternative hypothetical model in which *CDKN2X* expression levels in melanocytes may influence melanocyte differentiation in hybrid fish. If the *CDKN2X* gene is inadequately expressed in BC_1 hybrids lacking *X. maculatus* alleles, melanocytes might not fully differentiate into macromelanophores; and this lack of complete differentiation, coupled with *Xmrk-2* oncogene activation, could lead to a path of tumorigenesis. Such a scenario is paralleled by results derived from laboratory mouse studies, wherein overexpression of an activated H-RAS (G^{12V}) transgene in a *CDKN2A* null background resulted in a very high incidence of melanoma (60% at 6 mo of age; Chin et al. 1997). Furthermore, the same transgene in a hemizygous *CDKN2A* genetic background resulted in a low tumor incidence, and genetic analysis of the rare tumors that did occur revealed the presence of homozygous *CDKN2A* deletions (Chin et al. 1997). RAS proteins are activated within the cytoplasmic tyrosine kinase cascade by several proteins, including GRB2 and Shc, and these in turn are activated by receptor tyrosine kinase proteins (Chin et al. 1998). Because *Xmrk-2* has been shown to bind/activate both GRB2 and Shc (Wellbrock and Schartl 1999), it is not difficult to envision a parallel between the *RAS/CDKN2A* mouse model and a *RAS*-mediated antagonistic relation between *Xmrk-2* and *CDKN2X* within *Xiphophorus* fish. Obviously, numerous other hypotheses of involvement exist for both the *Xmrk-2* and *CDKN2X* genes that remain to be investigated, including potential use of transgenic or "knock-out" *Xiphophorus*, as the technologies become available (Chen et al. 2001). Further study of chromosomal regions and loci surrounding these genes is currently at the forefront of studies (Nanda et al. 2000; Volff et al. 2001 and unpublished) and will hopefully elucidate the precise molecular mechanisms of melanoma formation in these fishes.

Relation of Inducible *Xiphophorus* Tumor Models to the Gordon-Kosswig Model

The foregoing discussion highlights progress toward understanding the molecular basis of the two-gene hypothesis for segregation of melanoma in the Gordon-Kosswig model. However, researchers have also documented several tumor types that are not associated with inheritance of *CDKN2X* (or LG V) among the variety of *Xiphophorus* hybrid model crosses. Many of these model crosses require pretreatment of BC_1 animals with DNA-damaging agents (ultraviolet light [UV^1] or *N*-methyl-*N*-nitrosourea [MNU^1]) soon after birth to express tumor development. Depending on the cross and the inducing agent used, treated BC_1 hybrids exhibit from 2 to 30% tumor incidence at 6 to 8 mo of age (see below). Investigation of the genetics underlying these inducible *Xiphophorus* tumor models is a major focus of current *Xiphophorus* research.

The variability of pigment patterns and the modification of their expression after selective interspecies hybridization among *Xiphophorus* species present many interesting models for examining induced tumorigenesis of cells derived from the neural crest. An example of differences in pigment pattern expression on interspecies hybridization is shown in Plate 2. Because they have been derived from a single female, the *X. maculatus* lines Jp 163 A and Jp 163 B are very closely

related; however, they are separated and maintained as inbred lines (94 and 89 generations, respectively) based on their distinct macromelanophore pigment patterns. When *X. maculatus* harboring the spotted dorsal (Sd^1; strain Jp 163 A) or spotted side (Sp^1; strain Jp 163 B) macromelanophore pigment pattern are hybridized with another platyfish *Xiphophorus couchianus* (inbred 63 generations), the Sp pattern becomes enhanced in *Sp*-inheriting BC_1 hybrids and resembles the melanotic enhancement observed for Sd in the Gordon-Kosswig cross (Plates 1 and 2). However, when *X. maculatus* carrying the Sd pattern (i.e., Jp 163 A) are crossed with *X. couchianus*, the Sd character becomes largely suppressed (Plate 2A). This example illustrates that among *Xiphophorus* crosses, variability of pigment pattern expression in BC_1 hybrids can be experimentally manipulated to test comparative hypotheses regarding the nature of gene association in a wide array of tumor types (see Table 1).

UV-induced models that exhibit inheritance patterns similar to the Gordon-Kosswig melanoma cross are described below. Chemical (MNU) induction of tumors in the BC_1 hybrids, which appears to involve genetic mechanisms that may be quite different from UVB-induced tumorigenesis, is considered in the subsequent section.

Xiphophorus UV-induced Melanoma Models

Much of the historical work on tumor development in *Xiphophorus* pertains to the Gordon-Kosswig melanoma model wherein melanomas develop spontaneously in a predictable proportion of BC_1 hybrids due to the allelic segregation at two specific loci in the particular interspecific cross (Anders 1991; Schartl 1995). More recently, additional *Xiphophorus* backcross hybrid models have been developed wherein BC_1 animals normally develop tumors at a very low incidence (7%) but in which high incidence of melanoma can be induced readily by subjecting young fish (5 days after birth) to UV (Nairn et al. 1996c; Setlow and Woodhead 1994; Setlow et al. 1989, 1993). Researchers have used these UV-inducible tumor models to help define specific wavelengths that are melanomagenic and for use as comparative genetic models vis-à-vis the Gordon-Kosswig cross (Nairn et al. 1996c; Setlow and Woodhead 1994; Setlow et al. 1989, 1993).

Although controversial, it is believed that cutaneous malignant melanoma in humans often develops when genetically predisposed individuals are exposed to environmental agents, such as excessive sunlight. Cutaneous malignant melanoma incidence is greatly elevated, and onset is accelerated in certain families in which genetic predisposition is obvious, although these cases of strong heritability may account for only ~10% of malignancies (Goldstein et al. 1994). Nonheritable melanoma is an important public health concern because of an alarming recent increase in worldwide incidence, perhaps attributable to depletion of stratospheric ozone and the widespread practice of cosmetic skin tanning (deGruiji and Van der Leun 1993; Koh 1991; Rigel et al. 1987). From 1973 to 1990, the incidence of cutaneous malignant melanoma in the United States increased ~94%—more than that for any other cancer (Miller et al. 1993).

Although the maximal absorption for nucleic acids is in the UVC wavelengths (230-290 nm), UVB (290-320 nm) as well as UVA (320-400 nm) radiation may also alter genetic information and leave characteristic mutational "signatures" in altered genes (Drobetsky et al. 1995; Pollock et al. 1996; Wikonkal and Brash 1999). However, the relation between sunlight exposure and melanoma incidence is complex and may depend on the quantity and temporal specifics of exposure (de Gruijl 1999; Langley and Sober 1997). Hereditary factors also contribute to melanoma development in humans (Kamb et al. 1994; Langley and Sober 1997; Laporte 1998), and hypotheses attempting to correlate the dynamics of sunlight exposure and melanoma incidence must also include consideration of the genetic determinants underlying melanoma incidence. Although this caveat is generally recognized, it underscores the need for animal models for melanoma in which genetic components are easily recognized and subject to experimental manipulation and analysis.

The first UV-inducible *Xiphophorus* tumor models used BC_1 hybrid fishes produced from mating *X. maculatus* Jp 163 B carrying the *spotted side* (*Sp*) pigment pattern with the swordtail *X. helleri* (Setlow et al. 1989) (Plate 1B). This cross is very similar to the Gordon-Kosswig cross but differs in that the BC_1 hybrids do not develop tumors at appreciable incidences without UV treatment very soon after birth (Table 3). These BC_1 hybrid fish proved to be susceptible to development of neoplastic disease, which was inducible by exposure to wavelengths in the UVB range (Setlow et al. 1989). In addition, the melanoma incidence could be reduced by exposure to visible light immediately after the UVB treatment, indicating that photoreversal of UV-induced DNA damage played a role in the ultimate effects of UVB-induced carcinogenesis (Setlow et al. 1989). Later experiments using *Xiphophorus* BC_1 hybrid fish models and discrete wave-

Table 3 Comparison of melanoma tumor incidence with tumor-inducing treatments in *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) BC_1 hybrid fish[a,b]

Treatment	Fish with melanomas	Fish (total no.)	Incidence (%)
Control	6	83	7.2
UVB[b]	23	125	18.4
MNU[b]	25	68	36.8

[a]Incidence is calculated only for *Sp*-bearing (i.e., pigmented) fish that had been euthanized at a maximum age of 6 mo. Data from the UVB treatment group are partially derived from previous publications (Kazianis et al. 2001a; Nairn et al. 1996b,c, 2001).
[b]BC_1, first generation backcross hybrids; UVB, ultraviolet light B; MNU, *N*-methyl-*N*-nitrosourea.

lengths of UVA and visible light (405 and 436 nm) indicated that long wavelength light could also induce melanoma. This observation has considerable importance considering the composition of many sunscreens (Setlow and Woodhead 1994; Setlow et al. 1993). Because the majority of UV radiation reaching the earth surface is within the UVA range (Setlow 1974; Setlow et al. 1993), these experimental results prompted concern over the epidemiology of melanoma in humans and the use of certain sunscreens designed to minimize erythema induced by UVB radiation.

Genetic analyses of UVB-induced melanoma using the *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) (Plate 1B) model cross indicates that inheritance of the *Xmrk* and *CDKN2X* genes is strongly associated with BC_1 hybrid melanomagenesis (Table 2; Kazianis et al. 1998a,b; Nairn et al. 1996c). Similar to the Gordon-Kosswig model, all of the animals that develop melanoma inherit an *Xmrk-2* allele from the *X. maculatus* parent. In addition, approximately 80% of UVB-exposed BC_1 hybrid animals that develop melanoma inherit both *CDKN2X* alleles from the *X. helleri* parent. Furthermore, the degree of pigmentation (lightly vs. heavily pigmented, see legends in Plate 1 and 2) in BC_1 hybrids is also strongly associated with *CDKN2X* inheritance (Table 2). However, the fact that these animals develop melanoma more frequently than the nonirradiated controls indicates that other genes must also be segregating into certain BC_1 hybrids, predisposing a portion of them to the effects of UV radiation. In addition, it is important to note that association with *CDKN2X* inheritance in melanoma-bearing UVB-treated BC_1 hybrids is not complete. Mechanisms accounting for the ~20% of melanoma-bearing BC_1 fish that are genotypically heterozygous for *CDKN2X* may involve loss of function of the *X. maculatus* allele due to UV-induced mutagenesis or UV-induced regulatory modulation. These and other possibilities are currently under investigation.

Genetic analyses of the above-mentioned hybrid model and others indicate independent molecular genetic mechanisms leading to melanomas induced by UV wavelengths, as opposed to those arising spontaneously (or induced by 405 nm of light; Nairn et al. 1996c). The use and future exploitation of these and other *Xiphophorus* melanoma models for UV carcinogenesis is an exciting prospect, both for determination of the melanoma action spectrum and for continued genetic analysis of the hereditary factors involved in UV-induced melanoma formation and progression.

Chemical Carcinogen-induced Models

Less well developed than the spontaneous and UV-induced *Xiphophorus* tumor models, but no less important, are induced tumor models that use chemical (e.g., MNU) treatment as a tumor-inducing stimulus (Kazianis et al. 2001a; Schwab et al. 1978a,b, 1979). MNU is an alkylating agent that methylates DNA bases primarily at nucleophilic sites (N^7 and N^3 alkylpurines). The primary mutagenic lesion of MNU exposure is believed to be O^6 methylguanine (Friedberg et al. 1995). MNU induces numerous cancers in rodents, including mammary carcinomas and thyroid tumors in rats (Ohshima and Ward 1984; Zarbl et al. 1985) as well as thymic lymphomas in mice (Frei and Lawley 1980; Richie et al. 1996). This carcinogen also has been shown to induce a wide variety of tumors in *Xiphophorus* hybrids, including neuroblastomas, melanomas, fibrosarcomas, rhabdomyosarcomas at high incidence, and various carcinomas at a greatly reduced incidence (Schwab et al. 1978a,b, 1979).

Schwab and colleagues (1978a) were the first to indicate that chemically induced tumorigenesis in *Xiphophorus* possesses a strong genetic component. Scientists have shown that MNU treatment of 64 independent nonhybrid species/strains and derived hybrids induces neuroblastomas exclusively in BC_1 animals derived from one particular hybrid cross involving *Xiphophorus variatus* and carrying the Lineatus (Li) pigment pattern hybridized with *X. helleri* (Schwab et al. 1978a,b, 1979). However, these studies have not been developed past the initial description of tumor types and treatments.

We have recently developed several new MNU-inducible hybrid models that promise to further advance *Xiphophorus* as an important model for the study of chemical carcinogenesis (Kazianis et al. 2001a,b and unpublished). To provide a direct comparison between UVB and MNU tumor induction, we exposed *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) (Plate 1B) BC_1 hybrids to MNU. As a comparative example, the data in Tables 3 and 4 illustrate that the level of MNU induction of melanoma is higher than by UVR, and that there is no significant association with *CDKN2X* inheritance in BC_1 melanoma-bearing animals. This absence of association with *CDKN2X* suggests that the inheritance of different gene targets may be necessary to predispose BC_1 animals to UV or MNU tumor induction. Also, among the MNU-treated melanoma-bearing animals, approximately 20% exhibit multiple neoplastic lesions, often in distinct body areas, a situation that is not observed among hundreds of UVB-induced tumor-bearing fish. The UVB and MNU results comparatively indicate that multiple genetic routes may exist that lead to the same tumor (i.e., melanoma) even within BC_1 hybrids produced from pedigreed and highly inbred parental lines.

In a different model cross, *Xiphophorus andersi* × (*X. maculatus* Jp 163 B × *X. andersi*), the BC_1 hybrids exhibit enhancement of the spotted side pigment pattern nearly identical to the enhancement observed for the heavily pigmented hybrids shown in the *X. couchianus* backcross (Plates 2B and 3). Attempts at UVB induction of melanoma in such pigmented BC_1 hybrids have not been successful despite use of radiation protocols leading to substantial induction of melanoma in other model crosses (Nairn et al. 2001). However, MNU-induced tumorigenesis in the same hybrid model, *X. andersi* × (*X. maculatus* Jp 163 B × *X. andersi*), occurs at very high incidences (approaching 30% in BC_1 hybrids; Table 5). As is the case for the *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) model cross (Plate 1B and Table 4), genetic analyses did not show any association of melanoma

Table 4 Comparison of induction treatments and *CDKN2X* genotypic associations with melanoma formation in *X. helleri* × (*X. maculatus* Jp 163 B × *X. helleri*) BC_1 hybrids[a,b]

Treatment	Genotyped fish with melanomas	Heterozygotes	Homozygotes	Recombination (%)	LOD[b,c]
Control	49	8	41	16.3	5.3
UVB[b]	64	14	50	21.9	4.7
MNU[b]	29	12	17	41.4	0.2

[a]Individual BC_1 fish that developed melanomas were genotyped as being heterozygotes (one allelic copy from *X. helleri* and one from *X. maculatus*) or homozygotes (alleles from *X. helleri* only). Data derived from Kazianis et al. 2001a.
[b]BC_1, first generation backcross hybrids; LOD, log of odds; UVB, ultraviolet light B; MNU, *N*-methyl-*N*-nitrosourea.
[c]LOD score >3.0 is considered significant evidence for linkage/association.

development with inheritance of *CDKN2X* alleles (Table 5; Walter and Kazianis unpublished), which not only underscores the genetic complexity of melanoma but also attests to a major strength of using the *Xiphophorus* genetic system to identify the genetic mechanisms leading to tumor predisposition. If *CDKN2X* inheritance is not playing a role in MNU-induced tumorigenesis in the same model crosses where it is clearly associated with UVB-induced melanoma, what gene target(s) might be serving to predispose these hybrids to tumor development? Efforts are currently under way to perform extensive genotype analyses of MNU-treated BC_1 hybrids from the above-mentioned cross involving *X. andersi*. The robust genetics offered by interspecies hybridization will hopefully allow identification of factors associated with MNU tumor induction that will be of comparative interest.

A strictly MNU-induced tumor model that has recently been developed involves backcross hybrids using *X. maculatus Sd* (Jp 163 A) and the platyfish *X. couchianus* as the recurrent parent. As previously stated, when *X. maculatus* carrying the Sd pigment pattern are crossed to *X. couchianus,* the Sd macromelanophore pigment pattern becomes largely suppressed, whereas an erythrophore pattern referred to as dorsal red (Dr; Anders et al. 1973b; Kallman, 1975) becomes enhanced in BC_1 hybrids (Plate 2A). To determine whether

Table 5 Incidence of MNU-induced melanoma and lack of association of melanoma development with inheritance of LG V markers (linked to *CDKN2X* for MNU-treated *X. andersi* × (*X. maculatus* Jp 163 B × *X. andersi*) BC_1 hybrids[a]

	Melanoma incidence[b]		
Treatment	Fish with melanomas	Fish (total no.)	Incidence (%)[c]
Control	2	72	2.7
UVB[a]	0	40	<2.5
MNU[a]	47	158	29.7

	CDKN2X association of melanoma developing in MNU-treated BC_1 hybrids[c]		
Parental	Recombinant types	Recombination (%)	LOD[d]
22	17	43.6	0.14*

[a]BC_1, first generation backcross hybrids; LOD, log of odds; UVB, ultraviolet light B; MNU, *N*-methyl-*N*-nitrosourea. Data derived from Walter et al., unpublished.
[b]5-wk-old fish exposed to 1.0 mM MNU, 2 hr, on days 0, 2 , 4, and 6 and raised to the 12-mo experimental cutoff.
[c]Parental animals were tumor bearing and homozygous; recombinants were tumor bearing and heterozygous for *CDKN2X*.
[d]LOD score >3.0 is considered significant evidence of linkage/association.
*Not significant.

BC_1 animals that do not exhibit melanophore enhancement might nevertheless be predisposed to induced tumorigenesis, we exposed BC_1 animals from the *X. couchianus* × (*X. maculatus* Jp 163 A (Sd) × *X. couchianus*) backcross to MNU. In Table 6, a data set obtained from these experiments is shown.

MNU treatment of hybrid fish derived from crossing these two platyfish species resulted in the induction of several and varied neoplasms occurring at incidences between 2.8 and 6.6%. Even though the Sd pattern is suppressed in these BC_1 hybrids, a second melanophore pattern termed anal fin spot (Af^1) is expressed as a very small area of melanocytes located at the distal tip of the gonopodium (Plate 4A). Surprisingly, 10.4% of MNU-treated backcross hybrids exhibited extensive melanosis emanating from the melanocytes sequestered at the tip of the gonopodium. These melanocytes gradually expanded along the gonopodium and into the ventral viscera (Plate 4B). This enhanced melanosis occurred only in MNU-treated animals (0 instances observed in equal numbers of controls). This study identified three genetic preconditions for expression of Af melanosis in BC_1 hybrids: (1) Individuals must inherit an X chromosome derived from *X. maculatus* that carries the *Af* pigment pattern locus. (2) Affected fish are always males, inasmuch as the Af pigment pattern is sex limited and only male fish develop a gonopodium and have a melanocyte population restricted to its distal end. (3) As in the UVB-induced melanomas and the Gordon-Kosswig melanoma model, but unlike MNU-induced melanomas (see above) in other crosses, the progressive Af melanosis is significantly associated with the absence of inheritance of *X. maculatus CDKN2* alleles (Kazianis et al. 2001b). In this case, 100% of BC_1 fish developing Af melanosis were homozygotes for *X. couchianus CDKN2X* alleles. Conversely, the majority of individuals that did not develop severe Af melanosis, even after MNU treatment, were heterozygous for *CDKN2X*.

In addition to Af melanosis, discovery of schwannomas, fibrosarcomas, and retinoblastomas in the BC_1 hybrids, albeit at a lower incidence, creates the additional possibility of studying the genetic background underlying these neoplasms as well. We are currently initiating crosses to explore the genetic basis of these low-incidence tumors using hybrids between *X. maculatus* and *X. couchianus* and in several other crosses.

The results reviewed herein and other unpublished studies clearly indicate that MNU-induced melanomas can arise through genetic mechanisms distinct from those identified for UVB-induced tumorigensis, even within a specific *Xiphophorus* model crossing scheme in which both parental lines are extensively inbred. It is our hope that continued study of these various models will allow identification and isolation of genes implicated in MNU-induced melanomas and other cancers.

Table 6 Incidence and types of neoplastic lesions in *X. couchianus* × (*X. maculatus* Jp 163 A × *X. couchianus*) first generation backcross (BC_1) hybrid fish treated with *N*-methyl-*N*-nitrosourea (MNU)[a]

Lesion	MNU (N=105)	MNU (%)
Schwannoma	3	2.8
Fibrosarcoma	7	6.6
Retinoblastoma	4	3.8
Unrestricted melanosis[b]	11	10.4

[a]5-wk-old fish exposed to 1.0 mM of MNU, 2 hr, on days 0, 2 , 4, and 6. No neoplasms occurred in 171 untreated control fish. Data derived from Kazianis et al. 2001b.

[b]Melanosis derived from the Af pigment pattern is defined by aggregations of melanin-containing cells located outside the confines of the gonopodium. Such cells typically grow within the integument of the ventral and mid-ventral flanks of the fish (see text and Plate 4).

Spontaneous Melanoma Models in the Genus *Xiphophorus*

The involvement of LG V with melanoma susceptibility has been confirmed in several *Xiphophorus* hybrid crosses related to the Gordon-Kosswig cross (Ahuja et al. 1980; Kazianis et al. 1998b, 2001b; Morizot and Siciliano 1983). In all cases, these crosses involve *X. maculatus* from the Rio Jamapa, outcrossed to either *X. helleri* or *X. couchianus*. In these crosses, the involvement of a gene or genes in LG V as regulator(s) of melanotic hyperplasia is not restricted to fish with the Sd pigment pattern, but also occurs in those with Sp and Af, two distinct pigment patterns also located on the X chromosome of *X. maculatus*.

Genetic linkage analyses have also been conducted for several other *Xiphophorus* hybrid crosses. In several of these, BC_1 hybrids have exhibited extreme macromelanophore pigment pattern enhancement and melanoma formation that cannot be attributed to *CDKN2X* or LG V. Some of these crosses are summarized in Table 1. In one such example, *X. variatus* with a macromelanophore pattern referred to as Pu^2 is crossed to *X. helleri*. Although F_1 and subsequent BC_1 hybrids (to *X. helleri*) exhibit pigmentary hyperplasia and melanoma development, genetic analysis failed to implicate a *Diff* locus, as determined by analysis of several LG V markers (Kazianis et al. 1996). In contrast, researchers attributed phenotypic differences in Pu^2 expression in BC_1 hybrids to genotypic differences at a locus at the telomeric region of LG III (Kazianis et al. 1996; Morizot et al. 1998b and unpublished).

In addition to melanoma models induced by hybrid crossing, *Xiphophorus* fish also provide several relatively unexploited models of melanoma formation that do not involve interspecies hybridization (Borowsky 1973; Kallman 1971; Schartl et al. 1995). For example, *X. cortezi* fish with the Sc macromelanophore pattern can develop melanomas that are associated with aging (Kallman 1971; Schartl et al.

1995). The development of melanosis and melanoma also appears to be influenced by androgens, inasmuch as dominant males appear to develop the most extreme manifestations of melanosis and melanoma (Schartl et al. 1995). At the time of this writing, this model and several others that are associated with aging in *Xiphophorus* have not been studied extensively or with the benefit of modern applied research techniques and technologies.

Tumor Inducibility and DNA Repair Potential

The fact that different responses are observed using the same interspecies cross model that appears dependent on UV or MNU tumor induction protocols suggests that the action of these two agents may be tolerated by a given species or hybrid differentially. The parental species used in most tumor cross models are highly inbred fish lines. Thus, within feral populations of these species, a standard distribution of DNA repair phenotypes (i.e., capabilities) may exist; however, we may have unknowingly selected and genetically fixed them by inbreeding. Thus, different molecular elements and genes used in combating DNA damage within a given species may not be totally compatible with another species when placed in F_1 or BC_1 genetic backgrounds, which could result in modulated ability to clear DNA damage and a potentially tumorigenic genotype. To investigate this possibility, researchers have initiated DNA repair assays in an effort to determine the DNA repair capabilities of parental species/strains used in the tumor model crosses, as described below.

In a recently reported experiment, the swordtail *Xiphophorus signum* was exposed to UVB radiation. DNA from the skin and fin was then assayed at various times after exposure using radioimmune assays specific for the major UV-induced DNA photoproducts to determine the extents of nucleotide excision repair (Meador et al. 2000). The results indicated that induction of both the cyclobutane pyrimidine dimers (CPDs[1]) and 6-4 photoproducts ([(6-4)PDs[1]]) in addition to the calculated half-life (initial rate of repair) of CPD and (6-4)PD nucleotide excision repair or dark-repair exhibited values very comparable with those reported for rodents (Meador et al. 2000).

Investigation of nucleotide excision repair among *Xiphophorus* species using the UV photoproduct radioimmunoassay (Mitchell 1996; Mitchell et al. 1993) revealed highly variable repair capabilities (Mitchell et al. 2001 and unpublished). For example, researchers have determined the kinetics for CPD and (6-4)PD repair for several species, including *X. couchianus*, *X. maculatus* (strains Jp 163 A and Jp 163 B), *X. signum, X. variatus*, and *X. andersi*. They found the relative capacity to repair CPDs and (6-4)PDs to be comparable in *X. variatus*, *X. signum*, and *X. couchianus*; however, these species revealed reduced repair levels compared with *X. maculatus* and *X. andersi*. The parental lines, *X. maculatus* Jp 163 A and *X. andersi,* displayed very efficient repair of CPDs and (6-4)PDs with comparable rates of removal. These data are consistent with the reported lack of UVB-induced tumorigenicity observed in hybrid fish from *X. andersi* crosses (Plate 3 and Table 5).

Unlike UV exposure, treating cells with monofunctional alkylating agents (MNU) induces DNA damage that is principally processed by base excision repair (BER[1]). One of us with colleagues (Walter et al. 2001a,b) have used two distinct oligonucleotide-based assays—the first for uracil-*N*-glycosylase-initiated base excision repair and the second specifically for repair by O^6-methylguanine-DNA-methyltransferase (O^6-MGMT)—to begin assessing DNA repair capability among *Xiphophorus* parental fish lines exposed to alkylating agents.

Both BER and O^6 methylguanine-DNA-methyltransferase (O^6-MGMT[1]) assays in *Xiphophorus* fishes and F_1 interspecies hybrids have indicated that various tissues exhibit different levels of repair capability. For example, brain extracts generally exhibited greater BER and O^6-MGMT repair activity than gill and liver extracts. Although we did not observe differences between species in the ability of a given tissue to repair the O^6-methylguanine DNA lesion (O^6-MGMT repair), we did observe species-specific differences in BER capabilities.

Comparing BER activities between each of two parental lines and their F_1 hybrids indicated that *X. couchianus* possessed less BER repair capability in gill and liver extracts than *X. maculatus* Jp 163 A. Furthermore, the repair capacity in F_1 hybrids produced by mating *X. maculatus* Jp 163 A with *X. couchianus* is very similar to values obtained for the *X. couchianus* parent, including a decreased ability to perform BER (compared with *X. maculatus*) in both gill and liver tissues. In addition, these F_1 hybrids may repair less well in gill tissue than either parent species, suggesting that the hybrid genetic background (heterozygotic for all loci) produces a genetic condition whereby protein interactions leading to efficient BER are compromised in hybrid gill tissue. In MNU tumor-induction exposures, we have observed that *X. maculatus* Jp 163 A $\times$ *X. couchianus* F_1 hybrids are more sensitive to MNU toxicity than either parent species (Walter unpublished).

The DNA repair studies conducted to date have not assessed repair capability in individual BC_1 hybrid animals that do or do not develop induced tumors. However, scaling down these DNA repair assays so that DNA repair capability can be treated as a quantitative trait may provide valuable data to document the relations between DNA repair and tumorigenicity. The *Xiphophorus* tumor models may then be uniquely positioned to provide insight into both the evolution of DNA repair and the relations between DNA damage, DNA repair, and latent tumorigenesis.

Concluding Remarks

Based on the abundance of genetic differences among inbred strains of *Xiphophorus* species and the fact that interspecies hybrids are fertile, classical genetic analysis of *Xiphophorus*

backcross hybrids has revealed linkage of genetic markers with phenotypic traits that includes hyperplastic pigment cell proliferation and tumor formation. More than two decades before the isolation of the *Xmrk* gene (Wittbrodt et al. 1989; Zechel et al. 1988) and the more recent isolation of the *CDKN2X* gene (Kazianis et al. 1999; Nairn et al. 1996b), pioneering investigator Dr. Fritz Anders had documented the hypothetical existence of both loci (Anders 1967). With a multitude of tumor models, proven tumor inducibility resulting from physical or chemical treatment regimens, and differing genetic causality leading to phenotypically identical neoplasia, the small aquarium fishes of the genus *Xiphophorus* offer the scientific community a valuable tool. Undoubtedly, these genetic models will be further exploited by researchers and should contribute toward the general understanding of neoplasia.

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