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Molecular Genetic Response to Varied Wavelengths of Light in *Xiphophorus maculatus* Skin

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Abstract

Xiphophorus fishes represent a model often utilized to study UVB induced tumorigenesis. Recently, varied genetic responses to UVB exposure has been documented in the skin of female and male *Xiphophorus*, as have differences in UVB response in the skin of different parental species and for interspecies hybrids produced from crossing them. Additionally, it has been shown that exposure to “cool white” fluorescent light induces a shift in the genetic profiles of *Xiphophorus* skin that is nearly as robust as the UVB response, but involves a fundamentally different set of genes. Given these results and the use of *Xiphophorus* interspecies hybrids as an experimental model for UVB inducible melanoma, it is of interest to characterize genes that may be transcriptionally modulated in a wavelength specific manner.

The global molecular genetic response of skin upon exposure of the intact animal to specific wavelengths of light has not been investigated. Herein, we report results of RNA-Seq experiments from the skin of male *Xiphophorus maculatus* Jp 163 B following exposure to varied 50 nm wavelengths of light ranging from 300–600 nm. We identify two specific wavelength regions, 350–400 nm (88 genes) and 500–550 nm (276 genes) that exhibit transcriptional modulation of a significantly greater number of transcripts than any of the other 50 nm regions in the 300–600 nm range. Observed functional sets of genes modulated within these two transcriptionally active light regions suggest different mechanisms of gene modulation.

Keywords

Light wavelength; RNA-Seq; Differential gene expression; Skin; Circadian; Cellular stress; *Xiphophorus*

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Introduction

Life evolved under the full spectrum of the sun and thus it is likely to have become attuned to use of all wavelengths in diurnal cycling of gene expression. Sunlight encompasses both ultraviolet (UV) and visible light regions. While light is necessary for some biological processes, such as regulation of circadian rhythm (Cermakian et al., 2002) and vitamin D production (Lucock et al., 2014), it can also have adverse effects, such as skin cancer and photoaging (Berneburg et al., 2000; Diepgen and Mahler, 2002). Studies of biological consequences of exposure of *Xiphophorus* fishes to different regions of the UV spectrum (e.g., UVB or 290–320 nm and UVA or 320–400 nm) have documented induced DNA damage through a variety of different mechanisms (Ahmed and Setlow, 1993; Mitchell et al., 2001; Meader et al., 2007). UVB exposure may produce direct DNA damage (e.g. cyclobutane pyrimidine dimers and 6–4 photoproducts; Tzung and Runger, 1998; You et al., 2001), whereas UVA exposure is thought to generate reactive oxygen species (ROS) via absorption of light energy by cellular photosensitizers (Kielbassa et al., 1997; Wood et al., 2006; Swalwell et al., 2012). The differences in the mechanisms for induced DNA damage and its repair, suggest different gene sets may have evolved as a response to specific wavelengths of light. Although the genetic effects of UVB exposure in *Xiphophorus* skin have been reported (Yang et al., 2014), the global transcriptional response to visible light remains to be defined and the global molecular genetic response of skin after exposure to specific wavelengths of light have not been studied.

Among its many effects, light plays a crucial role in the regulation of the circadian clock. The circadian system has been shown to have an impact on behavioral, physiological, and molecular patterns based on the light-dark cycling of the sun (Kleitman, 1949; Reppert and Weaver, 2002; Hastings et al., 2007). The interaction of the transcription factors CLOCK and BMAL1 represent the core clock genes involved in maintaining the circadian rhythm (Gekakis et al., 1998). Subsequent transcription and translation of period (*per1*, *per2*, and *per3*), cryptochrome (*cry1* and *cry2*), and timeless proteins serve to inhibit CLOCK/BMAL1 activity, and thus constitute a negative regulatory loop (Gekakis et al., 1998; Sangoram et al., 1998; Griffin et al., 1999). There is ample evidence suggesting circadian cycles play a role in DNA repair and control of the cell cycle (Granda et al., 2005; Gery et al., 2006; Kondratov and Antoch, 2007), but the specific light wavelengths needed and what other transcriptional effects might be triggered by each wavelength is still under investigation.

Here we present RNA-Seq results from the skin of *Xiphophorus* Jp 163 B showing genes modulated by exposure to six specific 50 nm wavelength regions between 300 and 600 nm. We observed two 50 nm wavelength regions of light that exhibit substantially higher levels of transcriptional response (300–350 nm and 500–550 nm) than any of the other 50 nm wavelength regions tested. Light exposure in these two wavelength regions appears to induce circadian and cellular stress responses as well as activation of p53 and other cellular processes.

Methods and Materials

2.1 Fish Utilized

Xiphophorus maculatus Jp 163 B fish of pedigrees 105B and 105F were provided by the *Xiphophorus* Genetic Stock Center, Texas State University, San Marcos, TX 78666 (<http://www.xiphophorus.txstate.edu/>). All *X. maculatus* utilized were adult males between 10 to 11 months old and were in their 105th generation of inbreeding.

2.2 Specific Wavelength Exposure

X. maculatus Jp 163 B were exposed to 50 nm wavelength regions of light (300–350, 350–400, 400–450, 450–500, 500–550, and 550–600 nm) using a TLS-300X Series Tunable Light Source (Newport Corporation, Irvine, CA, USA) containing an Ushio 300 W Xenon Short Arc Lamp Model 6258. Light emitted was passed through an attached Cornerstone 130 Monochromator (Newport Corporation, Irvine, CA, USA) to define specific wavelengths. The bulb was burned in 15 min prior to all exposure treatments. The specific wavelengths were divided by 2 fiber optic light cables, allowing the fish to be exposed on both sides simultaneously to the defined wavelengths of light. Spectral distributions were made to determine the power output of each light source at specific wavelengths using a Newport 1918-R power meter (Newport Corporation, Irvine, CA, USA). The dose of all exposures was determined to be 10 kJ/m². The spectral distribution of the xenon light source was measured at full spectrum (0 nm) using an Ocean Optics STS 350–800 nm Microspectrometer (Ocean Optics Inc., Dundedin, FL, USA) and OceanView software v1.5 (<http://oceanoptics.com/product/oceanview/>). The Microspectrometer was calibrated to a known standard using Ocean Optics Halogen Calibrated Light Source HL-3P-CAL (Ocean Optics Inc., Dundedin, FL, USA). To cover each wavelength in each 50 nm regions, the monochromator was set to scan and repeat (i.e. loop) using Asoftech Automation (<http://www.asoftech.com/>) through the wavelengths of each region (1 nm/sec for 50 sec) for the duration of the light exposure.

Prior to light exposure, fish were placed into individual 125 mL flasks filled with 100 mL of filtered aquaria water and kept in the dark overnight for 12 hrs. Each fish was then placed in a 4 cm length × 1 cm wide × 4.5 cm height quartz cuvette filled with 14 mL of filtered aquaria water. The cuvette was then centered between the 2 fiber optic light cables and covered by a cardboard box to eliminate ambient light. After exposure, the fish was removed from the cuvette, rinsed with filtered aquaria water, placed back into a 125 mL flask filled with 100 mL of filtered aquaria water, and in the dark for 6 hours to allow for gene expression prior to sacrifice and tissue dissection. Unexposed control fish were placed in the dark overnight inside an individual 125 mL flask and were sacrificed and dissected without being subjected to light.

Fish were anesthetized by placing them in ice, sacrificed by cranial resection, and tissues dissected. Skin samples were immediately placed in 1.5 mL microcentrifuge tubes containing 300 µL TRI reagent (Sigma Inc., St Louis, MO, USA) and flash frozen in an ethanol dry ice bath. Remaining tissues were placed in individual 1.5 mL microcentrifuge tubes filled with 300 µL RNeasy lysis buffer (Qiagen, Crawley, UK) and flash frozen in an ethanol dry ice bath.

2.3 RNA Isolation

RNA isolation was performed following the Qiagen RNeasy RNA isolation protocol (Qiagen, Valencia, CA, USA). Skin samples harvested from fish were first homogenized using a hand held homogenizer in a 1.5 mL microcentrifuge tube while the sample remained frozen in TRI Reagent (Sigma Inc., St Louis, MO, USA). After homogenization, 300 μ L of fresh 4°C TRI Reagent was added to the samples followed by incubation (rt) for 5 min. Chloroform extraction was performed by adding 120 μ L chloroform and shaken for 15 sec. Samples were centrifuged (16,100 rcf for 5 min at 4°C) for phase partition. The aqueous layer was transferred to a new 1.5 mL microcentrifuge tube and a second chloroform extraction performed (300 μ L TRI Reagent, 60 μ L chloroform). After extraction, nucleic acids in the aqueous phase were precipitated with 500 μ L 70% EtOH in diethylpyrocarbonate (DEPC) treated water. The sample was then transferred to a Qiagen RNeasy mini spin column and on-column DNase treatment was performed for 15 min at 25°C. RNA samples were then washed and eluted in 100 μ L RNase free water. RNA concentration was measured with a Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY, USA). To further assess the RNA quality, a RNA integrity (RIN) score was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples processed for RNA sequencing had a RIN score above 8.

2.4 RNA Sequencing

For each 50 nm exposure, 2 biological replicates of *X. maculatus* Jp 163 B skin samples were sent to Beckman Coulter Genomics (Beckman Coulter, Inc., Atlanta, GA) for Illumina High-throughput Sequencing using the Illumina TruSeq mRNA Library Prep Kit on the HiSeq 2000 platform (Illumina, Inc., San Diego, CA, USA). RNA was sequenced (75 bp, paired-end [PE] reads) and the raw reads were trimmed and filtered using a custom Perl script (Garcia et al., 2012). The reads were truncated based on similarity to library adaptor sequences using custom Perl scripts (Garcia et al., 2012). Then, low-scoring sections of each read were removed, preserving the longest remaining fragment as previously described (Yang et al., 2014). Overlapping PE reads were merged using FLASH (Mago and Salzberg, 2011). FastQC was then used to assess the quality of the filtered reads to identify any potential deficiencies within the data for each sample (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

2.5 Computational Analysis

All filtered reads were mapped to the *X. maculatus* reference transcriptome using GSNAP (Wu and Nacu, 2010) and transcripts were annotated using BioMart (Ensembl v79). The percentage of reads mapped and unmapped as well as the coverage depth was calculated using SAMtools flagstat (Li et al., 2009). The R-Bioconductor (<http://www.bioconductor.org>) package DESeq (Anders and Huber, 2010) was used to determine differentially expressed genes with a fold change cut off ± 2 (p -adj ≤ 0.05) based on the comparison between normalized reads of exposed skin samples to unexposed skin samples.

Differentially expressed genes from each wavelength region were compared using a multi-Venn diagram generated by Bioinformatics Evolutionary Genomics (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) to identify shared and unique genes between

each region. Functional analyses of shared genes were performed using Ensembl Biomart (<http://www.ensembl.org/biomart/>) and characterized using GeneCards (<http://www.genecards.org/>).

To obtain a comprehensive understanding of the differentially modified genes in the 350–400 nm and 500–550 nm regions, *Xiphophorus* Ensemble transcript IDs were converted to human homolog gene IDs using Ensembl Biomart. Over-represented gene ontology (GO) terms were tested by R-Bioconductor package GStat (ontology = Biological Process, p-value cutoff = 0.05; Beissbarth and Speed, 2004).

ConsensusPathDB (<http://cpdb.molgen.mpg.de/CPDB>) was used to identify potential pathway and genetic associations for each wavelength region. Networks included predicted genes generated by ConsensusPathDB. Functional characterization of genes found at each wavelength region was performed using GO terms collected from the human homolog gene IDs. Uncharacterized genes appearing in the networks were manually categorized using GeneCard characterization. Heat maps were generated using R package gplots (Warnes et al., 2012) and a custom script to assess the differential expressions of genes shown within the network.

2.6 NanoString

Aliquots of the same *X. maculatus* Jp 163 B skin samples used in RNA-Seq were used in a NanoString nCounter assay (NanoString Technologies, Inc., Seattle, WA, USA). The nCounter assay was performed at the Baylor College of Medicine Microarray Core Facility (Houston, TX, USA) using 500 ng (7 μ L) RNA per sample. Hybridization protocols were strictly followed according to manufacturer's instructions (Geiss et al., 2008). Hybridized samples were incubated overnight at 65°C with custom probes in the NanoString Prep Station and immediately evaluated with the NanoString nCounter based on unique color coded signals. Data analyses were performed by lane normalization using a set of standard NanoString probes followed by sample normalization using a set of 10 housekeeping genes. Counts were generated by nCounter Digital Analyzer. Fold changes were calculated on normalized counts and plotted using Microsoft Excel. The two biological samples were averaged and standard deviations calculated. Seventeen genes (Table S1) were selected for NanoString gene expression analysis, 10 of which were housekeeping genes.

2.7 Quantitative real time PCR

Quantitative real time PCR (qRT-PCR) was performed as previously described (Walter et al., 2014). Briefly, isolated skin RNA was turned into cDNA using a High Capacity cDNA reverse transcription kit (Applied Bioscience, Carlsbad, CA, USA) following the manufacturer's instructions. Synthesized cDNA was utilized in qRT-PCR SYBR Green-based detection methods. Applied Biosystems 7500Fast systems (Applied Bioscience, Carlsbad, CA, USA) were used for all qRT-PCR reactions. PCR primers were designed using Geneious (Biomatters Ltd, Auckland, New Zealand) bioinformatics software.

All primer sets designed had 40–60% GC content and a T_m between 60–62°C with less than 1°C difference within each primer set. Primer lengths were designed to be 80–150 bp in

length and across at least one exon junction. The efficiency of all primers was tested in *X. maculatus* skin in triplicate 20 μ L reactions in a standard serial dilution series of 25, 2.5, 0.25, and 0.025 ng cDNA. Primers with 70–120% efficiency were selected for relative gene expression analysis.

Three biological replicates with 4 technical replicates of each sample were plated for expression analysis. To remain consistent with RNA-Seq and NanoString, 2 of the biological replicates were selected to calculate fold change expression relative to unexposed skin samples. 18S RNA was used as a normalization transcript for all samples. mRNA expressions relative to the 18S RNA endogenous control were calculated by applying 2^{-CT} . Calculated values represents the mRNA expression level for each target tested relative to the 18S RNA control. Fold change for each sample was determined relative to each respective unexposed sample and standard deviations were calculated with the average of 3 technical replicates for each biological replicate. Primers selected for differential expression analysis are shown in Table S2.

Results

3.1 Distribution of genes exhibiting differential expression after exposure to 50 nm wavelength regions between 300–600 nm

To assess how fish skin genetically responds to various wavelength exposures, RNA-Seq data from each of six 50 nm wavelength light treatments between 300–600 nm were mapped to a reference *X. maculatus* transcriptome (Ensembl v79; 20,218 transcripts). The number of reads obtained and the fraction mapped to the transcriptome for each sample that corresponds to a 50 nm wavelength exposure are presented in Table 1. The average fraction of filtered reads mapped to the transcriptome was 55% (46.3 to 58.2%), equating to about 61x coverage. DESeq analyses were performed to identify differentially expressed (DE) genes based on comparison to the RNA-Seq reads derived from the skin of unexposed fish ($\pm$ 2-fold cutoff, p -adj = 0.05) for each of the 50 nm wavelength regions tested.

To determine whether the cuvette or water would shift the wavelength and energy emitted from the xenon source, light spectrums were measured through the cuvette with and without water (Figure S1). Both spectrums were nearly identical, indicating neither the quartz cuvette nor water altered the wavelength reaching the fish skin. Furthermore, the energy emitted was only altered by 5 μ W when measured through water, indicating the volume of water used in the 1 cm path did not absorb a significant amount of energy.

As shown in Figure 1, RNA-Seq assessment of gene expression across wavelengths ranging from 300 to 600 nm shows two wavelength regions having much greater numbers of differentially expressed genes than any of the other regions. These regions are 350–400 nm with 88 DE genes (Table S3) and 500–550 nm with 276 DE genes (Table S4). Overall, DE genes in both of these wavelength regions exhibited up-modulation to a greater extent than down-modulation (Figure 1). Among the 88 DE genes in the 350–400 nm region, 82% (72 genes) were up-modulated and 18% (16 genes) were down-modulated. While at the 500–550 nm exposure, 61% (167 genes) were up-modulated and 39% (109 genes) were down-modulated (Figure 1). In contrast to these two wavelength regions, considerably fewer genes

were modulated following exposure to 400–450 nm and 450–500 nm light (3 and 25 DE genes), respectively (Tables S5 and S6).

3.2 Circadian DE genes and validation of RNA-Seq results

Modulation of genes involved with maintaining circadian rhythm that are well documented to be light responsive (Whitmore et al., 2000; Cermakian et al., 2002; Reppert and Weaver, 2002) were examined to test whether the dose of light exposure (10 kJ/m^2) at the various wavelength regions mimicked what had been previously observed in *Xiphophorus* skin (Walter et al., 2014; Yang et al., 2014; Walter et al., 2015). Six differentially expressed circadian genes were identified that met the statistical cut off (± 2 -fold cut-off, $p\text{-adj} = 0.05$) in at least one of the six 50 nm wavelength regions tested (*per2*, *per1b*, *per3*, *arntl1a* [also called *bmal1*], *clock*, and *bhlhe40*; Figure 2). While *per2*, *arntl1a* (*bmal1*), and *clock* were consistently up-modulated across all wavelengths tested; *per1*, *per3*, and *bhlhe40* were observed to be down-modulated across all wavelengths. Additionally, the expression of *per1* and *per3* became more down-modulated in fish skin as the wavelength increased (Figure 2). The expression pattern of circadian pathway related genes is consistent with previous reports (350–400 nm; *clock*, *arntl1a*, *per2*, etc., and 500–550 nm; *per1b*, *nr1d2b*, *arntl2*, etc.; Yang et al., 2014; Walter et al., 2015).

Validation of the RNA-Seq data was performed using two independent experimental technologies, NanoString nCounter analysis and qRT-PCR. Among the 7 experimental genes tested by NanoString, 4 genes (*ppp1r27*, *ybx2*, *clock*, and *per2*) identified as DE in at least one of the 50 nm wavelength regions were also assessed by RNA-Seq (Figure 3A–D). The remaining 3 genes (*tgm8*, *dnah7*, and *klhl38b*) were assessed using all three platforms; RNA-Seq, NanoString, and qRT-PCR (Figure 3E–G). An additional 2 genes (*atm* and *per1b*) were assessed using only RNA-Seq and qRT-PCR (Figure 3H–I).

Although exact fold change values between the three independent experimental platforms were not equal, the fold change trends in transcription were very similar among all the genes tested. Thus, both NanoString and qRT-PCR confirmed the expression patterns observed by RNA-Seq at each independent wavelength exposure tested.

3.3 Shared genetic response for 350–400 nm and 500–550 nm regions

As presented in Figure 1, different wavelengths of light induced different numbers of differentially expressed genes. However, some DE genes were also identified as responsive to more than one 50 nm wavelength region. Shared DE genes across different wavelength regions are shown in Figure 4. Adjacent wavelength regions were observed to share a larger fraction of DE genes (average $\approx 36\%$ shared) as one may expect. For example, 31 DE genes (59.6% of DE genes in 550–660 nm) were shared between 500–550 nm and 550–600 nm regions while only 6 DE genes (11.5% of DE genes in 550–600 nm) were shared between 450–500 nm and 550–600 nm wavelength regions (Figure 4B).

Examination of the number of shared DE genes between the two most transcriptionally responsive regions (300–350 and 500–550 nm) identified 11 shared DE genes (4% of DE genes in 500–550 nm; Table 2). Gene ontology (GO) and functional analyses of these 11

shared genes were examined across all other 50 nm wavelength regions (Figure 4C). While 3 genes (*per3*, *ppargc1a*, and *bhlhe40*) were identified to be involved in circadian rhythm pathways, the other 8 shared genes did not readily cluster into a functional or GO group and appear to be involved in disparate biological functions.

3.4 Unique DE genetic response in 350–400 nm and 500–550 nm regions

Differentially expressed genes exclusive to the 350–400 nm or 500–550 nm regions that could be assigned human homolog ID's were assessed for gene ontology function. Among the 88 differentially expressed genes identified in the 350–400 nm region, 78 could be assigned human homologue ID's and analyzed through the human genome database. Sixty-six genes readily clustered into 5 major categories: circadian rhythm (6 genes, p-value = 5.99e-05), epithelium development (13 genes, p-value = 6.10e-04), actin cytoskeleton organization (7 genes, p-value = 6.23e-03), transport (26 genes, p-value = 3.07e-02), and cellular metabolic process (53 genes, p-value = 2.47e-02). The remaining 11 genes did not cluster in any GO categories (Table 3). Additionally, GO categories with similar gene sets and function, such as regulation of cell proliferation (11 genes, p-value = 3.69e-02) and epithelium development, were grouped into broader categories (e.g. cell proliferation; Table 3).

Similar functionalization of the 276 DE genes in the 500–550 nm region identified 246 genes that could be assigned a human homologue ID. Clustering of these genes segregated 221 genes into 8 categories: circadian rhythm (7 genes, p-value = 4.12e-03), cell differentiation (60 genes, p-value = 4.82e-03), regulation of growth (14 genes, p-value = 2.05e-02), muscle structure development (23 genes, p-value = 3.43e-07), cytoskeleton organization (20 genes, p-value = 2.08e-02), metabolic process (167 genes, p-value = 6.50e-03), response to unfolded proteins (8 genes, p-value = 4.49e-04), and response to stimulus (126 genes, p-value = 8.69e-04). The remaining 25 genes did not sort into a functional category (Table 4). Smaller GO clusters, including epithelial cell proliferation (8 genes, p-value = 4.56e-02) and Wnt signaling pathway (11 genes, p-value = 5.63e-03), were grouped into broader categories with GO terms cell differentiation and regulation of growth. Genes that clustered in GO terms cellular lipid metabolic process (20 genes, p-value = 1.72e-02) and cellular response to lipids (14 genes, p-value = 1.02e-04) were grouped with metabolic process (Table 4).

To visualize pathways and genetic associations, the 88 DE genes at 350–400 nm and 276 genes at 500–550 nm were independently analyzed using ConsensusPathDB (Figure 5, top). The fold change values of each unique gene in these clusters are visualized in a heat map (Figure 5, bottom). As shown, two DE gene networks appear modulated at 350–400 nm exposure (Figure 5A) and 500–550 nm (Figure 5B) exposure, centered around *atm* and *atr* genes, respectively. Genes related to cell proliferation, metabolic process, and cytoskeleton were DE in both transcriptionally active regions (350–400 and 500–550 nm). In addition, the GO transport cluster was only observed in the 350–400 nm region while genes related to muscle differentiation were only observed in the 500–550 nm region. DE genes related to cellular stress were observed in both 350–400 and 500–550 nm regions. However, each

stress related gene cluster contained genes that are expected to have substantially different functions (see below).

Discussion

4.1 Gene induction at 350–400 nm and 500–550 nm regions

Light responsive transcription of gene encoding proteins involved with circadian cycling, transcriptional regulation, and various other biological processes (e.g. metabolism, cytoskeletal, and detoxification) have been documented in mammalian systems (Albrecht et al., 1997; Akhtar et al., 2002). In addition, recent studies have shown gene expression in fish skin to be light responsive (Walter et al., 2014; Boswell et al., 2015, Walter et al., 2015). However, these previous reports dealt exclusively with ultraviolet wavelengths, or more recently, with broad-spectrum fluorescent lights. Data presented in Figure 1 shows the distribution of differentially expressed genes in skin across the range of 300–600 nm is not uniform, but exhibits a transcriptional response that clusters around two regions: 350–400 nm and 500–550 nm. Additionally, we observe that fish exposed to 400–450 nm (violet light) and 450–500 nm (blue light) exhibit the least number of genes modulated in the 300–600 nm range. The low genetic response exhibited in the violet-blue region is interesting considering that certain proteins undergo photoactivation via blue light (Mitani et al., 1996). For example, photolyases are activated through photoreduction of flavin (Li et al., 1991). Also, the cryptochrome family proteins in vertebrates can undergo a similar photoactivation, but lack DNA repair capabilities and interact with proteins involved in the circadian pathway (Thompson and Sancar, 2002; Bouly et al., 2007).

Use of all wavelengths by fish skin to regulate homeostasis may not be surprising given our evolution, but such clustering of transcriptional response is novel and unexpected. Since many proteins and cofactors have been documented to be light responsive and absorb in the violet-blue region, the observed clustering of DE transcriptional response outside the violet-blue region (400 to 500 nm) in skin may suggest that intracellular biomolecules and photoreceptors that serve as primary light responsive effectors specifically absorb these violet-blue wavelengths of light to provide energy for cellular processes, while the transcriptional activators have conscripted the wavelength regions shown here to be transcriptionally active (350–400 and 500–550 nm) for gene regulation. It is currently unclear what intracellular antennae might absorb specifically in the transcriptional hotspots at 300–350 or 500–550 nm and what mechanism(s) might be involved to produce the observed modulated gene expression, but this presents an interesting avenue for future study.

4.2 Cell cycle progression and stress response after exposure to 350–400 nm light

Gene ontology analyses revealed differentially expressed genes were associated with circadian rhythm, epithelium development, cytoskeleton organization, and metabolic processes at both the 350–400 nm and 500–550 nm wavelength regions. While genes related to metabolic process, transport, and response to stimulus represented most of the DE genes, these broad GO terms encompass a wide array of overlapping cellular functions. Thus, for the purpose of discussion, we focus on the other major GO clusters.

In addition to DE genes related to epithelium development, the 350–400 nm region also presented a cluster of genes associated with regulation of cell proliferation. Among the 76 genes assigned with human homolog ID's, 21 genes were found to encode proteins involved in cell proliferation (*acer1*, *evpl*, *anxa1*, *atm*, *cxcl12*, *tgm1*, *tgm3*, *ezr*, *ca2*, *rab25*, *wnk2*, etc.; Table 3). Most (16 of 21) of the genes within this cluster were up-modulated, suggesting promotion of cell proliferation. Additionally, genes associated with the Akt and Wnt signaling pathway (*mlkl*, and *gna15.1*; Table 3) are also up-regulated, supporting active cell proliferation (for review, see Chang et al., 2003; Niehrs and Acebron, 2012). Our results are consistent with previous reports that have shown non-lethal exposure to UVA can up-regulate the Akt signaling pathway and promote cellular survival (He et al., 2008; see below).

Another DE gene GO category in the 350–400 nm region is related to cytoskeleton organization. Seven genes were clustered into this category (*limch1*, *iqsec2*, *fmn1*, *epb4114b*, *cxcl12*, *ezr*, *myh14*), 5 genes being up-modulated (Table 3). This suggests skin cells may be undergoing cytoskeletal remodeling due to the light exposure. Past reports have indicated cellular stress and damage, and in particular oxidative damage, may induce cytoskeletal remodeling and reorganization of endothelial cells and skin cells in mammals (Paladini et al., 1996; Houle et al., 2003). Thus, the cytoskeleton organization response observed in skin upon exposure to 350–400 nm wavelengths may indicate cells are undergoing stress concomitant with cell proliferation.

Fish exposed to 350–400 nm light were subjected to UVA (defined as 320–400 nm; Maverakis et al., 2010) exposure, which has been documented to produce reactive oxygen species (ROS) upon absorption by intracellular photosensitizers (Kielbassa et al., 1997; Wood et al., 2006). ROS production increases oxidative stress within the cell and may also induce DNA damage, leading to apoptosis (Simon et al., 2000; Lesser et al., 2001; Apel and Hirt, 2004). Therefore, stress induced by UVA exposure in production of ROS may explain the uptick in expression of genes associated with cellular reorganization. However, from the pattern of DE genes affected, we speculate that the dose used in the 350–400 nm exposure is not adequate to induce apoptosis.

4.3 Cellular stress response upon exposure to 500–550 nm light

At 500–550 nm, a functional response similar to the 350–400 nm exposure was observed in several up-modulated genes. p53 activators and p53 target genes, such as *hspb7*, *rrad*, *ankrd1a*, *gls2*, *sesn1*, and *hspa8* (Budanov and Karin, 2008; Hu et al., 2010; Kirschner et al., 2010; Kojic et al., 2010; Bansal et al., 2011; Lin et al., 2014; Zhang et al., 2014), are among the differentially expressed gene set. Up-regulation of these p53 target genes suggests an activated p53. Also, upon 500–550 nm exposure, genes known to respond to DNA damage (*tmem150b*, *zak* (*mrk*), and *nuak1*; Tosti, et al., 2004; Bensimon et al., 2011; Mershtik et al., 2015; Table 4) were up-modulated in the skin. While exposure to 500–550 nm light has not been documented to produce ROS or induce DNA damage, our data suggests *Xiphophorus* skin exposed to this particular wavelength region is responding in a manner consistent with cellular stress (Welch and Suhan, 1985; Schwartz and Rutter, 1998). This may be why the DE stress genes in the 350–400 nm exposure constitute a very different set of genes than

those observed at 500–550 nm. In addition, a group of 7 heat shock stress related genes is up-regulated exclusively after 500–550 nm exposure (*dnajb4* (*hsp40*), *hspb7*, *hspa1b*, *hspa1l*, *hspa4*, *hspa6*, *hspa8*). This set of DE genes supports our contention that 500–550 nm light initiates a stress response in *Xiphophorus* skin (Santoro, 2000; Table 4).

Within the metabolic process cluster, gene encoding proteins involved in lipid metabolism (20 genes) are observed to be modulated in skin after 500–550 nm exposure. Genes observed in the lipid metabolic process cluster encompass various processes, such as lipid transport, transferase activity, signaling, etc. Particularly, *acsl1a*, *lpin1*, *cpt1b*, *gpd1b*, and *acer2* are all found up-modulated and involved in lipid degradation (Table 4). While fatty acids and lipids are essential to maintaining various cellular components (e.g. cellular membrane and energy storage; Spector and Yorek, 1985), increased degradation of fatty acids can contribute to cellular stress (Schönfeld et al., 2010; Soardo et al., 2011). Consistent with this, lipid responsive and lipid metabolism genes are also observed to be differentially modulated upon 500–550 nm exposure (e.g. *ppargc1a*, *fbxo32*, *abca1*, *esrrb*, *ankrd1*, *tnc*, *lrp6*, *myod1*, *srebfl*, *nr4a3*, *znf703*, *trim63* and *nr1d2*; Table 4). Among the genes that encode proteins responsive to lipids, many were involved in transcriptional regulation of processes such as inflammatory response (e.g. *ankrd1a* and *nr4a3*; Liu et al., 2015; Murphy and Crean, 2015), lipid regulation (e.g. *abca1b* and *srebfl*; Oram and Lawn, 2001; Eberlé et al., 2004), and muscle protein ubiquitination (e.g. *fbxo32* and *trim63*; Lecker et al., 2004; Chen et al., 2012). The presence of these two correlated clusters of genes suggests that lipids play a role in the cellular stress response of fish skin.

Among the 276 DE genes in 500–550 nm light, one of the largest gene ontology cluster of genes are related to muscle differentiation (23 genes; *spep*, *chrnb1*, *wfikkn2*, *xirp2*, *coll1a1*, *xirp1*, *fhl1*, etc.; Table 4). Seventeen of these genes were up-modulated. Besides their involvement in muscle differentiation, these genes play a role in regulation and stabilization of cytoskeletal actin filaments. As previously discussed, cellular stress and damage can induce the reorganization of the cytoskeleton. While it is possible that this particular set of genes may be due to muscle contamination in the skin samples during dissection, we speculate these muscle DE genes are involved in cytoskeletal remodeling in response to stress induced by 500–550 nm exposure.

Besides an implication of cellular stress at 500–550 nm region, a cluster of DE genes involved in the Wnt pathway (11 genes; *celsr2*, *tnik*, *gata3*, *lrp6*, *myoc*, *calcoco1*, etc.) were observed. Eight of these 11 genes were down-modulated. The Wnt signaling pathway plays a major role in cell-cell signaling, cell proliferation, and migration (Chang et al., 2003; Niehrs and Acebron, 2012). Down-modulation of the Wnt signaling pathway suggests cell proliferation may be suppressed or slowed down. Thus, stress induced by 500–550 nm exposure may be suppressing cell proliferation in the *Xiphophorus* skin, in contrast to 350–400 nm exposure where we found up-regulated DE genes involved with promotion of cell proliferation.

4.4 Shared genetic response at both 350–400 nm and 500–550 nm regions

It has been reported that circadian rhythm regulates cell cycle control and response to DNA damage (Granda et al., 2005; Gery et al., 2006; Kondratov and Antoch, 2007).

ConsensusPathDB identified two main nodes (one at each wavelength region) centered on *atm* (at 350–400 nm) and *atr* (at 500–550 nm; Figure 5). Both ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) initiate a cascade of events in response to stress to either repair damaged DNA or initiate apoptosis (for reviews, see Tibbetts et al., 1999; Abraham, 2001; Liu et al., 2007; Malewicz and Perlmann, 2014). Contrary to the up-regulation of *atm* and *atr* in these cases, our data show a down-modulation of both *atm* and *atr*. However, transcriptional activation by *atm* and *atr* may occur rapidly and by the 6 hours post exposure, down-modulation of *atm* and *atr* may be due to a feedback inhibition while the targets of these transcription activators remain up-regulated. To test this, further studies will need to be performed to assess the time course of gene regulation after exposure to specific wavelengths of light. Moreover, recent studies indicate that *atm* and *atr* co-regulate cell proliferation with p14^{arf}, a secondary tumor suppressor found in human and mouse cells (Carlos et al., 2013; Velimezi et al., 2013). The *X. maculatus* genome does not encode this *arf* homolog. Nevertheless, we do not exclude the idea that fish may have evolved a similar tumor suppressor that has yet to be identified.

The cellular stress response observed at both peak wavelength regions suggests 350–400 nm and 500–550 nm light may be inducing cellular damage. However, cell proliferation appears to be occurring after exposure to 350–400 nm light, as suggested by the overall up-modulation of genes related to cell proliferation (Table 3). Conversely, skin exposed to 500–550 nm light is shown to have a different and more robust stress response, exhibiting down-modulation of Wnt pathway genes, and up-regulation of genes involved with cytoskeletal remodeling (Table 4). It has been previously reported that actin plays a role in apoptosis and that reception of apoptotic signals in the cell induces changes in the actin cytoskeleton (Desouza et al., 2012). Future studies may assess skin exposed to 500–550 nm light for cellular stress induced apoptosis.

Collectively, our data indicate 350–400 nm and 500–550 nm light exposures induce a greater differentially expressed gene response compared to other 50 nm wavelengths between 300–600 nm. Within these two peak regions, cell cycle regulators were observed affected, most notably *atm* and *atr*. Cell cycle progression within the 350–400 nm region, despite exposure to UVA light, appears to be continuing and is consistent with other reports (He et al., 2008).

In addition, p53 activation is implicated in the 500–550 nm region by the up-modulation of p53 target genes as well as the up-modulation of heat shock proteins and fatty acid oxidation genes. The genetic effects observed within the 500–550 nm exposure suggest production of a cell based stress response that includes cytoskeletal remodeling and possible apoptosis. In a recent report of differential gene expression, *Xiphophorus* skin after exposure to “cool white” fluorescent light, a robust suppression of genes involved in cell cycle progression was noted in Walter et al., 2015. A cellular stress response indicated in this region may contribute to the suppression of the cell cycle as was observed for fluorescent light, but clearly cell cycle suppression observed with fluorescent light is due to exposure of more than one 50 nm region (Walter et al, 2015). Based on results presented herein, the genes involved in this response may be reacting to large fractions of radiance in longer

wavelengths around 500 nm and above emitted by standard fluorescent lights. This remains to be examined in greater detail.

Although the global genetic effects of UV and fluorescent lights have been studied (Yang et al., 2014; Boswell et al., 2015; Lu et al., 2015; Walter et al., 2015), these studies employed broad spectrum light sources. With the increasing prevalent use of broad spectrum artificial lighting, these reports collectively began the elucidation of the role light may play in altering global gene expression profiles. The results presented here show *Xiphophorus* skin is responsive to specific wavelength regions within the broader spectrum from 300 to 600 nm. Given that all vertebrates evolved under the full spectrum of the sun, it is probable that cells have been tuned to cooperatively use a combination of wavelengths to maintain cell homeostasis. Thus, it is potentially impacting that spectra from light sources such as “cool white” fluorescent lamps exhibit many sharp peaks across an otherwise low emitting broad spectrum background. Herein we show the wavelength position of such peaks and valleys may indeed have direct effect on gene expression and thus biological function. Although this study focuses on the effects of single 50 nm wavelength regions, combinatorial effects upon co-exposure to different wavelength regions may be an interesting avenue for future investigation.”

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

X. maculatus skin exposed to 350–400 or 500–550 nm light shows higher differentially expressed genes compared to other wavelength regions. Bar and line graphs represent the number of genes that were ± 2 -fold (p -adj = 0.05) up-modulated (blue bars) and down-modulated (red bars) in adult male *X. maculatus* skin at each 50 nm wavelength region from 300–600 nm. The black line represents the total number of differentially expressed genes at each wavelength. Differentially expressed genes were determined by RNA-Seq methods from two biological replicates at each wavelength region. Two wavelength regions were observed to exhibit the greatest number of modulated genes (350–400 nm and 500–550 nm) with a decreased number of modulated genes in between (400–500 nm).

Figure 2.

Light responsive genes involved in circadian rhythm confirms changes in gene expression when exposed to various regions of light. Shown is a line graph representation of circadian rhythm genes with a ± 2 -fold (p -adj ≤ 0.05) in at least one wavelength region in adult male *X. maculatus* skin. Fold change was determined by RNA-Seq transcriptome analysis of two biological replicates at each wavelength region through comparison of exposed fish to basal expression of unexposed fish. Expression of light responsive genes was consistent with previous reports (Yang et al., 2014; Walter et al., 2015).

Figure 3.

Validation of RNA-Seq (blue) gene expression changes by comparison to two independent methods, NanoString (red) and qRT-PCR (green) in *X. maculatus* skin. Fold changes determined by both methods represent 2 biological replicates. NanoString samples were normalized to standard NanoString probes and a set of 10 housekeeping genes (Table S1). Gene counts were collected for each target gene and compared to basal levels of unexposed fish samples to determine fold change. All qRT-PCR samples were normalized to an 18S rRNA internal standard. mRNA expression of target genes was used in a ratio comparison to expression of unexposed fish to determine fold change. qRT-PCR data was tabulated from 2 biological replicates and 3 technical replicates for each sample at each exposure treatment. Y-axis has been adjusted to fit the data of each gene. List of primer sequences used for qRT-PCR can be found in Table S2. Gene comparisons of RNA-Seq and NanoString: (A) *ppp1r27*, (B) *ybx2*, (C) *clock*, and (D) *per2*. Gene comparisons of all three independent methods: (E) *tgm8*, (F) *dnah7*, and (G) *klhl38b*. Gene comparison of RNA-Seq and qRT-PCR: (H) *atm*, (I) *per1b*. Relative gene modulation trends were observed to be similar across all independent methods.

Figure 4.

Comparison of differentially expressed genes between different wavelength regions based on RNA-Seq analysis. Multi-venn diagram comparison of ± 2 -fold ($p\text{-adj} = 0.05$) differentially expressed genes between (A) 300–450 nm and (B) 450–600 nm wavelength regions. Diagram was generated through Bioinformatics Evolutionary Genomics. Differentially expressed genes were determined by RNA-Seq. (C) Line graph of fold change response for genes shared between 350–400 and 500–550 nm regions across wavelength regions from 300 nm to 600 nm. List of genes and description of shared genes are shown in Table 2.

Figure 5.

Protein and genetic associations of differentially expressed genes (± 2 -fold) of male *X. maculatus* skin exposed to (A) 350–400 nm (14 genes) and (B) 500–550 nm light (57 genes). Network was created through ConsensusPathDB (CPDB; Max Planck Institute for Molecular Genetics) gene analysis software. A total of (A) 88 and (B) 276 genes were queried and the following associations were made: protein (orange), genetic (blue), and biochemical (green) interactions. Dotted lines represent a physical interaction, solid arrows show substrate/product interaction, and solid lines with a circle represent enzyme interaction. Symbols correlate to different GO categories (triangle = metabolic process, diamond = cell proliferation, square = cytoskeletal/plasma membrane, star = muscle differentiation, pentagon = stress, and hexagon = transport) while the color of each symbol indicates fold change scaled to the heat map colors. Boxes in blue and purple represent predicted genes and proteins CPDB used to make associations. Heat maps of up-modulated (red) and down-modulated (green) genes are categorized by GO categories as well as their fold change to their respective wavelength region. Within the 350–400 nm region, genes were clustered into cell proliferation (3 genes), transport (4 genes), metabolic process (5 genes), cytoskeletal (2 genes). DE genes shown in the 500–550 nm region were grouped into cell proliferation (9 genes), metabolic process (31 genes), cytoskeletal (1 gene), muscle differentiation (12 genes), and stress (4 genes).

Table 1

Read depth and RNA-Seq statistics for wavelength specific exposures in male skin samples. All values were generated using Samtools flagstat (Li et al., 2009).

Wavelength Treatment (nm)	Reads Mapped (M)	Reads Unmapped (M)	% Mapped	Average Coverage (X)
0 – A	37.7	30.9	55.0	60.6
0 – B	33.9	26.5	56.1	54.6
300–350 – A	28.4	22.5	55.9	47.2
300–350 – B	26.2	21.2	55.3	43.8
350–400 – A	31.8	24.9	56.1	52.5
350–400 – B	26.2	20.9	55.6	44.0
400–450 – A	27.4	21.8	55.6	46.5
400–450 – B	28.2	22.6	55.5	47.5
450–500 – A	34.1	25.4	57.4	44.9
450–500 – B	34.1	25.9	56.8	57.4
500–550 – A	29.8	34.5	46.3	39.9
500–550 – B	34.7	26.6	56.2	56.0
550–600 – A	29.3	22.4	56.7	47.1
550–600 – B	84.1	60.4	58.2	93.4

M = Million

Differentially expressed genes shared between 350–400 and 500–550 nm regions. Presented are the Ensembl ID, their respective gene name, description, and their fold change expression in 350–400 and 500–550 nm regions. Description of genes includes what function and pathway each gene belonged to. Besides circadian genes, all other genes did not readily cluster into functional categories.

Table 2

Ensembl ID	Gene Name	Fold Change		Description
		350–400	500–550	
ENSXMAT00000018306	<i>iqsec2</i>	-2.70	-2.47	IQ motif and Sec7 domain 2 Cytoskeletal and Synaptic Organization
ENSXMAT000000004015	<i>per3</i>	-2.49	-3.67	period homolog 3 Circadian Rhythm
ENSXMAT000000009017	<i>bhlhe40</i>	-2.13	-2.24	basic helix-loop-helix family, member e40 Circadian Rhythm
ENSXMAT00000015322	<i>meml50b</i>	6.44	3.59	transmembrane protein 150B Damage-Regulated Autophagy Modulator
ENSXMAT00000001424	<i>camk1gb</i>	4.68	4.72	calcium/calmodulin-dependent protein kinase Igb Calcium Signal Cascade
ENSXMAT000000007789	<i>ppargc1a</i>	4.43	5.92	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha Metabolic Reprogramming
ENSXMAT00000015040	<i>emppl</i>	4.36	3.05	ethanolamine-phosphate phospho-lyase Generate ammonia, phosphate, and acetylaldehyde
ENSXMAT000000013238	<i>nr4a3</i>	4.30	2.93	nuclear receptor subfamily 4, group A, member 3 Transcriptional Activator of NBRE
ENSXMAT000000015051	<i>ostc</i>	4.24	3.52	oligosaccharyltransferase complex subunit
ENSXMAT000000002377	<i>esyt2</i>	2.75	3.57	extended synaptotagmin-like protein 2 Ca++ Regulated Membrane
ENSXMAT000000007180	<i>cxc112b</i>	2.10	2.47	chemokine (C-X-C motif) ligand 12b (stromal cell-derived factor 1) Chemoattractant of T-cells and monocytes

Table 3

GO terms and p-values (cutoff p-value = 0.05) of gene clusters at 350–400 nm by comparison with the human genome database. Listed are the 78 Human homolog ID's that were converted from *Xiphophorus* transcript ID's. Among the 78 genes, 67 genes were clustered into 5 major categories: circadian rhythm (6 genes, p-value = 5.99e-05), epithelium development (13 genes, p-value = 6.10e-04), actin cytoskeleton organization (7 genes, p-value = 6.23e-03), transport (26 genes, p-value = 3.07e-02), and cellular metabolic process (53 genes, p-value = 2.47e-02). The remaining 11 genes did not readily cluster into any GO category. In addition, Akt/Wnt signaling groups are not GO terms that were listed, thus they do not have a GO ID or p-value. These genes were manually grouped through literature. GO categories that were similar in gene set and function were grouped into a broader category shown on the left. GO ID for each GO term and the number of DE genes (count) that fall within each cluster are presented. Gene names and fold change associated with a particular GO term are listed. Genes listed may overlap into different GO categories due to multiple functions.

	GOBPID	p-value	Count	GO Term	Gene Name
Circadian	GO:0007623	5.99E-05	6	Circadian Rhythm	<i>ppargc1a</i> (4.43), <i>arntl</i> (2.70), <i>bhlhe40</i> (-2.13), <i>per3</i> (-2.49), <i>per2</i> (2.58), <i>clock</i> (2.99)
	GO:0060429	6.10E-04	13	Epithelium Development	<i>acer1</i> (2.63), <i>evpl</i> (2.81, 2.34), <i>anxa1</i> (2.22), <i>arntl</i> (2.70), <i>atm</i> (-2.99), <i>cycl12</i> (2.10), <i>tgml</i> (2.14), <i>tgml3</i> (5.36), <i>err</i> (2.24), <i>ca2</i> (2.83), <i>far4</i> (-2.62), <i>tgml5</i> (5.36), <i>clock</i> (2.99)
Cell Proliferation	GO:0042127	3.69E-02	11	Regulation of Cell Proliferation	<i>sgk2</i> (2.33), <i>ppargc1a</i> (4.43), <i>ctbp2</i> (-2.02), <i>anxa1</i> (2.22), <i>atm</i> (-2.99), <i>pdgfc</i> (-2.35), <i>rab25</i> (2.75), <i>cycl12</i> (2.10), <i>wink2</i> (-2.72), <i>capn1</i> (2.84), <i>per2</i> (2.58)
Cytoskeletal	GO:0030036	6.23E-03	7	Actin Cytoskeleton Organization	<i>limch1</i> (-2.44), <i>iqsec2</i> (-2.70), <i>fmm1</i> (2.17), <i>epb41l4b</i> (2.56), <i>cycl12</i> (2.10), <i>ezr</i> (2.24), <i>myh14</i> (2.30)
Transport	GO:0006810	3.07E-02	26	Transport	<i>sgk2</i> (2.33), <i>ppargc1a</i> (4.43), <i>ap1s3</i> (2.33), <i>ldtrap1</i> (2.89), <i>anxa1</i> (2.22), <i>tmem173</i> (-2.46), <i>tipr1</i> (2.32), <i>cal13</i> (2.83), <i>arntl</i> (2.70), <i>myo5b</i> (2.33), <i>bapry</i> (2.15), <i>rab25</i> (2.75), <i>rhbg</i> (3.45), <i>esyt2</i> (2.75), <i>cycl12</i> (2.10), <i>wink2</i> (-2.72), <i>snca</i> (2.83), <i>ezr</i> (2.24), <i>cal1</i> (2.83), <i>ca2</i> (2.83), <i>ca3</i> (2.83), <i>prss12</i> (2.16), <i>per2</i> (2.58), <i>cltc3</i> (2.11), <i>clock</i> (2.99), <i>osbp12</i> (2.02)
	GO:0044237	2.47E-02	53	Cellular metabolic process	<i>sgk2</i> (2.33), <i>ppargc1a</i> (4.43), <i>galnt5</i> (2.04), <i>tgml7</i> (5.36), <i>acer1</i> (2.63), <i>samd8</i> (2.16), <i>samd11</i> (2.97), <i>ctbp2</i> (-2.02), <i>mlkl</i> (3.71), <i>epb42</i> (2.56), <i>evpl</i> (2.81, 2.34), <i>faah</i> (-2.45), <i>iqsec2</i> (-2.70), <i>galnt3</i> (2.47), <i>hacl1</i> (-2.41), <i>ldtrap1</i> (2.89), <i>glde</i> (2.34), <i>gna15</i> (3.22), <i>anxa1</i> (2.22), <i>tmem173</i> (-2.46), <i>idh2</i> (2.04), <i>tgml6</i> (5.36), <i>tipr1</i> (2.32), <i>cal13</i> (2.83), <i>arntl</i> (2.70), <i>atm</i> (-2.99), <i>ybx2</i> (2.47), <i>hsd17b12</i> (2.24), <i>pdgfc</i> (-2.35), <i>camk1g</i> (4.68), <i>ostc</i> (4.24), <i>gripnat1</i> (2.34), <i>nadk</i> (2.01), <i>wink2</i> (-2.72), <i>snca</i> (2.83), <i>srms</i> (2.12), <i>tgml</i> (2.14), <i>tgml3</i> (5.36), <i>ezr</i> (2.24), <i>cal1</i> (2.83), <i>ca2</i> (2.83), <i>ca3</i> (2.83), <i>myh14</i> (2.30), <i>elov17</i> (2.38), <i>nr4a3</i> (4.30), <i>ppp1r14c</i> (2.02), <i>capn1</i> (2.84), <i>bhlhe40</i> (-2.13), <i>per3</i> (-2.49), <i>per2</i> (2.58), <i>tgml5</i> (5.36), <i>clock</i> (2.99), <i>gfp2</i> (2.11)
Metabolism	N/A	N/A	11	Not Clustered	<i>etnupl</i> (4.36), <i>evpl</i> (2.81, 2.34), <i>homer2</i> (2.10), <i>ifit44</i> (-3.84), <i>ifit44l</i> (-3.84), <i>limch1</i> (-2.44), <i>slc9a3r2</i> (2.42), <i>tlcd1</i> (2.06), <i>tlcd2</i> (2.20), <i>tmem150b</i> (6.44), <i>ENSG00000266997</i> (2.33)
	N/A	N/A	2	Akt/Wnt Signaling	<i>mlkl</i> (3.71), <i>gna15</i> (3.22)

Table 4

GO terms and p-values (cutoff p-value = 0.05) of gene clusters at 500–550 nm. Listed are the 246 Human homolog ID's that were converted from *Xiphophorus* transcript ID's. Among the 246 genes, 221 genes were clustered into 8 major categories: circadian rhythm (7 genes, p-value = 4.12e-03), cell differentiation (60 genes, p-value = 4.82e-03), regulation of growth (14 genes, p-value = 2.05e-02), muscle structure development (23 genes, p-value = 3.43e-07), cytoskeleton organization (20 genes, p-value = 2.08e-02), metabolic process (167 genes, p-value = 6.50e-03), response to unfolded proteins (8 genes, p-value = 4.49e-04), and response to stimulus (126 genes, p-value = 8.69). The remaining 25 genes did not readily cluster into any GO category. In addition, p53 interactions and DNA damage groups are not GO terms that were listed, thus they do not have a GO ID or p-value. These genes were manually grouped through literature. GO categories that were similar in gene set and function were grouped into a broader category shown on the left. GO ID for each GO term and the number of DE genes (count) that fall within each clusters are presented. Gene names and fold change associated with a particular GO term are listed. Genes listed may overlap into different GO categories due to multiple functions.

	GOBPID	p-value	Count	GO Term	Gene Name
Circadian	GO:0007623	4.12E-03	7	Circadian Rhythm	<i>ppargc1a</i> (5.92), <i>per1</i> (-5.16), <i>arntl2</i> (-2.66), <i>srebfl1</i> (-2.09), <i>bhlhe40</i> (-2.24), <i>per3</i> (-3.67), <i>nr1d2</i> (-3.54)
	GO:0030154	4.82E-03	60	Cell Differentiation	<i>spep</i> (2.41), <i>ppargc1a</i> (5.92), <i>chrb1</i> (2.69), <i>wfikn2</i> (3.12), <i>coll1a1</i> (-4.61), <i>crem</i> (3.60), <i>xirp1</i> (7.78), <i>abca1</i> (-2.16), <i>efp2</i> (2.26), <i>celsr2</i> (-4.08), <i>esrb</i> (2.60), <i>fh11</i> (2.58), <i>slirk3</i> (-2.63), <i>tnik</i> (-2.55), <i>fhna</i> (-2.20), <i>prtc2c</i> (-2.03), <i>clasp1</i> (-2.35), <i>obs1</i> (3.64), <i>gata3</i> (-3.13), <i>ankrd1</i> (4.81), <i>nmrk2</i> (5.29), <i>anpep</i> (2.04), <i>hsplb</i> (9.75), <i>hsplb8</i> (3.39), <i>myoc</i> (3.30), <i>myod1</i> (2.84), <i>agm1</i> (-2.62), <i>lrp6</i> (-2.16), <i>nmpl5</i> (-2.09), <i>myf6</i> (3.70), <i>myf6</i> (3.30), <i>myod1</i> (2.84), <i>nme1</i> (2.44), <i>nme1-nme2</i> (2.44), <i>npx1</i> (4.32), <i>rhcg</i> (-3.55), <i>fbxo40</i> (6.23), <i>cltc5</i> (3.75), <i>2.39</i> , <i>bcap29</i> (2.69), <i>mk12</i> (-2.25), <i>mk11</i> (-2.13), <i>bcl9</i> (-2.15), <i>scn1b</i> (2.45), <i>cxcl12</i> (2.47), <i>pbl1</i> (2.32), <i>wink1</i> (-2.74), <i>tpm1</i> (2.82), <i>kdm6a</i> (-2.09), <i>ppdpl</i> (5.15), <i>steap4</i> (2.11), <i>znf703</i> (-2.49), <i>kiaa1109</i> (-2.85), <i>tcap</i> (3.37), <i>prom1</i> (6.55), <i>nexn</i> (2.29), <i>lgi1</i> (2.95), <i>creb5</i> (3.88), <i>cul7</i> (-2.11), <i>nr1d2</i> (-3.54)
Cell Proliferation	GO:0040008	2.05E-02	14	Regulation of Growth	<i>esml</i> (2.35), <i>fam107a</i> (2.81), <i>fh11</i> (-2.20), <i>hsplb</i> (9.75), <i>agm1</i> (-2.62), <i>myod1</i> (2.84), <i>cltc5</i> (3.75), <i>2.39</i> , <i>cxcl12</i> (2.47), <i>kiaa1109</i> (-2.85), <i>atm</i> (-2.29), <i>wisp2</i> (2.06), <i>wisp1</i> (2.04), <i>lgi1</i> (2.95), <i>cd38</i> (3.93)
	GO:0050673	4.56E-02	8	Epithelial Cell Proliferation	<i>gata3</i> (-3.13), <i>hmox1</i> (2.36), <i>lrp6</i> (-2.16), <i>nme1</i> (2.44), <i>nme1-nme2</i> (2.44), <i>cxcl12</i> (2.47), <i>pbl1</i> (2.32), <i>znf703</i> (-2.49)
	GO:0016055	5.63E-03	11	Wnt Signaling Pathway	<i>celsr2</i> (-4.08), <i>tnik</i> (-2.55), <i>gata3</i> (-3.13), <i>lrp6</i> (-2.16), <i>myoc</i> (3.30), <i>calcoccl</i> (2.18), <i>bcl9</i> (-2.15), <i>kdm6a</i> (-2.09), <i>znf703</i> (-2.49), <i>wisp1</i> (2.04), <i>usp34</i> (-2.01)
	GO:0061061	3.43E-07	23	Muscle Structure Development	<i>spep</i> (2.41), <i>chrb1</i> (2.69), <i>wfikn2</i> (3.12), <i>xirp2</i> (3.12), <i>coll1a1</i> (-4.61), <i>xirp1</i> (7.78), <i>fh11</i> (2.58), <i>obs1</i> (3.64), <i>ankrd1</i> (4.81), <i>nmrk2</i> (5.29), <i>myoc</i> (2.27), <i>lrp6</i> (-2.16), <i>myf6</i> (3.70), <i>myod1</i> (2.84), <i>fbxo40</i> (6.23), <i>mk12</i> (-2.25), <i>mk11</i> (-2.13), <i>bcl9</i> (-2.15), <i>svil</i> (3.61), <i>tpm1</i> (2.82), <i>tcap</i> (3.37), <i>nexn</i> (2.29), <i>nr1d2</i> (-3.54)
Muscle Differentiation/Cytoskeleton	GO:0007010	2.08E-02	20	Cytoskeleton Organization	<i>tenm1</i> (-2.55), <i>xirp2</i> (3.12), <i>xirp1</i> (7.78), <i>tnik</i> (-2.55), <i>iqsec2</i> (-2.47), <i>cul9</i> (-2.11), <i>fhna</i> (-2.20), <i>clasp1</i> (-2.35), <i>obs1</i> (3.64), <i>coralc</i> (3.98), <i>ankrd1</i> (4.81), <i>myoc</i> (3.30), <i>cep192</i> (-2.06), <i>cxcl12</i> (2.47), <i>svil</i> (3.61), <i>tpm1</i> (2.82), <i>tcap</i> (3.37), <i>nexn</i> (2.29), <i>cul7</i> (-2.11)

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	GOBPID	p-value	Count	GO Term	Gene Name
Metabolism	GO:0044255	1.72E-02	20	Cellular Lipid Metabolic Process	<i>ppargc1a</i> (5.92), <i>cpt1b</i> (2.45), <i>crem</i> (3.60), <i>abca1</i> (-2.16), <i>acs1l</i> (2.78), <i>sng1</i> (-2.18), <i>lpin1</i> (2.78, 2.07), <i>ankrd1</i> (4.81), <i>gpd1</i> (2.41), <i>acer2</i> (2.25), <i>agrn</i> (-2.62), <i>mtip</i> (2.31), <i>pdx2</i> (2.46), <i>pik3c2b</i> (-2.29), <i>pigg</i> (-2.15), <i>srebf1</i> (-2.09), <i>pgap1</i> (2.08), <i>nmnr4</i> (-2.45), <i>dhrr3</i> (3.09), <i>ncor2</i> (-2.02)
	GO:0071396	1.02E-04	14	Cellular Response to Lipids	<i>ppargc1a</i> (5.92), <i>fbxo32</i> (8.15), <i>abca1</i> (-2.16), <i>esrrb</i> (2.60), <i>ankrd1</i> (4.81), <i>inc</i> (-2.55), <i>ltp6</i> (-2.16), <i>myod1</i> (2.84), <i>nmel</i> (2.44), <i>srebf1</i> (-2.09), <i>nr4a3</i> (2.93), <i>zfp703</i> (-2.49), <i>trim63</i> (7.01), <i>nrlid2</i> (-3.54)
Stress	GO:0006986	4.49E-04	8	Response to Unfolded Proteins	<i>dnajb4</i> (2.73), <i>hspb7</i> (3.41), <i>hspal1b</i> (9.75), <i>hspa11</i> (9.75), <i>hspa4</i> (2.20), <i>hspa6</i> (9.75), <i>hspa8</i> (2.59), <i>cul7</i> (-2.11)
	N/A	N/A	25	Not Clustered	<i>fam117a</i> (2.29), <i>flhrs</i> (-2.28), <i>filip1</i> (2.97), <i>gliscr1</i> (-2.04), <i>kbibd12</i> (5.04), <i>kbibd3</i> (3.63), <i>ktiaa0556</i> (-2.25), <i>klhl30</i> (4.93), <i>klhl38</i> (28.78, 3.59), <i>lnad2</i> (5.53), <i>lnad3</i> (3.46), <i>lrba</i> (-2.94), <i>lrcc2</i> (4.45), <i>lrcc30</i> (2.45), <i>mphosph9</i> (-2.17), <i>myoz3</i> (2.15), <i>prrl2</i> (-2.66), <i>smnl2</i> (2.26), <i>tmem150b</i> (2.59), <i>tmem179</i> (2.67), <i>txlib</i> (2.72), <i>ubap2</i> (-2.11), <i>ubn2</i> (-2.01), <i>vps13c</i> (-2.55), <i>vps13d</i> (-2.45)
	N/A	N/A	6	p53 Interactions	<i>hspb7</i> (3.41), <i>rrad</i> (7.29), <i>ankrd1</i> (4.81), <i>glis2</i> (3.63), <i>sesn1</i> (2.53), <i>hspa8</i> (2.59)
	N/A	N/A	3	DNA Damage	<i>tmem150b</i> (3.59), <i>zak</i> (2.58), <i>nuak1</i> (2.31)