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Proteomic Analyses of the *Xiphophorus* Gordon-Kosswig Melanoma Model

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Abstract

Interspecies hybridization between the platyfish *X. maculatus* Jp 163 A, and the swordtail *X. helleri* (Sarabia), generates F₁ hybrids with pronounced melanin pigmentation. Backcrossing of F₁ hybrids with the *X. helleri* parent results in 25% of progeny that will spontaneously develop melanoma. We have applied proteomic methods to this Gordon-Kosswig (G-K) melanoma model to identify candidate proteins that exhibit modulated expression in fin tissue due to interspecies hybridization and progression of hybrid tissues to spontaneous melanoma. Difference Gel Electrophoresis (DIGE) was used to minimize the variability commonly observed in quantitative analyses of comparative protein samples. Following identification of up- or down-regulated protein expression by DIGE, candidate protein spots were identified by mass spectrometric sequencing. Several protein expression differences displayed in interspecies hybrids were identified and compared to distinct differences that occur upon backcrossing and progression to melanoma. These studies are important for the identification of distinct biochemical pathways involved in the variety of *Xiphophorus* interspecies hybrid tumor models.

Keywords

Xiphophorus; Melanoma; DIGE; MALDI-TOF; Mass Spectrometry; Proteomics; Gene Regulation

1. Introduction

The genus *Xiphophorus* is comprised of 27 species of freshwater, livebearing platyfish and swordtails. Interspecies hybridization among *Xiphophorus* species has allowed research examining the underlying genetic events that correspond to inheritance of particular parental phenotypes. The first *Xiphophorus* interspecies cross shown to produce backcross hybrid progeny prone to melanoma development was published independently by two scientists, Myron Gordon and Kurt Kosswig, in the late 1920's (Gordon, 1927; Kosswig, 1928). The initial cross described by Gordon and Kosswig between the platyfish *X. maculatus* Jp 163

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A and the swordtail *X. helleri*. The F₁ interspecies hybrids from the cross exhibit more pronounced melanin pigmentation in the dorsal fin than either of the parents. When these F₁ hybrids are backcrossed to the *X. helleri* parent (i.e., *X. helleri* (x) [*X. maculatus* Jp 163 A (x) *X. helleri*]) 50% of the resulting backcross hybrid progeny (BC₁), are non-pigmented, 25% have pigment patterns resembling F₁ fish, and 25% develop severely enhanced melanization of the dorsal fin region that spontaneously develops into melanoma (Fig. 1). This interspecies cross, often termed the Gordon-Kosswig (G-K) melanoma model, has served an experimental system to study the genetics underlying melanoma production for over 70 years.

The *Sd*(spot dorsal) locus, responsible for pigmentation in the dorsal fin, was identified very early as a predictor of melanoma in this model (Gordon, 1927; Kosswig, 1928). Much later, a hypothetical tumor suppressor gene, *Diff*, was invoked to explain the absence of melanoma in parental *X. maculatus*. The *Diff* tumor suppressor is proposed to interact with a melanoma oncogene tightly linked to the *Sd* pigment pattern locus (Morizot and Siciliano, 1983). The oncogene *Xiphophorus* melanoma receptor tyrosine kinase-2 (*Xmrk-2*) is highly overexpressed in melanomas and melanotic (F₁ hybrid) fish (Wittbrod et al., 1989), whereas its related proto-oncogene *Xmrk-1* (Gutbrod and Schartl, 1999; Schartl, 1990) is expressed at low levels in all tissues. *Xmrk-2* is homologous to the human epidermal growth factor receptor gene (EGF-1) and has been shown to associate with phosphatidylinositol-3 kinase (PI3 kinase), which is known to be related to cell cycle function (Wellbrock et al., 1999). It is still unclear if *Xmrk-2* affects only PI3 or if there are other unknown kinases affected by *Xmrk-2* that contribute to the spontaneous melanoma development. A cyclin-dependent kinase inhibitor-2 (CDKN2X) has been cloned and mapped very close to the proposed *Diff* tumor suppressor (Kazianis et al., 1998; Nairn et al., 1996). The CDKN2X gene bears striking homology to the human p15 and p16 genes (*CDKN2B*, *CDKN2A*, respectively) which have been shown to be associated with familial (early onset) melanoma in humans (Landi et al., 2004). Additionally, p16 (*CDKN2A*), has been shown to be somatically mutated (UV signature) in human melanoma tumors. It is reasonable to hypothesize that interspecies hybridization in *Xiphophorus* may lead to global gene dysregulation brought about by interactions between two divergent (i.e. species) genomes. Alteration of pigment gene expression in cells (melanophores) derived from *X. maculatus* certainly occurs upon interspecies hybridization (Kazianis et al., 1996). This results in enhanced pigmentation, where fish are pre-disposed to tumor development (melanoma). However, one may envision many alternative mechanisms that might explain these observations (i.e. species-specific loss or gain of genes, modulated expression of existing alleles, alteration of protein complexes, etc.). For instance, there is evidence that cellular pathways (in particular DNA repair and cell cycles) are modulated within interspecies hybrids, relative to either parent (Walter et al., 2001; David et al., 2004; Mitchell et al., 2004). The initial interspecies producing F₁ hybrids might be expected to show gene or protein interaction effects, while the backcross interspecies hybrids may amplify certain traits due to loss of non-recurrent parental alleles.

We have applied proteomic methods to identify candidate proteins that exhibit modulated expression in fin tissue due to these two distinct biological events - interspecies hybridization, and progression to spontaneous melanoma using the G-K tumor model. Utilization of the *Xiphophorus* model system for these studies has particular value since changes in the proteome may be followed through successive crosses. Difference Gel Electrophoresis (DIGE) (Unlu et al., 1997; Alban et al., 2003) was used to minimize the variability commonly observed in quantitative analyses of comparative protein samples (Gustafsson et al., 2004). Following validation of up- or down-regulated protein abundance observed *via* DIGE, candidate protein spots were identified using MALDI-TOF/TOF mass spectrometry. Preliminary results from DIGE analyses suggest that about 30% of proteins

able to be analyzed exhibit different abundances in parental *versus* interspecies hybrid fin tissues. Several protein expression differences due to interspecies hybridization were identified and compared to distinct differences that occur upon further progression to melanoma. These studies represent a first step in identification of distinct biochemical pathways involved in the variety of *Xiphophorus* interspecies hybrid tumor models.

2. Materials and Methods

2.1. Reagents

All chemicals were analytical-grade or better and were purchased from Invitrogen (Grand Island, NY, USA), unless otherwise noted. Cyanine fluorescent dyes (CyDyes), pH 3-7 non-linear immobilized pH gradient (IPG) strips, isoelectric focusing (IEF) rehydration buffer, Bind-Silane, and Deep Purple stain were from Amersham (GE Healthcare, Piscataway, NJ, USA). HPLC-grade acetonitrile was purchased from Burdick-Jackson (Morristown, NJ, USA). Sequencing-grade trifluoroacetic acid (TFA), *N,N,N',N'*-tetramethylethylenediamine (TEMED), ProteoPrep Chaotropic Extraction Reagent 3, 4-sulfophenyl isothiocyanate, 2,4,6-trihydroxyacetophenone monohydrate (THAP), and α -cyanohydroxy-cinnamic acid (HCCA) were from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). ZipTip reverse-phase pipet tips were from Millipore (Bedford, MA, USA) and peptide standards used to calibrate the mass spectrometer were from Bruker Daltonics (Billerica, MA, USA).

2.2 Fin Sample Preparation

Dorsal fin samples from parental *X. maculatus* Jp 163 A (generation 102) (25 total), *Sd-helleri* F₁ hybrids (30 total), and *Sd-helleri* BC₁ hybrid tumors (16 total) were provided by the *Xiphophorus* Genetic Stock Center, Texas State University, San Marcos, TX, USA. Biological samples were collected on three separate occasions over a period of one year, resulting in three biological replicates per group. Each fish was anesthetized in 0.1% MS-222 until gill activity slowed appreciably then laid on a glass plate and their dorsal fins removed. Upon removal of the dorsal fin the fish were returned to their aquaria and after regeneration the dorsal fins (~ 2 months) were removed again. The dorsal fins were pooled to the extent necessary for obtaining sufficient protein for further analysis. Total proteins were extracted using 200 μ L Sigma ProteoPrep Chaotropic Extraction Reagent according to vendor instructions. Homogenized fin tissue (handheld pestle) samples were sonicated (5 bursts of 15-20 s) in an ice bath. Protein samples were centrifuged at 13,000 g for 30 min at room temperature (rt). The supernatant was transferred to a fresh microcentrifuge tube and reduced with 5 mM tributylphosphine. The samples were incubated for 1 h at rt, then alkylated with 15 mM iodoacetamide followed by incubation at rt for 1.5 h in the dark. Upon centrifugation (13,000 g for 15 min), the protein supernatant was transferred to a new centrifuge tube.

2.3 DIGE and Gel Imaging

CyDyes (Cy3, Cy5, and Cy2) were reconstituted to 1 mM in dimethylformamide (DMF). CyDye working solution (200 pmol/ μ L) was prepared by adding 4 μ L of DMF to a fresh microfuge tube followed by 1 μ L CyDye stock solution. This was done for each of the three dyes. DIGE labeling was performed for 50 μ g protein per gel according to the manufacturer's instructions. Samples were labeled with either Cy3 or Cy5 dye. Normalization was achieved by pooling 25 μ g of two respective samples and labeling with Cy2.

Once the fin protein samples were prepared, pH 3-7 non-linear Immobiline DryStrips were rehydrated with DeStreak rehydration solution containing IPG buffer and labeled protein.

The labeled protein mixtures contained either (1) *X. maculatus* Cy3-labeled sample or (2) *Sd-helleri* F₁ Cy5-labeled sample, and (3) Cy2-labeled standard sample. This dye labeling scheme was repeated on 6 gels. Two additional protein mixtures were prepared similarly except they contained “dye flipped” samples where *X. maculatus* was labeled with Cy5 and *Sd-helleri* was labeled with Cy3. A similar dye-labeling strategy was employed for gels comparing *Sd-helleri* and BC₁ hybrid tumors. All gels were rehydrated overnight at rt.

After rehydration, IEF strips containing protein samples were rinsed with water and loaded on an Amersham Ettan IPGphor II IEF unit for first dimension isoelectric focusing. The IEF strips were focused in the dark through the following run parameters; 300 V 50 μ A for 3 h, 600 V 50 μ A for 3 h, 1000 V 50 μ A for 3 h, and finally 8000 V 50 μ A for 7 h whereupon the unit was set at hold.

SDS-PAGE gels (12.5%, bis-tris, large format or 25.5 $\times$ 20.5 cm and 1 mm thick) harboring two fluorescently detectable reference markers attached to plates prior to gel casting were run employing an Ettan Dalt-6 Electrophoresis System (GE Healthcare). After focusing each IEF strip was equilibrated in 2 mL buffer (50 mM Tris-Cl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and a few grains of bromophenol blue) for 10 min, then transferred to the same buffer containing 2 mM iodoacetamide and equilibrated for 10 min. Once equilibrated the IEF strips were rinsed in 1X running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS, in distilled water) and immediately layered on the top of 12.5% SDS-PAGE gels. Each strip was sealed using 0.5% agarose overlay. All gels were run at 5 W/gel for 30 min followed by 17 W/gel for 4 h (until the dye front reached gel bottom).

Following the second dimensional separation each gel plate was rinsed with water and imaged using a tri-color laser imager (Amersham, Typhoon Trio imager). Gels were scanned using three emission filters corresponding to each of the three dyes (Cy2, Cy3, and Cy5) at 100 μ m pixel resolution. ImageQuant software was used to assess the gel images and determine protein spot patterns for the DeCyder image analysis software. From these comparisons specific spots were assigned as Proteins of Interest (POI's). POI's were assessed by the Student's *t*-test with a p-value of 0.05 and an average ratio of <1.5 to >1.5. POIs that passed all statistical criteria were further analyzed (see below). This statistical analysis process was repeated with both dye flip gels harboring proteins differentially labeled from the same sources. All POIs that were found in all three experiments across dye-flips were selected for further validation and subsequent identification. Validation of statistically significant POIs detected by the DeCyder software involved the key variables in Eq. (1) (Dell et al., 2002).

$$N=1+2C(CV/D) \quad (1)$$

where N is sample size, C is constant value (determined by α and β), CV is coefficient of variation, and D is the natural logarithm of observed ratio between two groups. (Dell et al., 2002, with some modifications). With $1-\beta = 0.8$ (80% power, type 2 error) and $\alpha = 0.05$ (type 1 error), the constant value is 7.85 (Gravetter and Wallnau, 2000). A coefficient of variation (CV) was determined for individual protein spots that were matched in all gels by first calculating an average spot volume from individual normalized spot volumes that were calculated in each gel. A deviation was then determined for each protein spot and the standard deviation was divided by the average normalized volume of each protein spot to determine a coefficient of variation for that individual protein spot. Those individual coefficients of variation for all the protein spots were pooled to obtain an average coefficient of variation that was used in equation 1.

2.4 Preparative Gels and Spot Picking

For identification of POI's by MALDI-TOF/TOF MS, four preparative PAGE gels were run with much higher protein loads (400 µg total protein) in order to excise sufficient quantities of the POI. The preparative gels were run in first and second dimensions exactly as detailed above for the analytical gels. After the second dimension, one glass plate was removed and the gel submerged into 500 mL fixing solution of 7.5% (v/v) acetic acid, 10% (v/v) methanol overnight at rt. The gels were rinsed in 500 mL solution of 35 mM sodium hydrogen carbonate, 300 mM sodium carbonate for 30 min, then immersed in water containing Deep Purple stain solution for 1 h in the dark with gentle agitation. The gels were destained with 7.5% acetic acid for 15 min with gentle agitation. After destaining, each gel was scanned using a Typhoon Trio with an emission filter corresponding to the Deep Purple Stain. The scanned image was at 100 µm pixel resolution (the maximum resolution needed for analysis by the DeCyder software). The image was manipulated as detailed previously for the analytical gels. POI's were excised from the gel using an Ettan Spot Picker (GE Healthcare).

2.5 Trypsin Digestion and Peptide Sulfonation

Excised protein spots were destained in 200 µL of 50 % acetonitrile (ACN)/100 mM ammonium bicarbonate (ABC) overnight. The gel plugs were then rinsed with 200 µL of 50% ACN/ 100 mM ABC and dehydrated for 5 min in 100% ACN at rt. After drying in a SpeedVac, gel pieces were resuspended in 25 µL of trypsin solution (Trypsin Gold, 1 µg/µL in 50mM acetic acid diluted in 40 mM ABC/10% ACN to 20 µg/mL). The samples were incubated at 37 °C overnight. The resulting peptides were extracted twice with 50 µL of 50% ACN/ 0.5% trifluoroacetic acid (TFA), dried in a SpeedVac and stored at -80 °C.

SPITC-modified peptides were prepared by dissolving peptide extracts in 2 µL of 10 mg/mL 4-sulfophenyl isothiocyanate (SPITC) in 20 mM sodium carbonate pH 9.5 that included 4 mM n-octyl-β-D-glucopyranoside (GDP) (Oehlers et al., 2007). The pH of the peptides was adjusted between pH 8 and 9. The samples were ZipTip-purified and redissolved in 1 µL of 10% ACN/ 0.1% TFA for mass spectrometric analysis.

2.6 MALDI-TOF/TOF-MS Analysis

MALDI matrix solutions were prepared by mixing 2,4,6-trihydroxyacetophenone (THAP, 20 mg/mL) and diammonium citrate (DAC, 10 mg/mL) in 50% ACN/50% water and combining in a 2:1 ratio with α-cyano-4-hydroxycinnamic acid (CHCA, 10 mg/mL) in 50% ACN/ 0.1% TFA. Peptides were spotted on a matt steel target plate and dried under a stream of air. Spots were washed with 1 µL of 0.1% TFA and dried.

All MALDI mass spectra were obtained with a Bruker Daltonics Autoflex II TOF/TOF (Billerica, MA, USA) mass spectrometer with a 337 nm nitrogen laser. Response was calibrated over a m/z 1000-3200 range using an external standard calibration mix (Bruker Daltonics). Peptide ion spectra for each sample spot were collected in reflecting mode at a frequency of 50 Hz (with matrix deflection of 500 Da) and precursor ions were selected for further post source decay (PSD) fragmentation. Each precursor ion was selected with a timed ion gate with a resolution of 100. PSD spectra of 2000 laser shots were collected for each precursor ion chosen. The SPITC labeling allowed *de novo* sequencing of target peptides (Oehlers et al., 2007) with >90% y-series ions for each peptide identified. Resolution was at least >1000 for the PSD spectra. PSD spectra were analyzed in FlexAnalysis v.2.0 and BioTools v.2.2 (Bruker Daltonics). Target peptides were then: (1) BLAST searched for short-nearly exact matches using the NCBI non-redundant databases of Vertebrata and *Danio rerio*, (2) searched against the Swiss-Prot database, and (3) searched using the FASTS algorithm to identify homologous protein sequences using unordered,

multiple short peptide sequences (FASTA Sequence Comparison at the University of Virginia, http://fasta.bioch.virginia.edu/fasta_www2/fasta_list2.shtml) (Mackey et al., 2002). Parameters for BLAST searches included: a maximum of one missed cleavage for trypsin digestion, acrylamide cysteine modification, and mass tolerance of ± 0.5 Da. Parameters for the FASTS searches were: MD20 matrix, (18:-29), ktup=2, and default statistics. Protein identifications were accepted on the basis of the lowest E values and highest scores and in most cases resulted from at least 2 distinct peptides.

3. Results

3.1 DIGE Comparison

Dorsal fin proteins were extracted from *X. maculatus* (Jp 163A), *Sd-helleri* F₁ hybrids, and BC₁ tumor-bearing fish and compared by DIGE proteomic analysis. An example DIGE comparison between *X. maculatus* and *Sd-helleri* F₁ is shown in Fig. 2. Multiple DIGE gel runs were performed with different labeling reactions using a dye-flip approach and mixtures of samples were labeled with Cy2 and used for normalization (Karp et al., 2004; Karp and Lilley, 2005). In order to distinguish proteins exhibiting modulated expression due to interspecies hybridization from changes occurring as a result of tumor development, two separate proteome comparisons were made. Dorsal fin proteins of the parental *X. maculatus* and the *Sd-helleri* F₁ interspecies hybrid were compared to identify protein expression differences that occurred simply due to hybridization of two distinct species (Fig. 1). Following this comparison, the dorsal fin proteins of the *Sd-helleri* F₁ interspecies hybrid and BC₁ tumor offspring were subsequently analyzed to identify protein with modulated abundance due to development of melanoma. In each proteome comparison, three separate analyses were performed using the DeCyder program: 6 gels of one dye labeling scheme (Cy3-labeled F₁ and Cy5-labeled *X. maculatus* or Cy3-labeled F₁ and Cy5-labeled BC₁); 6 gels of the opposite dye labeling scheme (Cy3-labeled *X. maculatus* and Cy5-labeled F₁ or Cy3-labeled BC₁ and Cy5-labeled F₁); and all 12 gels for both dye-labeling schemes from each proteome comparison (Table 1).

The average number of spots detected in gels comparing *X. maculatus* and *Sd-helleri* varied by 264 protein spots; in the gels comparing *Sd-helleri* and BC₁ tumor tissue the average spots detected varied by only 8 protein spots (Table 1, column 4). The number of spots matched between *all* gels (*i.e.* 12) in a particular analysis varied between 11-32% of the total spots detected (Table 1, column 6).

Protein abundance differences observed by DIGE analysis were considered to be statistically significant only if the protein of interest (POI) was observed in all gels of an analysis, exhibited an abundance ratio of at least ± 1.5 fold or greater, had a t-test score of $\alpha = <0.05$ and a power of 80% (Gravetter and Wallnau, 2000). The power requirement was included to address Type II error that results when an analysis fails to detect a change that is actually present. In particular, our aim was to address protein spot volume variability that occurs from gel to gel. The DeCyder software used for DIGE analysis only allows users to address the alpha requirement of the student's t-test; therefore analyses were initially conducted with the DeCyder software to obtain a range of POI's to be further subjected to the 80% power requirement (equation 1). Using an average coefficient of variation in Eq.1, the number of gels (N) required to observe statistically significant abundance ratio differences of ± 1.5 at 80% power and $\alpha = <0.05$ was calculated. For the proteome comparison of parental *X. maculatus* and interspecies hybrid *Sd-helleri*, 12 gels were required for identifying POI's under these conditions (Table 2A). Using all 12 gels in the proteome comparison of *Sd-helleri* and BC₁ tumor fish required that protein abundance ratios be ± 1.8 in order to be statistically significant (Table 2B). In order to detect abundance ratios of ± 1.5 in this case, approximately 20 replicate gels would be required. The increased variability observed in this

second proteome comparison may be related to the multitude of cell types in melanoma tumors, including normal, necrotic, and dead cells.

Thus, only ~11-13% of all spots observed in repeated dye flip DIGE gels (12 total gels, 6 gels in each direction) were suitable for further analysis (Table 1, column 6). Of these, ~30-35% of the proteins observed exhibited statistically significant differences in abundance ratios when comparing parental and interspecies hybrid dorsal fin proteins, or interspecies hybrid and backcross hybrid melanoma fin tissues. For all 12 DIGE gels in each proteome comparison 23 POI spots (9 up-regulated/14 down-regulated) were chosen for parental *X. maculatus* versus interspecies hybrid F₁, and 36 POI spots (23 up-regulated/13 down-regulated) were chosen for F₁ versus BC₁ tumor fish for identification using MALDI-TOF/TOF mass spectrometry.

3.2 Identification of Differentially Expressed Proteins

For both proteome comparisons, POI's excised from preparative gels were subjected to trypsin digestion and the resulting peptide fragments were SPITC-modified for subsequent *de novo* sequencing (Oehlers et al., 2005). Since the complete *Xiphophorus* genome was not available, amino acid sequences were BLAST searched using the NCBI non-redundant databases of *Vertebrata* and *Danio rerio* and the Swiss-Prot and FASTA databases for protein identification.

The comparison between parental *X. maculatus* and *Sd-helleri* F₁ was done to identify protein expression level changes resulting from interspecies hybridization. Of the 23 validated POI's in the proteome comparison between the *Xiphophorus* parental species and the interspecies hybrid, 19 POI's were identified with E values ranging from $2.8e^{-16}$ to 1.1; those identified with greatest confidence are listed in Table 3. In general, sequences with E values of less than 0.01 are considered to be homologous with the identified protein. The abundance differences ranged from nearly six-fold up-regulation of annexin to greater than two-fold down-regulation of NADH dehydrogenase. The identified proteins with the most negative E values and highest scores were actin, peroxiredoxin, glutathione-S-transferase, transferrin, and annexin.

Protein expression differences between F₁ hybrid melanin pigmented dorsal fin tissue and melanoma tissue from first generation backcross dorsal fins were observed for 36 validated POI's, of which 29 were identified with E values ranging from $2.9e^{-12}$ to 2.8; those identified with greatest confidence are listed in Table 4. Abundance ratios ranged from greater than four-fold up-regulation of dihydrotase to seven-fold down-regulation of cohesion. Identified proteins with the most negative E values and highest scores were enolase, actin, glyceraldehyde-3-phosphate dehydrogenase, peroxiredoxin, galectin, and rho GDP dissociation inhibitor.

4. Discussion

The proteome comparisons presented here - parental *X. maculatus* versus the F₁ interspecies hybrid *Sd-helleri* and the F₁ interspecies hybrid *Sd-helleri* versus backcross (BC₁) tumor fish - distinguished proteomic differences arising from interspecies hybridization and spontaneous melanoma. Although there have been proteomic studies on other aquatic fish models by our group (medaka, Oehlers et al., 2007), and others (Danio, Mehzoud et al., 2008; De Wit et al., 2008), these have primarily investigated changes in response to environmental stressors.

4.1 Comparison of parental *X. maculatus* and *Sd-helleri* F₁ interspecies hybrids

The most striking differences we observed when comparing protein expression in parental *X. maculatus* with the F₁ interspecies hybrid *Sd-helleri* were the differential regulation of transferrin, glutathione-S-transferase (GST), and peroxiredoxin (Table 3). Transferrin was down-regulated two-fold in the interspecies hybrid compared to parental *X. maculatus*. Transferrin mediates iron levels in the cell and a reduction of transferrin levels has been previously shown to induce apoptosis by a sodium ascorbate-mediated pathway (Lambert et al., 2005). In our study, two ROS proteins, GST and peroxiredoxin, were surprisingly affected in opposite ways in the dorsal fin cells of *Sd-helleri* compared to that of the parental, *X. maculatus*. GST was down-regulated nearly two-fold in the F₁ hybrid, while peroxiredoxin was up-regulated two-fold. The GST gene has been implicated in familial melanoma cases (Stahl et al., 2004) and is usually down-regulated (De Souza et al., 2006). Peroxiredoxin is known for its antioxidant properties but is also important for redox-dependent cell signaling (Groten et al., 2006). It may be that up-regulation of peroxiredoxin in the interspecies hybrid compensates to some degree for the partial loss of GST.

4.2 Comparison of *Sd-helleri* F₁ interspecies hybrids and BC₁ tumor-bearing fish

A number of up- and down-regulated proteins identified in the second proteome comparison are noteworthy. Annexin 5 and peroxiredoxin 6 were up-regulated in tumor-bearing BC₁ compared to F₁ hybrids. Annexin 5 is a commonly used apoptosis marker in melanoma studies (Zuidervaart et al., 2006). Peroxiredoxin 2 is usually expressed in melanocytes but lost in melanoma (Culp et al., 2006). However, the expression of peroxiredoxin 6 has been shown to be associated with metastatic progression of human breast cancer (Chang et al., 2007). Importantly, we observed up-regulation of glutathione peroxidase, another antioxidant protein, in the tumor-bearing fish. Glutathione peroxidase is usually down-regulated in human melanoma, in contrast to results obtained here, similar to what we observed with peroxiredoxin 6. This is another indicator of the increase in oxidative stress likely experienced by melanotic dorsal fin tissue in the backcross hybrid fish.

Additional important differentially expressed proteins identified in the proteome comparison between F₁ hybrids and tumor BC₁ fish are galectin, enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and Rho dissociation inhibitor protein. The differences in expression observed for these proteins were attributed to melanomagenesis. Galectins are β -galactoside specific lectins which play roles in several processes including RNA splicing, apoptosis, and neuronal and vascular development (Thijssen et al., 2006; Leffler, 2001). Recent studies have demonstrated in zebrafish and mouse models that galectin is required for angiogenesis and tumor outgrowth (Leffler, 2001). Our results demonstrating a 3-fold increase in galectin after progression to melanoma in the backcross tumor-bearing progeny are consistent with these previous studies.

Enolase is a neuron-specific serum protein that has been used in previous studies to gauge the severity of neuroblastomas in humans (Sandoval et al., 2006). We observed three times the amount of enolase in BC₁ melanoma than in *Sd-helleri* F₁ fin tissue. An increase in enolase has also been suggested as a possible diagnostic biomarker for acute brain infarctions (Selakovic et al., 2005). Thus, an increase in enolase abundance in *Xiphophorus* melanoma may be consistent with neural cell reorganization and indicates a potential area of cellular stress.

GAPDH was expressed two-fold higher in BC₁ melanoma than in *Sd-helleri* F₁ interspecies hybrids. GAPDH is involved in carbohydrate metabolism (Bosworth et al., 2005) and has been shown to be expressed two times higher in invasive melanoma cells compared to noninvasive melanoma (Stahl et al., 2004).

Rho dissociation inhibitor protein was down-regulated 2.3-fold in BC₁ tumor tissue compared to *Sd-helleri* F₁ fin. There are three types of Rho GTPases that regulate cell growth and cell migration (Wang et al., 2003). The use of RhoGAP-Rho chimeras to down-regulate Rho activity in an attempt to reverse growth and invasiveness of cancer cells has been suggested.

5. Summary

Common POI's identified between the first proteome comparison (parental *versus* interspecies hybrid) and the second proteome comparison (interspecies hybrid *versus* tumor-bearing fish) include actin, annexin 5, peroxiredoxin 6, and GST (Table 5). Differences in expression for these proteins were attributed to interspecies hybridization but several differences between the first and second proteome comparisons are noteworthy. Interestingly, actin, while up-regulated in the interspecies hybrid compared to parental fish, was down-regulated in the melanoma dorsal fin tissue compared to the interspecies hybrid. Levels of annexin 5 and peroxiredoxin 6 were up-regulated to an additional extent between tumor-bearing BC₁ compared to the upregulation observed in F₁ hybrids.

The up-regulation of peroxiredoxin 6 observed in combination with that of glutathione peroxidase is perhaps most important. A proteomic comparison using the same *Xiphophorus* spontaneous melanoma model has recently been reported (Lokaj et. al., 2009). In this study 65 differentially regulated proteins were identified that could be grouped into seven broad functional classifications. Those upregulated most strongly included cytoskeletal proteins and proteins related to the prevention of reactive oxygen species (ROS), including peroxiredoxin 2 and 6 and glutathione-S-transferase Mu3. Furthermore, upregulation of peroxiredoxin 2 and 6 was confirmed using Western blot analysis of healthy, benign, and tumor-bearing fish tissue. The authors concluded that overexpression of antioxidant proteins in malignant melanoma cells is important for counteracting the increased oxidative stress arising during melanomagenesis.

Our results are in agreement with the strong up-regulation of peroxiredoxin 2 and 6 found, suggesting this may be a fish-specific phenomena (Lokaj et. al., 2009). In any case, up-regulation of peroxiredoxin appears to be an important indicator of melanoma progression in the G-K *Xiphophorus* model.

In summary, dysregulation of cellular response to oxidative stress appears to be an important change that results from interspecies hybridization and one that further increases upon melanoma progression. In particular, our results confirm recent data indicating up-regulation of peroxiredoxin 6 is an indicator of melanoma progression. Proteins involved in neuronal organization, vascular development, cell growth and metabolism were found to be modulated in response to melanoma progression and not linked to interspecies hybridization in the current study. These represent important candidates for biomarkers of melanoma progression in the Gordon-Kosswig *Xiphophorus* melanoma model. With the completion of the *Xiphophorus* genome, we expect to identify a greater number of proteomic differences in this and related *Xiphophorus* interspecies hybrid systems with greater confidence in the near future. In particular, elucidation of genetic differences arising in another *Xiphophorus* tumor model, *Spandersi*, wherein BC₁ hybrids exhibit MNU induced tumor development but not UV-B induced tumorigenesis (Kazianis et al., 2001), may shed light on alternative genetic routes leading to melanoma.

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Abbreviations

DIGE	difference gel electrophoresis
MALDI-TOF/TOF MS	matrix assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry
POI	protein of interest

Figure 1.

The Gordon-Kosswig melanoma model. Interspecies crossing between *X. maculatus* and *X. helleri* generates *Sd-helleri* F_1 hybrids with pronounced melanin pigmentation on the dorsal fin. Backcrossing *Sd-helleri* F_1 with *X. helleri* results in 25% of progeny with dorsal fin pigmentation that spontaneously develops into melanoma.

Figure 2. Representative 2D-DIGE gel for the proteomic comparison between *X. maculatus* and *Sd-helleri* F₁.

Table 1

Spot data for 12 gel analyses between parental *X. maculatus* Jp163A, F₁ interspecies hybrids [*X. maculatus* (x) *X. helleri*], and BC₁ hybrids [to *X. helleri*]. All protein samples were dorsal fin fractionated on pH 3-7 DIGE gels.

Cy3	Cy5	#Gels	Spots Observed	Spots Matched to Master Gel	Spots Matched in All Gels	Spots of Interest *
F1	<i>X.mac</i>	6	879	655	113 (17%)	35 (31%)
<i>X.mac</i>	F1	6	794	616	200 (32%)	68 (34%)
F1	<i>X.mac</i>	12	615	615 (75%)	65 (11%)	23 (35%)
F1	Tumor	6	1250	908	233 (26%)	113 (48%)
Tumor	F1	6	1258	854	202 (24%)	91 (45%)
F1	Tumor	12	1254	850 (68%)	107 (13%)	36 (34%)

* +/- 1.5 abundance ratio for parental *versus* hybrid fish; +/- 1.8 abundance ratio for hybrid *versus* tumor-bearing fish

Table 2

A). Number of gels required to identify statistically significant proteins of interest in the proteomic comparison of parental *X. maculatus* versus F_1 interspecies hybrids [*X. maculatus* (x) *X. helleri*]. B). Number of gels required to identify statistically significant proteins of interest in the proteomic comparison of F_1 interspecies hybrids [*X. maculatus* (x) *X. helleri*] versus BC_1 (to *X. helleri*) tumor-bearing progeny.

A.			
Sample	Coefficient of Variation	Number of Gels Required for +/-1.5 Abundance	Abundance Ratio Significant for 12 Gel Replicates
Cy2	0.336	11.8	1.49
<i>X. mac</i>	0.339	12.0	1.50
F_1	0.351	13.0	1.52
B.			
Sample	Coefficient of Variation	Number of Gels Required for +/-1.5 Abundance	Abundance Ratio Significant for 12 Gel Replicates
Cy2	0.465	21.6	1.74
F_1	0.504	25.3	1.83
Tumor	0.478	22.8	1.77

Table 3

Most significant identified proteins that are up- or down regulated in F₁ interspecies hybrids relative to parental *X. maculatus* (out of 19).

F1 Abundance Ratio	Protein Match	Organism/E scores	Peptide sequence(s)
+5.99	Annexin (5)	gi-114786394 (Oreochromis, 4.6e-4) gi-47211293 (Tetradon, 3.4e-3)	EDA[I/L][I/L]Q[I/L][I/L]TAR
+1.95	Peroxiredoxin 6	gi-110589040 (Ictaurus, 1.1e-11) gi-41387146 (Danio, 7.6e-11)	DEAGTP[I/L]PFP[I/L][I/L]ADDQR PY[I/L]NGEVFNPFADTTSGR E[I/L]SVQ[I/L]GM[I/L]DPDER
+1.68	β-actin	gi-30038092 (Drosophila, 2.8e-16)	[I/L]WHHTFYNE[I/L]R AVFPS[I/L]VGR
+1.56	Put. Ferreredoxin	gi-56418995 (Geobacillus, 7.1e-2) - p#1	PDG[I/L]ED[I/L][I/L]GSRT [I/L]EYTADYSPY
-1.59	Precorrin-4	gi-83594713 (Rhodopirellula, 9.6e-2)	QPGHSSHYGVNAEQ[I/L]TS[I/L]JR
-1.60	Put. Transmemb. Protein	gi-32475051 (Rhodopirellula, 1.7e-2)	LEPSGASTGLDSDCLELR
-1.64	DNA binding Protein FIS	gi-87120521 (Marinomanas, 8.9e-2)	VFSVVEH[I/L]JR SHAPQDGV[I/L]NTR
-1.84	Glutathione-S-Transferase	gi156616388 (Danio, 6.9e-8)	A[I/L][I/L]HY[I/L]DGR MTQ[I/L]PA[I/L]SR
-1.84	Fatty Acid Binding Protein	gi-18874532 (Goose, 1.1e-2) [100%] gi-126321232 (Monodelphis, 4.8e-2)	SYE[I/L]PDGQ[I/L]T[I/L]G[I/L]JER
-2.20	Transferrin	gi-23305165 (Salmo, 3.1e-5)	EEGYGYAGAFR
-2.39	NADH dehydrogenase	gi-38491385 (Macaranga, 3.6e-2)	YQGH[I/L]NVQFQTR

Table 4

Most significant identified proteins that are up- or down regulated in melanoma tumor-bearing BC₁ fish relative to F₁ interspecies hybrids (out of 29).

BC ₁ Melanoma Abundance Ratio	Protein match	Organism/E scores	Peptide sequence(s)
+4.40	Dihydrorotase (homodimeric)	gi-152980376 (Janthinobacter, 3.5e-3) gi-134093512 (Herminimonas, 0.1)	QFDSFNG DPAPDMFDR
+3.19	Galectin	gi-7340066 (Paralichthys [flounder], 4.4e-6)	ST[I/L]HFA[I/L]R YSV[I/L]SFEGDAR FTPAEFVVT[I/L]TAR
+2.76	Enolase Enolase β/α	gi-20981682 (Human, 1.6e-4) p#1 gi-41394393 (Limulus, 9.1e-3) p#1-3	STG[I/L]YEA[I/L]E[I/L]R DVAAGCVH[I/L]R YPFEQPR
+2.69	Enolase γ	gi-20981682 (Human, 2.9e-12)	GVMVSHR PSGASTG[I/L]YEA[I/L]E[I/L]R SGWSA[I/L]R
+2.69	Annexin (max2) Annexin 5	gi-157278487 (Oryzias, 2.4e-2) gi-41107552 (Danio, 8.6e-2)	V[I/L]VE[I/L][I/L]SSR MEEG[I/L]NETDAQ
+2.43	Peroxisredoxin 2	gi-1717797 (1.7e-07) gi-50539996 (Danio, 3.4e-07)	Q[I/L]T[I/L]ND[I/L]PVGR [I/L]QEDEG[I/L]AYR
+2.34	Peroxisredoxin 6	gi-110589040 (Ictalurus, 4.4e-06), gi-47193903 (Tetradon, 2.9e-05)	E[I/L]SVQ[I/L]GM[I/L]DPDER AFANEAGTP[I/L]PFPI[I/L][I/L]ADDQR
+2.37	Glutathione Peroxidase	gi47229604 (Tetradon, 1.6e-2)	TH[I/L][I/L]ECVFSF[I/L][I/L][I/L]R
+1.97	Glutathione Peroxidase	gi-55960528 (Hypophthalmichthys, 1.3e-2) gi-47229604 (Tetradon, 2.5e-2)	[I/L][I/L][I/L]WSPVSR GDSWEP
+2.15	Serine/Thre.Kinase B-Raf Proto-Oncog..	gi-125486 (Rous viral, 1.2-4) gi-50403720 (Human, 2.1e-4)	DAPSQ[I/L]AH[I/L]R
+1.89	Glyceraldehyde-3-P-dehydrogenase	gi-119655542 (Tetradon, 2.2e-11),	VPVADVSVVD[I/L]T [I/L][I/L]SWYDNEYGYSNR
+1.86	Adenylate Kinase	gi-125153 (Yeast, 3.5e-2) [100%] gi-149046993 (Rat, 0.89) [100%]	[I/L][I/L]DGFPR
-1.88	Glutathione-S-Transferase	gi-158347524 (Goldfish, 1.2)	V[I/L][I/L]HYFDGR
-1.99	β-actin	gi-45505238 (Ictalurus, 3.0e-11)	[I/L]AANVE[I/L]VVHR SYE[I/L]PDGQ[I/L]T[I/L]GNER

BC1 Melanoma Abundance Ratio	Protein match	Organism/E scores	Peptide sequence(s)
-2.53	Actin-1	gi-113212 (ABSG, 2.4e-10) gi-1703159 (SPOLI, 3.3e-10)	AVFPS[I/L]VVPPPR [I/L]DAHHTFYNE[I/L]R
-2.34	Rho GDP dissociation inhibitor(GDI α)	gi-39645438 (Danio, 1.4e-4)	ADPTAPNVQVTR
-7.30	Cohesion Coactosin-like	gi-156403161 (Nematostella, 9.4e-3) p#1 gi-148640194 (Platypus, 8.8e-3) p#2 gi-126304707 (Monodelphis, 1.3e-2) p#2	ENEMDD[I/L]P[I/L]FGFVR CDN[I/L]P[I/L]FGFP F[I/L],T)I/L]TW[I/L]GER

Table 5

Peptides identified that appear modulated due to interspecies hybridization between *X. maculatus* and *X. helleri* and during subsequent progression to melanoma.

<i>X. maculatus</i> vs. F ₁ hybrid		F ₁ hybrid vs. BC ₁ melanoma	
Annexin 5	+5.99	Annexin (max2) Annexin 5	+2.69
		Peroxiredoxin 2	+2.43
Peroxiredoxin 6	+1.95	Peroxiredoxin 6	+2.34
β-actin	+1.68	β-actin	-1.99
		Actin-1	-2.47
		Actin-1	-2.53
Glutathione-S-transferase	-1.84	Glutathione-S-transferase	-1.88