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UVB-induced gene expression in the skin of *Xiphophorus maculatus* Jp 163 B[★]

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Abstract

Xiphophorus fish and interspecies hybrids represent long-standing models to study the genetics underlying spontaneous and induced tumorigenesis. The recent release of the *Xiphophorus maculatus* genome sequence will allow global genetic regulation studies of genes involved in the inherited susceptibility to UVB-induced melanoma within select backcross hybrids. As a first step toward this goal, we report results of an RNA-Seq approach to identify genes and pathways showing modulated transcription within the skin of *X. maculatus* Jp 163 B upon UVB exposure. *X. maculatus* Jp 163 B were exposed to various doses of UVB followed by RNA-Seq analysis at each dose to investigate overall gene expression in each sample. A total of 357 genes with a minimum expression change of 4-fold ($p\text{-adj} < 0.05$) were identified as responsive to UVB. The molecular genetic response of *Xiphophorus* skin to UVB exposure permitted assessment of; (1) the basal expression level of each transcript for each skin sample, (2) the changes in expression levels for each gene in the transcriptome upon exposure to increasing doses of UVB, and (3) clusters of genes that exhibit similar patterns of change in expression upon UVB exposure. These data provide a foundation for understanding the molecular genetic response of fish skin to UVB exposure.

Keywords

Fishes; Gene expression; RNA-Seq; Transcriptome; Ultraviolet light; UVB; *Xiphophorus*

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1. Introduction

The impact of solar ultraviolet (UV) radiation on living organisms has been intensely studied for many years (Kadekaro et al., 2003). In humans, UV exposure has been linked with photoaging and increased risk of skin cancers including cutaneous malignant melanoma (Armstrong et al., 1997; Jemal et al., 2006). Although many factors have been suggested to be responsible for this increase, there remains a poor understanding of the precise molecular genetic responses that organisms execute upon exposure to UV. This lack of molecular genetic understanding hampers the efforts to identify the primary underlying genotypes that may increase susceptibility to UV-induced skin cancer.

It is common knowledge that UVB exposure of skin may result in a large array of induced DNA photoproducts, either by direct damage (e.g., cyclobutane pyrimidine dimers [CPDs] and 6–4 pyrimidine pyrimidine photoproducts [6–4 PPs]) (Goodsell, 2001), or indirectly due to UV-induced formation of oxidative free radicals (Black, 1987; Tyrrell, 1995). To improve our understanding of the environmental components of UV exposure and skin cancer, several animal models have been employed including mouse (Merlino and Noonan, 2003), the South American opossum (Kusewitt et al., 1991) and *Xiphophorus* fishes (Walter and Kazianis, 2001). Of these model organisms, *Xiphophorus* fishes have been shown to be a particularly valuable model to assess UVB-induced DNA damage, DNA repair, and induced melanomagenesis (Nairn et al., 2001; David et al., 2004; Patton et al., 2010).

Xiphophorus fish interspecies hybrids have been studied since the 1920s (Gordon, 1927; Kosswig, 1928) and several UV-induced melanoma models have been described (Nairn et al., 2001). One of these *Xiphophorus* interspecies hybrid melanoma models is produced by crossing *Xiphophorus maculatus* Jp 163 B with *Xiphophorus couchianus* to produce fertile F₁ interspecies hybrids that are then backcrossed to the *X. couchianus* parental line (Nairn et al., 2001; Mitchell et al., 2010). The *X. maculatus* Jp 163 B line carries the *Sp* (spot side) pigment pattern resulting in macromelanophore pigment spots on the side of the animals. When this parental line is crossed with *X. couchianus*, the F₁ interspecies hybrids develop highly enhanced melanocyte pigmentation compared to the *X. maculatus* parent (Fig. 1). If the *X. maculatus* Jp 163 B (×) *X. couchianus* F₁ hybrids are backcrossed to *X. couchianus* (the BC₁), 50% of the progeny that inherit the *Sp* pigment patterns show enhanced melanization, and of these melanoma may be induced at relatively high frequencies by exposure to UVB just after birth (i.e., 5 days post birth; Walter and Kazianis, 2001). Induced melanoma in such cases is scored in BC₁ hybrids at about 6–9 months of age as nodular lesions. Studies using this *Xiphophorus* model support a role for direct UVB-induced DNA damage (i.e., CPDs and 6–4 PPs) as pivotal to melanoma development; however, other reports suggest melanomagenic affects in *Xiphophorus* skin may also result from UVB-induced oxidative free radicals (Mitchell et al., 1993; Wood et al., 2006; Mitchell et al., 2010).

The recent availability of the *X. maculatus* genome sequence assembly and its annotation (Schartl et al., 2013) provides tools that allow profiling of the global molecular genetic responses within the intact animal. Understanding the global molecular genetic responses of *Xiphophorus* skin to UVB exposure may promote a better understanding of the complex

events that comprise a melanoma-susceptible genotype and illuminate relationships between genetic pathways that may be altered by interspecies hybridization and/or induced by various types of cellular damage. With this in mind, we performed RNA-Seq studies detailing the molecular genetic changes in the skin of *X. maculatus* Jp 163 B after exposure to UVB. We present expression profiling results, reveal gene clusters that exhibit similar UVB-affected expression patterns, and show motifs common among some clusters. These findings increase our understanding of the global genetic response to UVB exposure in the skin of intact *X. maculatus* and provide a foundation to build hypotheses regarding the inherited susceptibility to UVB-induced melanoma within interspecies hybrids.

2. Material and methods

2.1. Fish

X. maculatus Jp 163 B fish were obtained from the *Xiphophorus* Genetic Stock Center (XGSC, <http://www.xiphophorus.txstate.edu>), Texas State University, San Marcos, TX, USA. The *X. maculatus* Jp 163 B utilized in this study were mature adult males (≈ 9 months old) in the 101st generation of full-sibling inbreeding. A complete strain description is available at <http://www.xiphophorus.txstate.edu/publications-data/stockcentermanual.html>.

2.2. UVB exposure

Three mature adult male fish, from generation 101 of full-sibling inbreeding, were used for each UVB exposure dose (0, 8, 16 and 32 kJ/m² UVB) in these experiments. Prior to experimental treatments, fish were placed in the dark for 14 h. For the UVB exposure, they were exposed to UVB light in UV-transparent cuvettes (9 cm $\times$ 7.5 cm $\times$ 1.5 cm) in about 95 mL of water suspended 10 cm between two banks of four unfiltered narrow spectrum UVB lamps (Philips, TL 20W/01 RS SLV) mounted horizontally on each side of a wooden exposure chamber. The TL 20W/01 RS SLV lamps emit a narrow waveband between 305 and 315 nm and peaks at 311 nm. Fluence was determined on each side of the chamber by using an IL-1400A Radiometer/Photometer coupled to a SEL 240/UVB detector containing a 280 nm Sharp Cutoff Filter (International Light, Newburyport, MA, USA) to be 12.2 J m⁻² s⁻¹. Dosages of 8, 16, and 32 kJ/m² UVB were achieved by varying the exposure time. Sham fish went through the same procedure as the 16 kJ/m² fish, but in this case the UVB lights were turned off. After UVB or sham exposure, all fish were incubated in the dark for 6 h to allow time for transcriptional response and then sacrificed for dissection and RNA isolation.

2.3. RNA isolation and RNA-Seq

For total RNA isolation, each skin sample was homogenized in TriReagent (Sigma Inc., St. Louis, MO, USA) using a handheld tissue disruptor. Following homogenization, 0.2 mL chloroform was added and the samples vigorously shaken and subjected to centrifugation at 12,000 g for 15 min at 4 °C. RNA was purified using a RNeasy mini RNA isolation kit (Qiagen, Valencia, CA, USA). Any residual DNA was eliminated by performing column DNase digestion at 37 °C (30 min). The integrity of RNA was assessed by gel electrophoresis (2% agarose in TAE running buffer) and the concentration was determined using a spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA).

RNA quality was verified on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) to confirm that RIN scores were above 8 prior to sequencing. *X. maculatus* Jp 163 B skin biological replicate RNAs were independently sequenced by using the Illumina High-Seq platform (100 bps, paired-end [PE] reads; Illumina, Inc., San Diego, CA, USA). About 120–165 million raw 100 bp PE reads were generated for each sample (Table 1). Raw reads were filtered and trimmed based on quality scores by using a custom filtration algorithm that removed low-scoring sections of each read and preserved the longest remaining fragment (Garcia et al., 2012). All raw reads were subsequently truncated by similarity to library adaptor sequences using a custom Perl script. Overlapping PE reads were merged using FLASH (Magoc and Salzberg, 2011). Detailed statistics on raw and filtered reads are shown in Table 1.

2.4. NGS data analysis

All filtered reads were mapped to the *X. maculatus* reference transcriptome (Ensembl V70) using Bowtie2 (Langmead and Salzberg, 2012). Read-mapping statistics were determined with an in-house Perl script. DESeq (Anders and Huber, 2010) in the Bioconductor R package (<http://www.bioconductor.org/>) was used to determine differentially expressed genes with fold-change cutoff at 4 ($p\text{-adj} < 0.05$).

All genes were grouped into clusters by using Bayesian Hierarchical clustering algorithm as implemented in the BHC package (Savage et al., 2011). These clusters were then manually merged into ten groups, each having a distinct expression change profile. Since interacting genes may be expected to show non-random patterns in cell localization, molecular function, and/or biological process, we performed a gene ontology (GO) enrichment test with Ontologizer (Bauer et al., 2008) and GeneMania (Warde-Farley et al., 2010) for each cluster. Genes without assigned symbols were queried against the human transcriptome (version GRCh37.p11) by using BLASTP (Altschul et al., 1990) and the reciprocal best hit E-values less than $1e-5$ were used to assign orthologous human counterparts.

2.5. Upstream motif identification and analysis

Three kilobase regions upstream from the start codon for all differentially expressed genes were extracted from the *X. maculatus* Ensembl genome assembly for motif identification. Differentially expressed genes were segregated into up and down groups for each sample by the direction of expression change, producing a total of six clusters including; up in 8 kJ/m², down in 8 kJ/m², up in 16 kJ/m², down in 16 kJ/m², up in 32 kJ/m², and down in 32 kJ/m². Motif occurrences are determined by scanning the upstream regions in each group with entries in the tax_group “vertebrates” from the Jaspar database (Stormo, 2000) using MOODS (Korhonen et al., 2009) ($E\text{-value} = 1e-5$). To obtain a background distribution, similar scanning was carried out on the upstream regions of all transcripts in the genome. Statistical significance for each of the identified motifs was calculated by using hypergeometric distribution.

2.6. RNA-Seq gene expression validation by Nanostring Technology

RNA-Seq derived expression changes for a selected group of genes were validated with Nanostring nCounter Analysis System (NanoString Technologies, Seattle, WA, USA) and

quantitative real-time PCR (qRT-PCR). Briefly, in the Nanostring nCounter system, extracted skin total RNA (500 ng) was annealed, ligated, purified, and then hybridized with probes (Integrated DNA Technologies, Coralville, IA, USA; Table 2) individually designed for each selected transcript. After the removal of unbound probes, the purified complexes were bound to the image surface and elongated by the nCounter Prep Station. The number of complexes were counted by a Digital Analyzer and standardized to positive and negative controls to determine read significance and background threshold, and normalized by selected standard internal controls in each sample. The $\log_2(\text{FoldChange})$ values for each transcript were calculated and compared to the RNA-Seq counterparts.

2.7. RNA-Seq validation by qRT-PCR

Total RNA from each tissue was used in cDNA synthesis employing a High Capacity cDNA reverse transcription kit (Applied Bioscience, Carlsbad, CA). The reaction was performed in two steps, a DNase I treatment was performed and deactivated by the addition of 25 mM EDTA at 65 °C for 10 min. Reverse transcription was performed by adding 100 mM dNTPs, 10X RT random primers, RNase inhibitor, and Multiscribe Reverse Transcriptase in a 20 μL reaction employing standard thermocycler conditions (25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min). Each sample was diluted to a final volume of 500 μL and analyzed for expression by qRT-PCR utilizing SYBR Green-based detection on an Applied Biosystems 7500Fast system (Applied Bioscience, Carlsbad, CA, USA). PCR primers were designed using Geneious (Biomatters Ltd, Auckland, New Zealand) software. All primers were designed with T_m values between 60 and 62 °C in 50 mM Na, 3 mM K, and 0.8 mM dNTPs. Primer lengths were limited to 18–25 bp long with 55–60% GC content, and not more than 1 °C between T_m s for each primer in a set. In addition, all amplicons were limited to 100–150 bps in length and were designed to cross at least one exon junction. Primers specific for the *Xiphophorus* transcripts (Table 3) were tested in triplicate efficiency in a 20 μL reaction consisting of a standard serial dilution series of either 250, 25, 2.5, 0.25 ng cDNA, 0.5 μM of each primer, and 10 μL SYBR Green ready mix (Applied Bioscience, Carlsbad CA). Each reaction was subjected to 40 cycles each at 95 °C for 20 s, 95 °C for 15 s, and 60 °C for 30 s, before being subjected to melting curve analysis. Amplified products were also analyzed for size by agarose gel electrophoresis (2% agarose in TAE running buffer). The 18S rRNA was selected as the transcript for normalization of all samples. Efficiencies were determined and only primers with accepted values between 70 and 120% were used for downstream analysis. Once the efficiency was established, the primers were used to test relative expression of each gene from cDNAs (25 ng) produced from *X. maculatus* skin from four technical replicates. All plates were normalized against the 18S rRNA transcript to determine CT values and fold changes were determined for samples exposed to doses of UVB light relative to sham (CT).

3. Results and discussion

3.1. Assessment of UV damage

The amount of UV-induced photodamage was assessed by a radio-immunoassay (RIA) for cyclobutane pyrimidine dimers (CPDs), the most prevalent direct photoproduct from UV irradiation (Mitchell, 2006). The RIA experiment was carried out on DNA isolated from the

same skin sample as used in the RNA-Seq experiment. The result demonstrated a positive correlation between the dose of UV and the amount of dimers formed as shown in Fig. 2.

3.2. UVB-responsive genes

Gene expression values from three UVB exposed samples (8, 16, and 32 kJ/m²) were compared to sham treatments to identify genes differentially expressed upon exposure of fish to UVB light. All genes with an expression change 4-fold or greater were considered significantly differentially expressed, hence termed “UVB responsive genes”, in the UVB-treated samples ($p\text{-adj} < 0.05$). Fig. 3 provides a general overview of the UVB responsive genes and a Venn diagram indicating genes that show significantly different expression from sham in more than one UVB dose.

As shown in Fig. 3, the number of genes that are significantly up-expressed at our statistical cut-off nearly doubled from 8 to 16 kJ/m², but is dramatically reduced in the 32 kJ/m² sample. On the other side, the number of down-regulated genes exhibited an opposite trend, with the fewest in the 16 kJ/m² sample (23 genes) and the most in the 32 kJ/m² (40 genes). These data suggest that the fish were capable of organizing genetic responses under low and moderate UVB irradiation (8 and 16 kJ/m²), hence the dramatic increase in up-expressed genes. However, at our high dose of UV irradiation at 32 kJ/m², severe UV-induced cell damage began to impair cell functionality and the underlying repair network, leading to a loss of up-expressed genes and a large increase in down-expressed genes.

3.3. Gene length differences in UVB-induced gene expression

An inverse correlation between UVB exposure and the lengths of UVB-responsive genes in *Homo sapiens* was recently reported (McKay et al., 2004), suggesting that UV-induced DNA damage may serve as a molecular dosimeter, signaling cells containing irreparable UV damage for elimination. Specifically, McKay et al. (2004) found that UV differentially expressed genes were on average shorter and had fewer introns as the UV dose increased. To examine this phenomenon, we compared the pools of UVB-responsive up- or down-expressed genes to test if the lengths of these genes, or their transcripts, differed significantly between different UVB doses (e.g., whether the average gene length and/or transcript length of the up-expressed genes in the 8 kJ/m² sample is statistically larger than those in the 16 kJ/m² sample). A Student's *t*-test was used to look for significant differences in the length of genes and transcripts between all possible sample combinations (8 kJ/m² vs. 16 kJ/m², 16 kJ/m² vs. 32 kJ/m², and 8 kJ/m² vs. 32 kJ/m²). There are significant differences in the gene length for up-expressed genes between the 8 and 32 kJ/m² samples and between the 16 and 32 kJ/m² samples (Table 2, left yellow cells). The up-expressed transcript lengths also trended similarly to the up-expressed gene lengths, but missed statistical significance at the 5% level (p -values of 0.0577 and 0.108). The comparisons of down-regulated genes and transcripts did not reveal any length differences significant at the 5% level (Table 4, blue cells), although several scores approached the 10% significance cut-off. The gene and transcript sizes of all up-expressed genes in each sample are plotted in Fig. 4. The log₂ representation was chosen for consistency with the previous study of human genes (McKay et al., 2004) and the number above each bar shows the mean in kilobase pairs (kbps) in the corresponding group.

In Table 4 the up-expressed genes at 8 kJ/m² and 16 kJ/m² are significantly longer than those up-expressed at 32 kJ/m², in agreement with earlier findings (McKay et al., 2004). Likewise, the down-regulated genes at the higher dose (32 kJ/m²) have a longer average length than the ones in the lower dose (8 kJ/m²), although this difference is not statistically significant ($p = 0.132$) at our cut-off.

3.4. Clustering and functional annotation

UVB-responsive genes were clustered by log₂ (FoldChange) values using the BHC package in R (Savage et al., 2011). A group of 38 clusters was initially produced; these were then consolidated by merging clusters with the same expression change pattern resulting in 10 clusters having a minimum number of 5 genes and with distinct expression patterns (Fig. 5). The full list of all genes and clusters is given in Supplementary Table S1. Here, we limit our discussion to the clusters listed in Table 5. These clusters are named based on the expression pattern at each dose. For each of the 8, 16, and 32 kJ/m² samples, genes in each cluster may be up (↑) if they show up-expression responses to the UVB treatment relative to sham, or down (↓) for down-regulated responses, or (=) if there was no significant difference from the sham. For example, the cluster of genes showing significant up-expression in only 8 and 16 kJ/m² UVB-exposed samples is named ↑↑=, and the cluster of genes designated ↑== only shows significantly up-expression in the 8 kJ/m² sample and not in the other two (16 and 32 kJ/m²) samples. Regardless of the degree of expression change, all genes with a p -adj > 0.05 were considered to have no significant change (=). These genes can still appear to be significantly changed in the non-significant samples, hence the occasional high log₂(FoldChange) values in the non-significant samples in Fig. 5.

3.4.1. Cluster 1—genes up-expressed at 8 kJ/m² (↑==)—Cluster 1 consists of genes that exhibit UVB induced up-expression in the 8 kJ/m² dose sample only. Of this set of 96 genes, 45 were able to be assigned as unique human orthologs, allowing functional analysis using GeneMANIA to be performed (Warde-Farley et al., 2010). The top five enriched functions are shown in Table 6.

This set of genes is clearly involved in protein activation cascades and this overlaps significantly with the sets of genes involved in the inflammatory response and the other three most enriched functions (peptidase inhibitor activity, peptidase regulatory activity, and enzyme inhibitor activity). After 8 kJ/m² of UV irradiation, *X. maculatus* increases transcription of genes in both the inflammatory response and protein activation cascades (C3, C5, CFI, CFP, KNG1, and SERPING1). C3 and C5 are components of the complement system. C3 is known to play a major role in the activation of the system while C5 is associated with initiating assembly of the late complement components into a membrane attack complex (Cianflone et al., 1989; Baldo et al., 1993). CFP is a complement factor that stabilizes the C3- and C5-convertase enzyme complexes and aids in the formation of membrane attack complex (Pruitt et al., 2009). The up-expression of SERPING1 is especially interesting since it inhibits activation of the C1 complex in the complement system. The simultaneous up-expression of SERPING1, C3, C5, and their related factors, but not C1, suggests the activation of the complement system via the alternative, rather than the classical pathway, where the involvement of C1 complex is essential.

The genes involved in all three remaining functions (A2ML1, ITIH2, ITIH4, SERPINA1, SERPINA10, SERPINC1, SERPIND1, and SERPING1) are all serine protease inhibitors. In addition, A2ML1 may inhibit the other three classes of proteases. Previous studies have shown that ITIH proteins are involved in inflammation and carcinogenesis, functioning as tumor suppressors (Hamm et al., 2008). A2ML1 has been identified as a major antigen in autoimmune disorders such as paraneoplastic pemphigus (Numata et al., 2013) and as a novel correlate of HIV-1 resistance in humans (Burgener et al., 2011). Other than SERPING1, the rest of the SERPIN members (SERPINA1, SERPINA10, SERPINC1, and SERPIND1) are involved in the blood coagulation cascade through the inhibition of thrombin and related factors.

Taken together, the uniquely up-expressed genes in the 8 kJ/m² sample are functionally enriched in the activation of complement and immune system components involved in the stress response. Although activation of immune response genes is also observed in other clusters, the enrichment of peptidase inhibitor activity genes is only present in this low-damage cluster.

3.4.2. Cluster 2—genes up-expressed at 16 kJ/m² (= ↑ =)—Cluster 2 consists of genes uniquely up-expressed in the 16 kJ/m² sample. It is the largest cluster of like expressed genes (170) of which 128 HUGO gene symbols were able to be retrieved from Ensembl as human orthologs. Several biological process functions are enriched in this group of genes, such as digestion (FDR = 8.7e-6), organic acid transport (FDR = 0.04), and carboxylic acid metabolic processes (FDR = 0.04). Here, we limit our discussion to the genes annotated as digestion-related in the human, which include LCT, CTRL, ACE2, SLC15A1, SCTR, PLA2G1B, SI, FABP2 and MEP1B. Because of the innate immune activities of the digestive system and a strong resemblance between human intestinal epithelia and fish epithelia, it is reasonable to expect digestion genes to play a role in the immune response in fish skin. For example, CTRL (Chymotrypsin-like protease) has been shown to induce endothelial activation, a pro-inflammatory and pro-coagulant state of the endothelial cells, in humans (Gu et al., 2009). Although the function of ACE2 is not clear, it has been shown to be involved in the response to viral infection (Donoghue et al., 2000; Tipnis et al., 2000). SLC15A1, more widely known as PEPT1, is localized to the intestinal epithelium and regulates di- and tri-peptide uptake in humans (Pruitt et al., 2009). However, its up-expression has also been recently identified in cancer cells (Tai et al., 2013). SCTR, a pancreatic secretion receptor, is localized to epithelial cells within the pancreatic and biliary duct and is involved in secretory regulation of the stomach and pancreas in mammals (Ulrich et al., 1998). In fish, SCTR may be regulating secretions from the mucus secretory glands located in fish skin. PLA2G1B (Phospholipase A2, Group1B) is a known digestive enzyme secreted by the pancreas of mammals (Seilhamer et al., 1986) and recent studies have also shown that it plays a role in inflammatory and immune responses (Jo et al., 2004; Seroussi et al., 2013). Highly expressed in kidney and intestines in mammals (Pruitt et al., 2009), MEP1B, is a membrane metallopeptidase that binds to several proinflammatory cytokines, such as IL1B and IL18, also suggesting a role in immune response (Becker-Pauly et al., 2011). Thus, although the annotated functions of genes in this group in mammals are related to digestion, when applied to fish skin it appears likely that they are involved in

inflammatory response, mucus secretion and associated immune response upon external insult.

3.4.3. Cluster 3—genes up-expressed at 32 kJ/m² (= = ↑)—Cluster 3 involves genes that exhibit up-regulated transcription only after 32 kJ/m² (i.e., = = ↑). This higher UVB dose appeared to be quite damaging and resulted in a major shift in the types and numbers of genes affected (Fig. 2a). Only five genes exhibited unique up-expression in the 32 kJ/m² sample, and four of these had human homologs (TNNT2, TNNI1, MYBPC3, and BAIAP2L). All of these genes are involved in cytoskeletal remodeling and cell restructuring. TNNI1 (Troponin I Type 1) and TNNT2 (Troponin I Type 2) are the inhibitory subunits of troponin that grants calcium-sensitivity to actomyosin ATPase activity in striated muscle. MYBPC3 encodes the cardiac isoform of myosin-binding protein C, another muscle protein that in humans is exclusively expressed in heart. BAIAP2L2 encodes a protein that binds phosphoinositides and promotes the formation of membrane structures. Our data suggest that these genes in fish may be conscripted in damaged skin to allow reorganization of the epidermis after cell death for recovery after UVB.

3.4.4. Cluster 4—genes up-expressed in 8 and 16 kJ/m² (↑↑ =)—Due to severe damage in fish skin after 32 kJ/m² UVB and very different sets of genes affected compared to the lower doses, we focus on UVB up-expressed genes in both the lower dose points (8 and 16 kJ/m²). Cluster 4 is made of 13 genes of which 11 were able to have HUGO gene symbols identified (APOA4, ENDOU, APOB, MAT1A, MUC2, PLAC8, TMIGD1, CYP2J2, LGALS4, TBX4, and TSPAN1).

Although a GO test on this cluster failed to return statistically significant enrichments compared to the overall genome (FDR [false discovery rate] < 0.05), a closer look reveals that the majority of these genes are likely to be involved in the fish skin immune response. Specifically, APOA4 has been shown to bind to low density lipoprotein (LDL) and to act as a site-specific antioxidant (Wong et al., 2007). APOB is also up-expressed in response to viral infection (Leong and Chow, 2006). MUC2 protein, secreted by fish goblet cells, is prominent in the internal organs of mammals. Since mucus on the fish epidermis is one of most rapid and important components of fish immune system, it is reasonable to assume that MUC2 plays an important role in response to UVB insult. Although PLAC8 (also known as ONZIN) is mainly enriched in mammalian placenta (Galaviz-Hernandez et al., 2003), it is also expressed in many adult cell types, particularly in immune cells (Rissoan et al., 2002). Its role in the immune system was confirmed in knockout mice that display deficiencies in neutrophil function (Ledford et al., 2007). TMIGD1, (Transmembrane and Immunoglobulin Domain Containing 1) also has immune system function and is expressed by intestinal epithelial cells in humans. LGALS4 (galectin-4) has been shown to activate T-cells by secretion of pro-inflammatory cytokines (Hokama et al., 2004) and has also been shown to play a role in tumor progression and metastasis in pancreatic cancer in vitro (Belo et al., 2013). Although CYPs (cytochrome P450s) are well known for their role in aryl hydrocarbon metabolism in the liver, studies have shown that it also has an essential role in vitamin D signaling, which regulates hundreds of genes in many physiological functions including the immune response (Schartl et al., 2013). Furthermore, our finding also

correlates with the previous study showing that CYP 1 genes appear to be up-expressed at moderate dose (20.1 kJ/m²) but not at higher dose (26.8 kJ/m²) in UVB exposure of developing zebrafish (Behrendt et al., 2010). From methionine and ATP, MAT1A catalyzes the formation of S-adenosylmethionine, which is the main methyl donor for a vast number of methylation processes (Chiang et al., 1996). Expansion of liver cancer stem cells has been correlated with MAT1A deficiency by knockout mice (Rountree et al., 2008).

Taken together, almost all the genes in this cluster are involved directly or indirectly in immune response. The cluster clearly suggests that an immune response is triggered by UV-induced stress in the fish skin.

3.5. Motif identification in UVB responsive genes

To better understand the genetic regulation of the UVB response in *Xiphophorus* skin, we searched the upstream regions of the UVB-responsive genes for known vertebrate regulatory motifs available in the Jasp database (Stormo, 2000). A background distribution for each motif in the genome was obtained by analyzing the upstream regions of all 20,498 genes. For these analyses, upstream gene regions are defined as 3 kb prior to the translational start codon. The presence of each motif was also sought in the upstream regions of only the genes shown by our analysis to be UVB responsive. The statistical significance for the motifs present in the upstream regions of the UVB-responsive genes was then calculated by hypergeometric distribution in regard to the background frequency. All statistically enriched motifs in the UV-responsive genes (p -value < 0.05) are listed in Table 7.

Prrx2 is a DNA-binding protein with expression localized to proliferating fetal fibroblasts and the developing dermal layer. Generally Prrx2-regulated genes are down-regulated in adult skin. The reduced expression of the Prrx2-targeted genes in UVB-exposed fish skin may suggest an inhibition to cellular proliferation, preventing further replication of UV-damaged cells in the skin in *X. maculatus*. NR1H2, also known as liver X receptor beta (LXRB) is a ubiquitously expressed member in the LXR family that plays key roles in controlling transcriptional programs in lipid homeostasis and importantly in this study, inflammation (Korf et al., 2009). The FOX, forkhead box transcription factor family is involved in regulation of genes involved in cell growth, proliferation, and differentiation. Although FOXF2 is only expressed in lungs of mammals (Pruitt et al., 2009), it has recently been shown to have possible roles in tumor suppression. Specifically, FOX expression is linked to tumor suppression via inhibition of the Wnt signaling pathway (van den Brink and Rubin, 2013). The up-expression of FOXF2 target genes may be linked to the resistance to melanoma exhibited by *X. maculatus* Jp 163 B. Further experimental analysis on interspecies hybrids that are susceptible to UVB induced melanoma would be warranted to determine if this is significant.

3.6. Nanostring and RNA-Seq expression validation

AMY1C, CPA1, EPDR1, JDP2, JUNA, SERPINA3, and TGM2 were selected for validation by Nanostring Technology and A2ML1, ACTC1, ASTL, BHMT, ELA2, GRN1, and HPX for validation by qRT-PCR. The selections were made to cover a variety of UVB induced patterns, such as up-in-all (AMY1C, CPA1, EPDR1, and ELA2), down-in-all (TGM2 and

JDP2), and mixed changes (SERPINA3 and HPX). The $\log_2(\text{FoldChange})$ values, compared with the respective basal expression, for both validation methods (Nanostring or qRT-PCR) are shown in Fig. 6. Apart from expected slight variances in degree of change, the modulated expression patterns observed in the bioinformatic analyses of RNA-Seq treatments were perfectly reflected by the two independent methodologies represented by Nanostring and qRT-PCR.

4. Conclusions

The molecular genetic response of *X. maculatus* Jp 163 B skin to varied UVB exposure is detailed herein. The fish with low UV irradiation (8 kJ/m²) appeared to increase expression of regulatory genes that may trigger activation of the inflammation and stress response genes and begin cell repair. The most expansive UV-induced response occurred in the fish exposed to 16 kJ/m² of UVB light, with more than 200 genes differentially expressed ($p\text{-adj} < 0.05$, fold change ≥ 4). Fish homologs to the genes expressed in the mammalian digestive tract are up-expressed after UVB in the fish skin and likely carry out functions involved in an immune response. A set of known regulatory motifs is enriched in the upstream regions of UVB-responsive genes, hinting at molecular mechanisms for the co-regulation of various UVB-responsive gene sets. The highest dose of UV irradiation (32 kJ/m²) delivers substantial damage to the fish skin, leading to an overall extensive down regulation of many genes preventing a genetic response. Overall, the higher the UVB dose the shorter the UVB responsive genes and their transcripts, as has also been observed in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Interspecies hybrid model between *X. maculatus* Jp 163 B and *X. couchianus*. Fish (A), (B), and (C) are *X. maculatus* Jp 163 B, *X. couchianus*, and F_1 hybrid respectively.

Fig. 2. Average CPDs per Mbp of *X. maculatus* Jp 163 B skin DNA. Error bars represent the standard error of the mean.

Fig. 3. Overview of RNA-Seq based assessment of UVB-responsive genes. The number of UVB-responsive genes for each sample is shown in (A). The numbers of shared up-down-expressed genes among the three experimental UVB exposures are shown in (B) and (C) respectively. The total number of genes in each sample is given in parentheses below each dose label.

Fig. 4. Mean gene and transcript sizes of the up-expressed genes in all three samples with the standard error of the mean. The mean in kilobase pair (kBp) is shown in the text above each bar.

Fig. 5.

Overview of the ten clusters with distinct expression change patterns. Each cluster is represented by a heat map in which each gene is represented by a row of three colored bars (one each for 8, 16, and 32 kJ/m²) for which the color represents $\log_2(\text{FoldChange})$ relative to sham. The ten clusters of genes are separated vertically by white space and are labeled with a series of three symbols to the right of each cluster. The symbols used in the labels are one of an upward red arrow, a horizontal bar, or a downward green arrow, and these indicate up-expression, no change, or down-expression respectively. The three symbols from left to right indicate the expression changes in 8, 16, and 32 kJ/m² samples respectively. The total number of genes in each cluster is given in parentheses at the end of each cluster symbol. Genes with $\log_2(\text{FoldChange})$ greater than 2 are still considered non-significant if their adjusted p-value is greater than 0.05, hence the occasional high $\log_2(\text{FoldChange})$ values in the non-significant samples.

Fig. 6.

Validation of RNA-Seq gene expression changes using two independent methodologies, Nanostring and qRT-PCR. (A) Shows the $\log_2(\text{FoldChange})$ of 7 genes validated by Nanostring and (B) shows the $\log_2(\text{FoldChange})$ of 7 genes validated by qRT-PCR. N8, N16, and N32 represent the $\log_2(\text{FoldChange})$ values determined by Nanostring Technology at 8, 16, and 32 kJ/m^2 , respectively. R8, R16, and R32 represent the values determined by qRT-PCR; and T8, T16, and T32 represent the values derived from RNA-Seq transcriptome analysis.

Table 1

RNA-Seq read statistics.

UV dose	Raw data		Processed reads		Expected coverage
	Read count	Total read length	Read count	Total read length	
Sham	165 M	16.5 B	120 M	12.0 B	230x
8 kJ/m ²	167 M	16.7 B	127 M	12.5 B	240x
16 kJ/m ²	155 M	15.5 B	120 M	11.7 B	225x
32 kJ/m ²	128 M	12.8 B	92 M	9.5 B	182x

M = million, B = billion.

Table 2

Genes and probe sequences used in the Nanostring nCounter analysis. The last five genes were used as expression controls.

Gene	Probe sequence
AMY1C	GGAGTCAGCATGGATAGCAATAAAAGGATCTTCCTCTGTATTGCTGATGTCGATTGCTGCATTCCGCTCAACGCTTGAGGAAGTA
JDP2	TAAGTGTGTAAACAATTTGTGAATATGTGCTTCTCATGACGCCAGCGCCACCTACGTATATATCCAAGTGTTATGTCCGACGGC
CPA1	CCATATCCAAGATGGCTTGACATCTTCAATCATGGTAGAGTACTCAAGGCATTTGGAATGATGTGTACTGGGAATAAGACGACG
EPDR1	GGCTTGGCAGATTTCTCCATTTTTCGTCGTAACAGAAAGCAGGAGGGAACCTTGACGTAGATTGCTATCAGGTTACGATGACTGC
SERPINA3	CGACATGAAGATGTTTTTCCGGGAGCGGTTCTACCATTTATGTTTCTGTCGCTATGCAGACGAGCTGGCAGAGGAGAGAAATCA
TGM2	AGCACCAAGGGTTGCAGAGTAGGATGAAATCTCCCACCACAAAACCTTCCATTGCAACCATGTGAAGTAATGTGAGCGTACTT
JUNA	GCTTGAAGTTTTTCGGGTCGTTTAGGTTTCAGGGTCATGGTTTGTTCAGTCAGCAAGAAGGAGTATGGAACCTTATAGCAAGAGAG
GAPDH	GACCTCACCTTTGAAGCGGCCGTGAGTGGAATCATACTGGAACATGTAGACTTGAGAGGAGTTGATAGTGGTAAACAAACATTAGC
PSMD4	CTTCTCAAAGTTGATGATATCCACGTTACCTTCTCCTTTTCAAGCGCCCGGAATCGGCATTTTCGCATTCTTAGGATCTAAA
B-actin	CCAACCCTACTCAAGATCAGGCAGACAGAAATCTCAAACATGTGCGCTTCTAGGACGCAAACTCACTTGAAGAAGTGAAAGCGAG
ATRAID	CAGACTCAGAGTGATTTGGCTTTGCGTCTCTGGGTAATCCACAACAGGAAGTGAAGGCTGTAAAGCTGTAGCAACTCTTCCACGA
EF1 α	TCTGACCGTTCTTGGAGATACCAGCCTCAAACCTCACCACACAAGAATCCCTGCTAGCTGAAGGAGGGTCAAAC

Table 3*Xiphophorus* specific qRT-PCR primer sequences.

Gene	Forward (5'-3')	Reverse (3'-5')	Amplicon length	Efficiency (%)
A2ML1	GGAGTTTCTTGAATGCATCCAGTTC	CGTTTCCTCGTACCTCAACCTC	86	103
ELA2	AGTTTGTGGTCCTTGCGTTG	CGTTACTGTTGTACTGCAGGG	147	98.1
BHMT	TCGATCTGCCTGAATTCCCC	CAGCTTTGTAGGCCTCCCTG	90	104.5
ASTL	GGAGAGAGCCAACAGAGACC	GTCCGCTTCAGACCGTACAG	85	95.6
GRN1	ACTCCCTGTCCTGATTTGTCG	CTTGTCAGTACAGCACACAGC	90	99.4
ACTC1	AGGTCTGTCACTCTTTCTACAACG	TTGGCTTTGGGGTTGAGCG	91	117.6
HPX	ACCCGGACACAAAACGATC	TCCTCGTTTCTGCGACTGC	90	112.5

Table 4

Student's *t*-test scores for comparisons of the length of transcripts and genes (introns included) among all three UVB doses.

	Gene length (bps)			Transcript Length (bps)		
	8 kJ/m ²	16 kJ/m ²	32 kJ/m ²	8 kJ/m ²	16 kJ/m ²	32 kJ/m ²
8 kJ/m ²		0.177	0.0263		0.834	0.108
16 kJ/m ²	0.406		9.86E-05	0.101		0.05777
32 kJ/m ²	0.377	0.132		0.812	0.759	

The yellow cells show the scores of the up-expressed genes or transcripts, and the blue cells the down-regulated ones. Statistically significant scores (*p* < 0.05) are shown in red. The score for comparison of transcript lengths between 8 kJ/m² and 32 kJ/m² is shown in orange because it approached the significance cutoff of 0.05.

Table 5

Subsets of interesting gene clusters similarly responsive to UVB exposure in *X. maculatus* Jp 163 B skin.

ID	Number of genes	Number of distinct human homologs	Expression change pattern (8–16–32 kJ/m ²)	Enriched biological process GO functions
1	71	45	↑ = =	Stress-related response
2	170	126	= ↑ =	Carboxylic acid metabolism, lipid metabolism
3	5	4	= = ↑	none
4	13	11	↑↑ =	Immune response/ion binding

“↑” represents up-expression relative to the sham; “↓” down-expression; “=” non-significant change.

Table 6

The top five enriched functions identified in Cluster 1 by GeneMANIA.

Function name	FDR	Number of genes	Genes (HUGO symbols)
Protein activation cascade	7.13E-07	7	C3, C5, CFI, CFP, F10, KNG1, SERPING1
Peptidase inhibitor activity	7.13E-07	8	A2ML1, ITIH2, ITIH4, SERPINA1, SERPINA10, SERPINC1, SERPIND1, SERPING1
Inflammatory response	2.72E-06	10	APOA1, C3, C5, CFI, CFP, CRP, KNG1, ITIH4, LYZ, SERPING1
Peptidase regulator activity	2.90E-06	8	A2ML1, ITIH2, ITIH4, SERPINA1, SERPINA10, SERPINC1, SERPIND1, SERPING1
Enzyme inhibitor activity	3.06E-06	9	A2ML1, ANGPTL3, ITIH2, ITIH4, SERPINA1, SERPINA10, SERPINC1, SERPIND1, SERPING1

FDR: False Discovery Rate.

Table 7

Motifs identified in the upstream regions of the UVB-responsive genes.

Sample dose (kJ/m ²)	Gene group	Motif ID	p-value	Motif regulator name	Related functions
8	Down	MA0075.1	0.041	Prrx2	Cellular proliferation
8	Up	MA0115.1	0.003	NR1H2::RXRA	Inflammatory response
8	Up	MA0030.1	0.047	FOXF2	Tumor suppression
16	Up	MA0141.1	0.002	Esrrb	Cell growth and differentiation
16	Up	MA0140.1	0.002	Tal1::Gata1	Haematopoietic processes
16	Up	MA0114.1	0.004	HNF4A	Liver, kidney, and intestine development
16	Up	MA0071.1	0.002	RORA_1	Organogenesis and differentiation