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Dieldrin augments mTOR signaling and regulates genes associated with cardiovascular disease in the adult zebrafish heart (Danio rerio)

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List of non-standard abbreviations:

ACE, angiotensin converting enzyme

ANOVA, analysis of variance

cdkn1b, cyclin dependent kinase inhibitor 1 b

cRNA, complementary RNA

DEG, differentially expressed genes

hsc70, heat shock cognate 70 kDa

kcnh2a, potassium voltage-gated channel, subfamily H (eag-related), member 2a

kcnj11l, potassium inwardly-rectifying channel, subfamily J, member 11, like

LAMP-2A, lysosomal associated membrane protein 2 A

LC3, microtubule associate protein, light chain 3

MCOLN, mucolipin

mTOR, mechanistic target of rapamycin

myl9b, myosin, light chain 9b, regulatory

OCP , Organochlorine Pesticides

phospho-, phosphorylated

RIN, RNA integrity number

s6k, ribosomal S6 kinase, 70 kDa

SNEA, sub-network enrichment analysis

ABSTRACT:

Dieldrin is a legacy organochlorine pesticide that is persistent in the environment, despite being discontinued from use in North America since the 1970s. Some epidemiological studies suggest that exposure to dieldrin is associated with increased risks of neurodegenerative disease and breast cancer by inducing inflammatory responses in tissues as well as oxidative stress. However, the direct effects of organochlorine pesticides on the heart have not been adequately addressed to date given that these chemicals are detectable in human serum and are environmentally persistent, thus individuals may show latent adverse effects in the cardiovasculature system due to chronic, low dose exposure over time**.** Our objective was to determine whether low level exposure to dieldrin at an environmentally relevant dose results in aberrant molecular signaling in the vertebrate heart. Using transcriptomic profiling and immunoblotting, we determined the global gene and targeted protein expression response to dieldrin treatment, and show that dieldrin effects gene networks in the heart that are associated to the development of cardiovascular disease, specifically cardiac arrest and ventricular fibrillation. We report that genes regulating inflammatory responses, a significant risk factor for cardiovascular disease, are upregulated by dieldrin while transcripts related to lysosomal function are significantly downregulated. To verify these findings, proteins in these pathways were examined with immunoblotting, and our results suggest that dieldrin constitutively activates Akt/mTOR signalling and downregulates lysosomal genes, participating in autophagy. Our data demonstrate that dieldrin induces genes associated with cardiovascular dysfunction and compromised lysosomal physiology, thereby identifying a novel mechanism for pesticide-induced diseases.

Introduction

The widespread use of pesticides negatively impacts our environment, thereby rendering human health susceptible to chronic diseases, such as obesity, cancer, and neurodegeneration (Alavanja et al., 2004; Lee et al., 2014). Dieldrin, an organochlorine pesticide, is a persistent chemical that was banned from agricultural use in the 1970s and was withdrawn from residential use by the 1990s. However, due to a long half-life, dieldrin persists in the environment (Jorgenson, 2001) and has been associated to human diseases such as Alzheimer's disease, (Singh et al., 2012; Singh et al., 2013) Parkinson's disease, (Fleming et al., 1994; Weisskopf et al., 2010; Baltazar et al., 2014) and obesity (Cupul-Uicab et al., 2013). Acute (Hung et al., 2015) and chronic pesticide exposure (Sergeev and Carpenter, 2005; Kim et al., 2015) are also associated with cardiovascular disease outcomes. Notably pre-natal exposure to pesticides disrupts cardiovascular development with significant relation to congenital heart disease (Gorini et al., 2014). Thus, studies are warranted to determine the mechanisms related to pesticide-induced cardiotoxicity in both the juvenile and adult heart.

Exposure to dieldrin and several other organochlorine pesticides (OCP) have been associated to cardiovascular disease. For instance, elevated circulating levels of OCPs are associated with peripheral artery disease (Min et al., 2011), insulin resistance (Lee et al., 2011) and cardiovascular disease development (Ljunggren et al., 2014). Additionally, sub-lethal endosulfan exposure in rabbits results in myocardial apoptosis (Ozmen, 2013), while chronic lindane exposure in rats led to oxidative stress and loss of myofibril integrity (Ananya et al., 2005). At the molecular level, dieldrin exhibits toxicity by targeting mitochondria and the endoplasmic reticulum (Kitazawa et al., 2003).

Dieldrin exposure also activates Protein Kinase B (Akt) /mTOR (mechanistic target of rapamycin) pathway, a major regulator of cell growth and proliferation (Briz et al., 2011). The Akt/mTOR pathway also inhibits lysosomal autophagy (Jung et al., 2010). Autophagy, an evolutionarily conserved cellular mechanism for the lysosomal degradation of cytosolic components including proteins and organelles, is known to respond to cell stress, including exposure to environmental pollution, and failure of this response could result in proteotoxicity (Moore et al., 2006). Autophagy ensures cardiomyocyte survival by clearing toxic protein aggregates that accumulate in the cell during cardiac stress (Martinet et al., 2009). However, whether dieldrin exposure induces cardiac stress by altering autophagy is currently unknown.

We hypothesized that activation of the Akt/mTOR pathway by dieldrin suppresses lysosomal gene expression and autophagy signaling. To examine the cardiac effects of dieldrin, we used adult zebrafish (*Danio rerio*), a freshwater teleost that is a popular model for studying human diseases (Lieschke and Currie, 2007) and for screening and validating drugs and chemicals inducing cardiotoxicity (Milan et al., 2006; McGrath and Li, 2008; Liu et al., 2014). At the genetic level, approximately 70 percent of human genes have a zebrafish orthologue, and 82 percent of human genes with a known relation to morbidity have at least one zebrafish orthologue (Howe et al., 2013), thus the zebrafish is a relevant model for human disease. Here, we show that dietary dieldrin exposure is sufficient to elevate mTOR signaling and its downstream targets, reducing transcripts related to autophagy, and inducing aberrations in transcripts governing functional outcomes in the zebrafish heart.

Methods

Experimental design

Six month old zebrafish (AB strain) were fed feed mixed with dieldrin at different concentrations for 21 days. Water parameters (mean ± standard deviation) during the experiment were as follows; temperature = 25.5ºC (±1.5), dissolved oxygen = 84.1% (± 8.4), and pH = 6.54 (±0.57). Fish were housed with a photoperiod of 16 hours light and 8 hours dark. All procedures involving animals conformed to Canadian Council on Animal Care (CCAC) standards for fish handling and were approved by University of New Brunswick, Saint John animal care committee. In order to ensure fish were only exposed to dieldrin in the diet, 50 percent of the water in the tanks was changed every three days, and uneaten food and fish waste product were removed. Additionally activated carbon was added to the tanks to prevent exposure to dieldrin from the water which is effective (Bandala et al., 2006). Dieldrin was incorporated into the feed through dissolution in olive oil and then mixed with the diet, while control diet was coated with olive oil only. Analytical chemistry analysis for feed has been published elsewhere (Cowie et al., 2017). Briefly, control feed contained no detectable dieldrin whereas mean dieldrin in feed was 0.03, 0.15, 1.8 µg/g of dry-weight feed for the low, medium and high dose, respectively. The treatments were significantly different from the control group and in this manuscript; the feed concentrations are used to describe treatments. Dieldrin was also measured in the whole animals. Dieldrin in zebrafish fed the control diet was at the detection limit whereas concentration (µg dieldrin/g dry weight) in fish was 0.011 in low, 0.058 medium, and 0.47 and high treatments. The mean wet weight concentrations of dieldrin in whole zebrafish were 3.50, 17.6, and 148 ng/g wet weight

fed the 0.03, 0.15, and 1.8 µg/g doses, respectively. Both the medium and high dose treatments were significantly different than control fish after 21 days of treatment while the lowest group was not different from controls. After 21 days, the fish were euthanized using a sodium bicarbonate buffered solution of 250 mg/L Tricaine (Sigma-Aldrich, St. Louis, MO, USA) and subsequent severing of the spinal cord. Hearts were dissected from the animals, flash frozen in dry ice, and stored at -80^0 C until processed.

Expression profiling in dieldrin treated zebrafish hearts

RNA extraction from whole zebrafish hearts

Female zebrafish hearts were pooled into 15 tubes. Final sample sizes for microarray analysis were as follows: Control group (n=5), fish fed the 0.15 µg/g dose diet (n=6), and (n=4) fish fed the 1.8 µg/g dose diet. Consideration was made for keeping the pooled masses even across samples. RNA was extracted using the Qiagen RNeasy® Mini Kit (Qiagen, Toronto, Canada). The concentration of RNA was measured using the BioTek Synergy H4 and Take3 plate by assessing absorbance at 260 nm. RNA quality was evaluated using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA, Cat#: G2940CA), which produces an RNA Integrity Number (RIN). RINs greater than 7.5 were deemed acceptable for microarray analysis (Kiewe et al., 2009). The mean (±SD) RNA Integrity Number (RIN) was 9.75 (± 0.21) for female heart pools and samples ranged in RIN values from 9.4 – 10.

Sample labelling

RNA samples were labelled with Cyanine-3 using the Agilent Low Input Quick Amp Labeling Kit for one-color microarray (Cat#: 5190-2305) according to manufacturer

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instructions, starting with 45 ng of RNA input. Quantification of the labelled cRNA was performed using the Nanodrop 2000 Spectrophotometer (Nanodrop; Thermo Sciencific, Buckinghamshire, UK). Cyanine 3 dye concentration (pmol/µl), and cRNA concentration were measured for each sample. The total yield of cRNA in µg, and specific activity of Cy3 (concentration of Cy3/concentration of cRNA) were calculated to determine whether labelling and amplification was successful. Microarray analysis with Agilent 4x44k microarrays proceeded if the total yield of cRNA was greater than 1.65 µg and the specific activity of Cy3 was greater than 6 pmol per µg of cRNA; criteria met by all samples.

Microarray hybridization and analysis

Microarray hybridization followed the Agilent One-Color Microarray-Based Gene Expression Analysis protocol for Agilent 4x44k zebrafish v3 microarray slides. Following hybridization, slides were scanned with the Agilent Microarray Scanner at a resolution of 5µm. Raw expression data along with tif images were extracted by Agilent Feature Extraction Software (v10.7.3.1). All microarray data reported in this study follow established guidelines “Minimum Information About a Microarray Experiment (MIAME)” (http://www.ncbi.nlm.nih.gov/geo/info/MIAME) and are located in the Gene Expression Omnibus (GEO, Gene Ontology Consortium, 2000) (GSE87294). The arrays were quality control checked using a distribution analysis that plots the intensity distributions of each microarray slide to ensure these distributions are relatively equal. One microarray in the control group show localized intensity outliers in the center of the array and these features were manually removed from the analysis. Box plots of normalized intensity also showed that the intensity distribution was not different across slides. The

microarray data were analyzed using JMP® Genomics (v6) to identify differentially-expressed genes (DEGs) using a one-way ANOVA followed by a FDR (5%). Data were first normalized using Quantile normalization and probes falling below the detection limit of the microarrays (2.5) were assigned an intensity value = 2.5. Bioinformatic analysis (Sub-network enrichment analysis and expression targets) was performed in Pathway Studio 9.0 (Ariadne, Rockville, MD, USA, now Elsevier) using the ResNet 9.0, a mammalian database curated by Ariadne (Nikitin et al., 2003).

Gel electrophoresis and immunoblotting

Frozen whole zebrafish hearts (80 in total) were pooled into tubes on the basis of the tank they originated from while keeping sexes separate, i.e. two separate lysates (male or female) were generated from one tank (five fish), for a total of four female and four male biological replicates per treatment. The pooled hearts were homogenized in 60 µl of ice cold lysis buffer as previously described (Bartlett et al., 2016). The homogenates were allowed to settle on ice before being centrifuged for 30 minutes at 1200 g. The supernatant was aspirated from the pellet and stored in 1.5 ml centrifuge tubes at -80^0 C.

Total protein concentration in pooled heart samples was estimated using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA, Cat#: 23225). Protein samples were size fractionated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Criterion Any KD pre-cast gradient gels (Bio-Rad, Hercules, California, USA, Cat#: 567-8125) before being electroblotted onto a nitrocellulose membrane. Uniformity and effectiveness of transfer

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was assessed by Pierce reversible protein stain kit for nitrocellulose membranes (Thermo Fisher Scientific Inc., Waltham, MA, USA) and imaged with the Bio-Rad Chemidoc MP imaging system (Bio-Rad, Hercules, California, USA) before the stain was washed away. Blotted proteins were characterized using the primary antibodies: phospho-mTOR, Ser2448 (Cell Signaling #2971), phospho-Akt, Ser473 (Cell Signaling #9271), phospho-s6k, Thr389 (Cell Signaling #9205), s6k (Cell Signaling #2708), LAMP-2A (Abcam #AB18528), hsc70 (Abcam #AB2788), and Cathepsin D (Santa Cruz Biotechnology #SC-6486).Immunoblots were developed with Pierce SuperSignal West Dura Substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA, Cat#: 34075) and imaged with the Bio-Rad Chemidoc MP imaging system. Normalized signal intensity of quantified immunoblots was analysed in GraphPad Prism 6 (GraphPad, La Jolla, CA, USA). A two-way analysis of variance (ANOVA) was conducted to test effect of sex on dieldrin treatment, however the factor was not significant, nor was there an interaction. One-way ANOVA was used to determine if the groups (comprised of two male samples and two female samples) were significantly different, and if so, Dunnett's post-hoc test or unpaired two-tailed t-tests were used to compare individual groups.

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Results

Transcriptomics analysis of the zebrafish heart following dieldrin exposure

Analysis of gene expression in response to dieldrin treatment resulted in 627 differentially expressed transcripts by either the measured 1.8 µg/g dose or 0.15 µg/g dose of dieldrin treatment ($p<0.01$). Furthermore, between the 1.8 µg/g dose and the 0.15 µg/g dose of dieldrin, there were 47 transcripts differentially expressed ($p<0.01$). Transcripts significantly regulated by both doses include *myl9b* which is involved in muscle contraction, and *cdkn1b*, involved in cell cycle arrest. Furthermore, two transcripts which are associated with endosome regulation, *mucolipin-3* and *map1lc3a* were downregulated in the heart following both treatment doses of dieldrin. All microarray gene expression data can be found in supplementary appendix 1. After applying a stringent false discovery rate of 5%, one transcript in the 1.8 µg/g dieldrin treatment group, *sphingosine-1-phosphate receptor 5b*, was significantly different compared to the control group. Pathway analysis however was conducted on all the transcriptomic data as per our established methods (Cowie et al., 2017).

Subnetwork enrichment analysis (SNEA) revealed that gene networks associated with 225 diseases were altered in the heart by either the 1.8 µg/g or 0.15 µg/g dose of dieldrin, and gene networks associated with 39 diseases were altered in zebrafish heart by both doses. All SNEA for diseases can be found in supplementary appendix 2. Several of the disease networks identified by SNEA analysis pertained to cardiovascular pathophysiology, including artery calcification, rheumatic heart disease, angina pectoris and cardiac arrest (Figure 1C, Table 1). Specific genes related to cardiac arrest

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included the *fos* (FBJ murine osteosarcoma viral oncogene homolog, fold change = 4.42, p = 0.001) and *jun* (fold change = 2.04, p = 0.005) complex, two proteins which dimerize to form a transcription factor regulating cell proliferation. Furthermore, angiotensin converting enzyme (*ace*) expression, a positive regulator of blood pressure, was increased > 2 fold (fold change = 2.11, p= 0.04), while *oxytocin*, a negative regulator of blood pressure was decreased >2 fold (fold change = -2.14, p = 0.048, Figure 1A). Transcripts related to ventricular fibrillation were also identified as being altered by dieldrin. These genes included *ace*, as well as catecholamine receptor *adrenoceptor β 2ab* (fold change = 2.99, p = 0.021). Two potassium channels specific to myocytes, which regulate membrane potential, were also upregulated with dieldrin including *potassium inwardly-rectifying channel, subfamily J, member 11, like* (*kcnj11l*) was increased (fold change = 1.61, p = 0.03) along with *potassium voltage-gated channel, subfamily H (eag-related), member 2a, kcnh2a* (fold change = 1.33, p = 0.049, Figure 1B).

Dieldrin treatment alters inflammatory and lysosomal processes

Analysis of microarray results by SNEA revealed that 196 cell processes were regulated by the 1.8 µg/g dose of dieldrin while 129 cell processes were regulated by the 0.15 µg/g dose in zebrafish hearts at $p<0.05$. SNEA analysis showed that 56 cell processes were regulated by both the 0.15 µg/g and 1.8 µg/g doses. All SNEA for cell processes can be found in supplementary appendix 2. Cell processes which we determined to be related to cardiovascular metabolism (Table 2) were further investigated to identify their component genes. Processes regulating inflammatory stress were significantly upregulated by dieldrin (median change = 1.0008, p = 0.041).

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Specific genes that were altered by the 1.8 µg/g dose of dieldrin treatment include the positive mediators of inflammation: *interleukin 6* (fold change = 1.46, p = 0.034) and *TNFα-induced protein 2* (fold change = 1.24, p = 0.020). Negative regulators of inflammation were downregulated in the heart by the 1.8 µg/g dose of dieldrin such as Nuclear Factor-κB repressing factor (fold change = -1.15, p =0.047) and Nuclear Factor-κB inhibitor interacting RAS-like (fold change = -1.2, p=0.042, Figure 3A). Enrichment analysis also identified several processes related to lipid metabolism that were significantly altered in the heart by 1.8 µg/g dose of dieldrin (Figure 3). Other cell processes identified as being differentially upregulated in the heart by the 1.8 µg/g dose of dieldrin include heart muscle growth and muscle development, along with a down regulation of processes involved in myocyte function (Table 2), which taken together with the diseases identified as being differential regulated, indicate that dieldrin exposure render zebrafish cardiomyocyte susceptible to injury.

SNEA analysis also identified gene networks related to protein degradation, specifically those pertaining to lysosomal lumen acidification and autophagy. Transcripts that play a role in these processes are differentially regulated by dieldrin (Table 2). Significantly altered transcripts related to lysosomal lumen acidification include the lysosomal H+ transport proteins: *atp6v1ba*, *atp6v1e1*, and *atp6ap1*, which were all significantly ($p < 0.05$) downregulated in the heart by the 1.8 µg/g dose of dieldrin. Furthermore, two membrane proteins required for lysosomal trafficking and acidification, mucolipin1 (*mcoln1*) and mucolipin3 (*mcoln3*), are downregulated in the heart following the 1.8 µg/g dose of dieldrin (Fold change = -1.49 and -1.50, p = 0.039 and 0.004, respectively), while MCOLN3 is also downregulated in the heart by the 0.15 µg/g

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treatment (Fold change= -1.42, p= 0.005). We observed that the expression of genes involved in lysosomal autophagy were significantly downregulated in the heart in response to dieldrin and these included microtubule associated protein 1, light chain 3 A and B (*LC3A/B*). *LC3A* was decreased in the heart by both doses of dieldrin (1.8 µg/g dose: Fold change = -1.21, p = 0.009; 0.15 µg/g dose: Fold change = -1.26, p = 0.001) while *LC3B* was decreased in the fish fed 0.15 µg/g dieldrin in the feed (Fold change = -1.23, p = 0.047).

Dieldrin treatment increases mTOR signalling

Given that genes critical for lysosomal function was identified by microarray analysis as being suppressed at the transcript level by dieldrin, we more closely examined the pathway that regulates the lysosome (Akt/mTOR) at the protein level. Immunoblotting for the mechanistic target of rapamycin (mTOR) and its upstream and downstream targets revealed hyper-phosphorylation and activation of the pathway in dieldrin treated zebrafish hearts (Figure 4). Akt/protein kinase b, an upstream activator of mTOR, shows a significant ($F(3,12)=5.502$, $p = 0.013$) increase in phosphorylation at serine 473 in fish fed both the 1.8 µg/g ($p < 0.05$) and 0.15 µg/g ($p < 0.01$) treatment groups compared to control (Figure 4B). Likewise, mTOR phosphorylation was significantly ($F(3, 12)=3.517$, $p = 0.049$) increased in hearts from the 1.8 µg/g group ($p < 0.05$) compared to control (Figure 4C). The downstream target of mTOR, ribosomal s6 kinase (p70s6k) is also significantly ($F(3,12)= 4.709$, $p = 0.021$) hyper-phosphorylated in zebrafish from the 1.8 µg/g treatment group ($p < 0.01$) compared to control (Figure 4D). These results correspond to our microarray findings, which indicated that expression targets of p70S6K and p90S6K (downstream targets of mTOR), negative regulators of

autophagy, are upregulated in fish in the 1.8 µg/g treatment group (Figure 5). All expression target data can be found in supplementary appendix 2.

To test our hypothesis that dieldrin alters lysosomal function, which was suggested by the microarray analysis, we immunoblotted for autophagy related targets (Figure 6). We determined that the expression of cathepsin D, a lysosomal protease has significantly reduced protein expression ($F(3,12)=3.662$, $p=0.044$) in zebrafish from both the 1.8 µg/g and 0.15 µg/g dieldrin treatment groups compared to the control ($p<0.05$, t-test, Figure 6B). Immunoblotting for lysosomal associated membrane protein 2A (LAMP-2A) and heat shock cognate of 70 kDa (hsc70) showed that they were unchanged in fish from the dieldrin treated groups compared to the control (Figure 6C/D). However, the gene expression analysis identified macroautophagy transcripts to be differentially regulated, while we were only able to examine the expression of chaperone mediated autophagy proteins.

Discussion

The overall goal of this study was to determine if dieldrin at a low, environmentally relevant dose results in cardiac injury. To achieve this, we examined the effect of a 3 week dieldrin (model legacy OCP) exposure in hearts of zebrafish. The total body burden of dieldrin achieved by the three week feeding is comparable to what is observed in some wild fish. Dieldrin total body burden in largemouth bass after four months of exposure at a contaminated site was roughly 500 ng/g wet weight (Martyniuk et al., 2010b) compared to our study in which we saw a total body burden of ~150 ng/g wet weight after three weeks of feeding a 1.8 µg/g dose. It is more difficult to compare the concentrations achieved in our study to human health as most human data is based upon serum, breast milk, and individual tissue concentrations (Brock et al., 1998; Jorgenson, 2001; Fujii et al., 2012), while our study measured whole body burden exclusively. Another area of caution in this study is the sex-specific effect of dieldrin. Our gene expression analysis was performed on hearts from adult female zebrafish, while the western blot analysis included both male and female fish. Given that previous studies have identified a sexually dimorphic response to dieldrin (Martyniuk et al., 2013), future experiments should investigate whether the same transcriptome networks are altered in male fish.

This is the first study to reveal that gene networks associated with cardiovascular disease, along with several cellular processes related to the development of cardiac injury, are altered by three weeks of dieldrin treatment in adult zebrafish. Together these data suggest that dieldrin causes cardiomyocyte injury which is due to altered status of growth, metabolic dysregulation, and lysosomal stress.

Our first aim was to characterize the global gene expression response in female zebrafish hearts to 21 day dieldrin treatment, and use bioinformatics approaches to relate the gene expression data to cell processes and diseases. This is one of the few reports on the effect of dieldrin in the vertebrate heart. Others have reported that aldrin, dieldrin and endrin all inhibit cardiac sarcoplasmic reticulum calcium pump activity in a dose dependent manner (Mehrotra et al., 1989). Interestingly, our data also indicate that target genes regulating ion pump function are downregulated by dieldrin treatment. In the current study, we demonstrate via functional enrichment analysis of microarray data that alterations in gene networks regulating the susceptibility to cardiac arrest, ventricular fibrillation, and arteriole stiffness are associated with dieldrin exposure.

Functional enrichment analysis identified several genes which are related to ventricular fibrillation (VF) lethal form of cardiac arrhythmia. Notably *kcnj11l*, also known as Kir6.2, part of a family of potassium-ATP channels, was upregulated by dieldrin (Medeiros-Domingo et al., 2010). Interestingly, dieldrin also increased the expression of the voltage-gated potassium channel *kcnh2*. Gain of function mutations for KCNH2 result in arrhythmias such as arterial fibrillation, and short or long QT syndromes (Hong et al., 2005). Carriers of KCNH2 gain of function mutations have an increased chance of ventricular arrhythmias resulting in sudden cardiac death (Brugada et al., 2004). Furthermore, others found that downregulation of Kir6.2 resulted in protection of ischemia induced VF, (Tsuburaya et al., 2011). We observe that the 1.8 µg/g dose of dieldrin also increases genes in networks related to cellular dedifferentiation in addition to muscle development and growth, which are processes characteristic of zebrafish heart regeneration in response to injury (Jopling et al., 2010).

Preclinical evidence from mammalian cardiovascular research indicates that vasculo-muscular stiffness and injury in the heart is an outcome of excess inflammation. In our study transcripts related to inflammatory response were shown to be modulated in the zebrafish heart after 21-day treatment with dieldrin. Previous reports have suggested that acute dieldrin injection or long-term exposure activates a transcriptional inflammatory response in the largemouth bass hypothalamus (Martyniuk et al., 2010a; Martyniuk et al., 2010b). Likewise, dieldrin was able to induce pro-inflammatory cytokine production in human neutrophils, along with the activation of other neutrophil functions (Pelletier et al., 2001). Our microarray results indicate that interleukin 6 (IL-6) is upregulated in response to dieldrin treatment. Notably our data also show that *fos* and *jun* are both significantly upregulated in response to dieldrin, which combine to form activator protein 1 (AP-1), that induces muscle differentiation and proliferation (Shaulian and Karin, 2002; Moore-Carrasco et al., 2006). AP-1 overexpression is associated with cardiac hypertrophy (Herzig et al., 1997) and chronic heart failure (Frantz et al., 2003). Interestingly, *ace* is upregulated by dieldrin in our results, and angiotensin II both activates and induces expression of AP-1 (Herzig et al., 1997; van Wamel et al., 2001). ACE was also identified as a gene related to the development of arterial stiffness in zebrafish treated with the high dose of dieldrin. An inflammatory response initiated by dieldrin treatment may be another mechanism of AP-1 activation, considering proinflammatory cytokines induce expression of both FOS and JUN (Karin et al., 1997).

Downstream cellular effects of dieldrin involve changes in mitochondrial and the endoplasmic reticulum. Prolonged oxidative stress from dieldrin exposure may result in mitochondrial dysfunction and release of cytochrome C into the cytosol leading to

apoptosis (Kitazawa et al., 2003). Likewise, treatment with endosulfan, which is in the same chemical class as dieldrin, results in increased caspase-3 expression in the heart (Ozmen, 2013). In another proposed mechanism, dieldrin causes endoplasmic reticulum stress, which leads to decreases in endoplasmic reticulum associated protein degradation, resulting in proteotoxicity and cell death (Sun et al., 2005). We examined whether disruption of lysosomal function and autophagy is a plausible outcome of dieldrin exposure in zebrafish hearts. Functional enrichment analysis identified alteration in gene transcripts that regulate lysosomal lumen acidification wherein examinations of individual genes responsible for lysosomal acidification are downregulated in the heart. Our search identified that three lysosomal v-ATPases have significantly reduced expression in fish fed the high dieldrin dose. Lysosomal v-ATPase uses energy from ATP hydrolysis to pump protons into the lysosomal lumen, thereby regulating the lysosomal acidity (Forgac, 2007). Bafilomycin, a selective v-ATPase inhibitor, inhibits lysosomal acidification and protein degradation (Yoshimori et al., 1991). Further supporting this hypothesis is our finding that *mcoln1* and *mcoln3* are downregulated by dieldrin treatment. Loss of function mutations in MCOLN1 result in mucolipidosis type IV, which is characterized by a build-up of lysosomes containing lipids (Chen et al., 1998) and increased lysosomal lumen pH has also been identified (Bach et al., 1999) while MCOLN3 inhibition results in defects in lysosomal acidification and impaired autophagosome maturation (Lelouvier and Puertollano, 2011). Furthermore, our microarray data revealed that microtubule associated proteins 1, light chain 3A and B (LC3A/B), are downregulated in the heart by dieldrin treatment, which are necessary for proper autophagosome maturation (Tanida, 2011). These data

suggest that lysosomal function and cellular trafficking is disrupted by dieldrin treatment. There is only one other report on the effect of dieldrin on lysosome function, which suggest that dieldrin treatment in rat livers upregulates protease activity (Kohli et al., 1977).

Given that microarray analysis highlighted several lysosomal proteins with reduced expression, we examined the mTOR/Akt axis which is known to inhibit lysosomal autophagy. Indeed mTOR and AKT signaling were upregulated in dieldrin treated hearts. Previous reports have also described dieldrin as an Akt activator mediated by activation of the G-Protein coupled estrogen receptor (Briz et al., 2011). The mTOR pathway acts as a negative regulator of lysosomal biogenesis and autophagy gene expression through inactivation of TFEB by phosphorylation, preventing its translocation into the nucleus (Roczniak-Ferguson et al., 2012). Recent studies show that autophagy provides protection to the heart by clearing toxic protein aggregates that accumulate in the cell during cardiac stress (Martinet et al., 2009). Our data show that dieldrin blocks this process, potentially contributing to cardiac injury.

In summary, using a zebrafish model, we characterized cardiac specific pathological signalling of dieldrin. Underlying molecular changes in dieldrin exposed hearts include inflammation and disruption of lysosomal signaling. Furthermore, dieldrin regulates genes which are related to the development of cardiac arrest and ventricular fibrillation, among many other disease associations. Future work should aim to characterize cell autonomous effect of dieldrin specifically using isolated cardiomyocytes to determine the direct action of dieldrin on the cardiac cells and its physiological impact.

AUTHOR CONTRIBUTIONS

T.P., L.S., and C.M. designed the research. L.S. and A.C. conducted the experiments. T.P., C.M., and P.C.K. contributed reagents and analytical tools. L.S, A.C. and C.M. analyzed the data. P.C.K. provided intellectual inputs, technical assistance, reviewed and proof read manuscript. L.S., A.C., C.M., and T.P wrote the paper. Dr. Thomas Pulinilkunnil is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

References

Alavanja MC, Hoppin JA and Kamel F (2004) Health effects of chronic pesticide exposure: cancer and neurotoxicity. *Annu Rev Public Health* **25**:155-197.

Ananya R, Subeena S, Kumar DA, Kumar DT and Kumar MS (2005) Oxidative stress and histopathological changes in the heart following orallindane (gamma hexachlorohexane) administration in rats. *Medical science monitor* **11**:BR325-BR329.

Bach G, Chen C-S and Pagano RE (1999) Elevated lysosomal pH in Mucolipidosis type IV cells. *Clinica Chimica Acta* **280**:173-179.

Baltazar MT, Dinis-Oliveira RJ, de Lourdes Bastos M, Tsatsakis AM, Duarte JA and Carvalho F (2014) Pesticides exposure as etiological factors of Parkinson's disease and other neurodegenerative diseases--a mechanistic approach. *Toxicol Lett* **230**:85-103.

Bandala ER, Andres-Octaviano J, Pastrana P and Torres LG (2006) Removal of Aldrin, Dieldrin, Heptachlor, and Heptachlor Epoxide Using Activated Carbon and/or Pseudomonas fluorescens Free Cell Cultures. *Journal of Environmental Science and Health, Part B* **41**:553-569.

Bartlett JJ, Trivedi PC, Yeung P, Kienesberger PC and Pulinilkunnil T (2016) Doxorubicin Impairs Cardiomyocyte Viability by Suppressing Transcription Factor EB Expression and Disrupting Autophagy. *Biochem J*.

Briz V, Molina-Molina J-M, Sánchez-Redondo S, Fernández MF, Grimalt JO, Olea N, Rodríguez-Farré E and Suñol C (2011) Differential Estrogenic Effects of the

Persistent Organochlorine Pesticides Dieldrin, Endosulfan, and Lindane in Primary Neuronal Cultures. *Toxicological Sciences* **120**:413-427.

Brock JW, Melnyk LJ, Caudill SP, Needham LL and Bond AE (1998) Serum Levels of Several Organochlorine Pesticides in Farmers Correspond With Dietary Exposure and Local Use History. *Toxicology and Industrial Health* **14**:275-289.

Brugada R, Hong K, Dumaine R, Cordeiro J, Gaita F, Borggrefe M, Menendez TM, Brugada J, Pollevick GD, Wolpert C, Burashnikov E, Matsuo K, Wu YS, Guerchicoff A, Bianchi F, Giustetto C, Schimpf R, Brugada P and Antzelevitch C (2004) Sudden death associated with short-QT syndrome linked to mutations in HERG. *Circulation* **109**:30-35.

Chen C-S, Bach G and Pagano RE (1998) Abnormal transport along the lysosomal pathway in Mucolipidosis, type IV disease. *Proceedings of the National Academy of Sciences* **95**:6373-6378.

Cowie AM, Sarty KI, Mercer A, Koh J, Kidd KA and Martyniuk CJ (2017) Molecular networks related to the immune system and mitochondria are targets for the pesticide dieldrin in the zebrafish (Danio rerio) central nervous system. *Journal of Proteomics* **157**:71-82.

Cupul-Uicab LA, Klebanoff MA, Brock JW and Longnecker MP (2013) Prenatal exposure to persistent organochlorines and childhood obesity in the US collaborative perinatal project. *Environ Health Perspect* **121**:1103-1109.

Fleming L, Mann JB, Bean J, Briggle T and Sanchez-Ramos JR (1994) Parkinson's disease and brain levels of organochlorine pesticides. *Annals of Neurology* **36**:100-103.

Forgac M (2007) Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nat Rev Mol Cell Biol* **8**:917-929.

Frantz S, Fraccarollo D, Wagner H, Behr TM, Jung P, Angermann CE, Ertl G and Bauersachs J (2003) Sustained activation of nuclear factor kappa B and activator protein 1 in chronic heart failure. *Cardiovascular Research* **57**:749-756.

Fujii Y, Ito Y, Harada KH, Hitomi T, Koizumi A and Haraguchi K (2012) Comparative survey of levels of chlorinated cyclodiene pesticides in breast milk from some cities of China, Korea and Japan. *Chemosphere* **89**:452-457.

Gorini F, Chiappa E, Gargani L and Picano E (2014) Potential Effects of Environmental Chemical Contamination in Congenital Heart Disease. *Pediatr Cardiol* **35**:559-568.

Herzig TC, Jobe SM, Aoki H, Molkentin JD, Cowley AW, Jr., Izumo S and Markham BE (1997) Angiotensin II type1a receptor gene expression in the heart: AP-1 and GATA-4 participate in the response to pressure overload. *Proc Natl Acad Sci U S A* **94**:7543-7548.

Hong KUI, Bjerregaard P, Gussak I and Brugada R (2005) Short QT Syndrome and Atrial Fibrillation Caused by Mutation in KCNH2. *Journal of Cardiovascular Electrophysiology* **16**:394-396.

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch G-J, White S, Chow W, Kilian B, Quintais LT, Guerra-Assuncao JA, Zhou Y, Gu Y, Yen J, Vogel J-H, Eyre T, Redmond S, Banerjee R, Chi J, Fu B, Langley E, Maguire SF, Laird GK, Lloyd D,

Kenyon E, Donaldson S, Sehra H, Almeida-King J, Loveland J, Trevanion S, Jones M, Quail M, Willey D, Hunt A, Burton J, Sims S, McLay K, Plumb B, Davis J, Clee C, Oliver K, Clark R, Riddle C, Eliott D, Threadgold G, Harden G, Ware D, Mortimer B, Kerry G, Heath P, Phillimore B, Tracey A, Corby N, Dunn M, Johnson C, Wood J, Clark S, Pelan S, Griffiths G, Smith M, Glithero R, Howden P, Barker N, Stevens C, Harley J, Holt K, Panagiotidis G, Lovell J, Beasley H, Henderson C, Gordon D, Auger K, Wright D, Collins J, Raisen C, Dyer L, Leung K, Robertson L, Ambridge K, Leongamornlert D, McGuire S, Gilderthorp R, Griffiths C, Manthravadi D, Nichol S, Barker G, Whitehead S, Kay M, et al. (2013) The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **496**:498-503.

Hung D-Z, Yang H-J, Li Y-F, Lin C-L, Chang S-Y, Sung F-C and Tai SCW (2015) The Long-Term Effects of Organophosphates Poisoning as a Risk Factor of CVDs: A Nationwide Population-Based Cohort Study. *PLoS ONE* **10**:e0137632.

Jopling C, Sleep E, Raya M, Martí M, Raya A and Belmonte JCI (2010) Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**:606-609.

Jorgenson JL (2001) Aldrin and dieldrin: a review of research on their production, environmental deposition and fate, bioaccumulation, toxicology, and epidemiology in the United States. *Environmental health perspectives* **109**:113.

Jung CH, Ro S-H, Cao J, Otto NM and Kim D-H (2010) mTOR regulation of autophagy. *FEBS Letters* **584**:1287-1295.

Karin M, Liu Z-g and Zandi E (1997) AP-1 function and regulation. *Current Opinion in Cell Biology* **9**:240-246.

Kiewe P, Gueller S, Komor M, Stroux A, Thiel E and Hofmann W-K (2009) Prediction of qualitative outcome of oligonucleotide microarray hybridization by measurement of RNA integrity using the 2100 Bioanalyzer™ capillary electrophoresis system. *Ann Hematol* **88**:1177-1183.

Kim S-A, Kim K-S, Lee Y-M, Jacobs DR and Lee D-H (2015) Associations of organochlorine pesticides and polychlorinated biphenyls with total, cardiovascular, and cancer mortality in elders with differing fat mass. *Environmental Research* **138**:1-7.

Kitazawa M, Anantharam V and Kanthasamy AG (2003) Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cδ in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. *Neuroscience* **119**:945-964.

Kohli KK, Siddiqui FA and Venkitasubramanian TA (1977) Effect of dieldrin on the stability of lysosomes in the rat liver. *Bull Environ Contam Toxicol* **18**:617-623.

Lee D-H, Porta M, Jacobs DR and Vandenberg LN (2014) Chlorinated Persistent Organic Pollutants, Obesity, and Type 2 Diabetes. *Endocrine Reviews* **35**:557-601.

Lee D-H, Steffes MW, Sjödin A, Jones RS, Needham LL and Jacobs DR, Jr. (2011) Low Dose Organochlorine Pesticides and Polychlorinated Biphenyls Predict Obesity, Dyslipidemia, and Insulin Resistance among People Free of Diabetes. *PLoS ONE* **6**:e15977.

Lelouvier B and Puertollano R (2011) Mucolipin-3 Regulates Luminal Calcium, Acidification, and Membrane Fusion in the Endosomal Pathway. *Journal of Biological Chemistry* **286**:9826-9832.

Lieschke GJ and Currie PD (2007) Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* **8**:353-367.

Liu Y, Asnani A, Zou L, Bentley VL, Yu M, Wang Y, Dellaire G, Sarkar KS, Dai M, Chen HH, Sosnovik DE, Shin JT, Haber DA, Berman JN, Chao W and Peterson RT (2014) Visnagin protects against doxorubicin-induced cardiomyopathy through modulation of mitochondrial malate dehydrogenase. *Science Translational Medicine* **6**:266ra170.

Ljunggren SA, Helmfrid I, Salihovic S, van Bavel B, Wingren G, Lindahl M and Karlsson H (2014) Persistent organic pollutants distribution in lipoprotein fractions in relation to cardiovascular disease and cancer. *Environment International* **65**:93-99.

Martinet W, Agostinis P, Vanhoecke B, Dewaele M and de Meyer G (2009) Autophagy in disease: a double-edged sword with therapeutic potential. *Clinical science* **116**:697-712.

Martyniuk CJ, Doperalski NJ, Kroll KJ, Barber DS and Denslow ND (2013) Sexually dimorphic transcriptomic responses in the teleostean hypothalamus: a case study with the organochlorine pesticide dieldrin. *Neurotoxicology* **34**:105-117.

Martyniuk CJ, Feswick A, Spade DJ, Kroll KJ, Barber DS and Denslow ND (2010a) Effects of acute dieldrin exposure on neurotransmitters and global gene

transcription in largemouth bass (Micropterus salmoides) hypothalamus. *NeuroToxicology* **31**:356-366.

Martyniuk CJ, Kroll KJ, Doperalski NJ, Barber DS and Denslow ND (2010b) Genomic and Proteomic Responses to Environmentally Relevant Exposures to Dieldrin: Indicators of Neurodegeneration? *Toxicological Sciences* **117**:190-199.

McGrath P and Li C-Q (2008) Zebrafish: a predictive model for assessing drug-induced toxicity. *Drug Discovery Today* **13**:394-401.

Medeiros-Domingo A, Tan B-H, Crotti L, Tester DJ, Eckhardt L, Cuoretti A, Kroboth SL, Song C, Zhou Q, Kopp D, Schwartz PJ, Makielski JC and Ackerman MJ (2010) Gain-of-Function Mutation, S422L, in the KCNJ8-Encoded Cardiac K(ATP) Channel Kir6.1 as a Pathogenic Substrate for J Wave Syndromes. *Heart rhythm : the official journal of the Heart Rhythm Society* **7**:1466-1471.

Mehrotra BD, Moorthy KS, Ravichandra Reddy S and Desaiah D (1989) Effects of cyclodiene compounds on calcium pump activity in rat brain and heart. *Toxicology* **54**:17-29.

Milan DJ, Jones IL, Ellinor PT and MacRae CA (2006) In vivo recording of adult zebrafish electrocardiogram and assessment of drug-induced QT prolongation. *American Journal of Physiology - Heart and Circulatory Physiology* **291**:H269-H273.

Min JY, Cho JS, Lee KJ, Park JB, Park SG, Kim JY and Min KB (2011) Potential role for organochlorine pesticides in the prevalence of peripheral arterial diseases in obese persons: results from the National Health and Nutrition Examination Survey 1999-2004. *Atherosclerosis* **218**:200-206.

Moore-Carrasco R, García-Martínez C, Busquets S, Ametller E, Barreiro E, López-Soriano FJ and Argilés JM (2006) The AP-1/CJUN signaling cascade is involved in muscle differentiation: Implications in muscle wasting during cancer cachexia. *FEBS Letters* **580**:691-696.

Moore MN, Allen JI and Somerfield PJ (2006) Autophagy: Role in surviving environmental stress. *Marine Environmental Research* **62, Supplement 1**:S420-S425.

Nikitin A, Egorov S, Daraselia N and Mazo I (2003) Pathway studio—the analysis and navigation of molecular networks. *Bioinformatics* **19**:2155-2157.

Ozmen O (2013) Cardiotoxicity and Apoptotic Activity in Subacute Endosulfan Toxicity and the Protective Effect of Vitamin C in Rabbits: A Pathological Study. **32**:53-58.

Pelletier M, Roberge CJ, Gauthier M, Vandal K, Tessier PA and Girard D (2001) Activation of human neutrophils in vitro and dieldrin-induced neutrophilic inflammation in vivo. *Journal of Leukocyte Biology* **70**:367-373.

Roczniak-Ferguson A, Petit CS, Froehlich F, Qian S, Ky J, Angarola B, Walther TC and Ferguson SM (2012) The Transcription Factor TFEB Links mTORC1 Signaling to Transcriptional Control of Lysosome Homeostasis. *Science signaling* **5**:ra42-ra42.

Sergeev AV and Carpenter DO (2005) Hospitalization Rates for Coronary Heart Disease in Relation to Residence near Areas Contaminated with Persistent Organic Pollutants and Other Pollutants. *Environmental Health Perspectives* **113**:756-761.

Shaulian E and Karin M (2002) AP-1 as a regulator of cell life and death. *Nat Cell Biol* **4**:E131-E136.

Singh N, Chhillar N, Banerjee B, Bala K, Basu M and Mustafa M (2013) Organochlorine pesticide levels and risk of Alzheimer's disease in north Indian population. *Hum Exp Toxicol* **32**:24-30.

Singh NK, Chhillar N, Banerjee BD, Bala K, Mukherjee AK, Mustafa MD and Mitrabasu (2012) Gene-environment interaction in Alzheimer's disease. *Am J Alzheimers Dis Other Demen* **27**:496-503.

Sun F, Anantharam V, Latchoumycandane C, Kanthasamy A and Kanthasamy AG (2005) Dieldrin Induces Ubiquitin-Proteasome Dysfunction in α-Synuclein Overexpressing Dopaminergic Neuronal Cells and Enhances Susceptibility to Apoptotic Cell Death. *Journal of Pharmacology and Experimental Therapeutics* **315**:69-79.

Tanida I (2011) Autophagosome formation and molecular mechanism of autophagy. *Antioxid Redox Signal* **14**:2201-2214.

Tsuburaya R, Yasuda S, Ito Y, Shiroto T, Gao JY, Ito K and Shimokawa H (2011) Eicosapentaenoic acid reduces ischemic ventricular fibrillation via altering monophasic action potential in pigs. *Journal of Molecular and Cellular Cardiology* **51**:329-336.

van Wamel AET, Ruwhof C, van der Valk-Kokshoorn LJM, Schriern P and van der Laarse A (2001) The role of angiotensin II, endothelin-1 and transforming growth factor-β as autocrine/paracrine mediators of stretch-induced cardiomyocyte hypertrophy. *Mol Cell Biochem* **218**:113-124.

Weisskopf MG, Knekt P, O'Reilly EJ, Lyytinen J, Reunanen A, Laden F, Altshul L and Ascherio A (2010) Persistent organochlorine pesticides in serum and risk of Parkinson disease. *Neurology* **74**:1055-1061.

Yoshimori T, Yamamoto A, Moriyama Y, Futai M and Tashiro Y (1991) Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *Journal of Biological Chemistry* **266**:17707-17712.

FOOTNOTES

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Figure Legends

Figure 1. Mean log2 intensity plus standard error of the mean (SEM) of genes related to diseases as identified by gene expression microarrays and sub-network enrichment analysis in females zebrafish hearts treated with a 1.8 µg/g (black bars) or 0.15 µg/g (gray bars) dose of dieldrin or vehicle control (Veh, white bars) for 21 days, with *p < 0.05, # p <0.01 compared to the control. A) Gene regulating cardiac arrest separated into positive and negative regulators B) Genes positively regulating ventricular fibrillation. C) Expression changes of genes related to cardiovascular diseases which were differentially expressed by the 1.8 µg/g dose of dieldrin in the adult zebrafish heart. Red signifies upregulation, blue signifies downregulation, definitions of abbreviations can be found in supplementary appendix 3.

Figure 2: Mean log2 intensity plus standard error of the mean (SEM) for genes related to cell processes as identified by gene expression microarrays and sub-network enrichment analysis in females zebrafish hearts treated with a 1.8 µg/g (black bars) or 0.15 µg/g (gray bars) dose of dieldrin or vehicle control (Veh, white bars) for 21 days, with *p < 0.05, # p <0.01 compared to the contro. A) Genes regulating inflammation separated into positive and negative regulators B) Gene regulating lysosomal function and acidification.

Figure 3. Expression changes of genes related to lipid metabolism which were differentially expressed by the 1.8 µg/g dose of dieldrin in the adult zebrafish heart. Red

signifies upregulation, blue signifies downregulation, definitions of abbreviations can be found in supplementary appendix 3.

Figure 4: Immunoblot analysis of Akt/mTOR pathway protein phosphorylation in pooled zebrafish hearts treated with three different concentrations of dieldrin or the control for 21 days. *p<0.05, #p<0.01 A) Immunoblot images of protein targets in the four different concentrations, along with protein stain loading control separated by treatment group and sex (F: Female, M: Male) B) phospho-Akt (Ser473) immunoblot quantification after correction to protein stain C) phospho-mTOR (Ser2448) immunoblot quantification after correction to protein stain D) quantification of phospho-p70s6k immunoblot by correcting the quantity to unphosphorylated p70s6k, mean densitometric units plus SEM, n = 4 per treatment group.

Figure 5. Expression targets of p90RSK (Ribosomal S6 kinase, 90 Kilodaltons) and p70RSK (Ribosomal S6 kinase, 70 Kilodaltons) which were differentially expressed by the 1.8 µg/g dose of dieldrin in the adult zebrafish heart. Red signifies upregulation, blue signifies downregulation, definitions of abbreviations can be found in supplementary appendix 3.

Figure 6: Immunoblot analysis of autophagy proteins in pooled zebrafish hearts treated with four different concentrations of dieldrin (control, 0.03, 0.15, 1.8 µg/g feed) for 21 days. *p<0.05 A) Immunoblot images of protein targets in the four different concentrations, along with protein stain loading control separated by treatment group

and sex (F: Female, M: Male) B) Cathepsin D immunoblot quantification after correction to protein stain C) LAMP-2A immunoblot quantification after correction to protein stain D) HSC70 immunoblot quantification after correction to protein stain, graphs represent mean densitometric units plus SEM, n = 4 per treatment group.

Tables

Table 1. Diseases identified with sub-network enrichment analysis from transcriptomics of female zebrafish. Hearts from zebrafish treated with the 1.8 µg/g or 0.15 µg/g doses of dieldrin compared to control after 21 days which are relevant to cardiovascular disease and cardiac physiology. Shown are the number of genes in the network, number of measured genes on the zebrafish platform, median fold change of the network, and p-value.

Name	Total Neighbors	# of Measured Neighbors	Median change	p-value
Cardiovascular diseases regulated by the high dose				
Arterial stiffness	32	23	1.04	0.003
Heart Arrest	45	36	1.16	0.009
Aortic Diseases	44	26	1.25	0.013
Rheumatic Heart Disease	11	9	1.38	0.013
Artery rhythm	10	7	-1.17	0.017
Angina Pectoris	28	22	-1.11	0.019
Weight Gain	179	136	1.02	0.039
Cardiogenic Shock	7	5	-1.31	0.042
Diabetes Mellitus	20	12	1.11	0.043
Cardiovascular diseases regulated by the medium dose				
Ventricular Fibrillation	43	30	1.17	0.006
Venous Thromboembolism	16	15	1.02	0.034
Carotid Artery Diseases	18	14	1.04	0.034

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Table 2. Cell processes identified with sub-network enrichment analysis from transcriptomics of female zebrafish hearts treated with the 1.8 µg/g or 0.15 µg/g doses of dieldrin compared to control after 21 days.

Name	Total Neighbors	# of Measured Neighbors	Median fold change		p-value	
			1.8 µg/g	**0.15 µg/g**	**1.8 µg/g**	**0.15 µg/g**
Cell processes regulated by both 1.8 and 0.15 µg/g doses						
Endocrine changes	15	14	1.3	-1.15	0.0059	0.0048
Muscle metabolism	37	36	-1.19	-1.15	0.0079	0.031
Lipid modification	12	9	-1.2	-1.22	0.0085	0.0067
Lipid export	93	67	-1.07	-1.07	0.021	0.0012
Lipoprotein metabolism	103	67	1.01	-1.03	0.025	0.042
Cell processes regulated by 1.8 µg dieldrin/g food						
Heart muscle growth	5	5	1.5	-	0.0039	-
Muscle development	178	148	1.01	-	0.0065	-
Myocyte function	93	74	-1.01	-	0.0071	-
Fatty acids import	84	64	-1.07	-	0.014	-
Hormone metabolism	30	18	1.2	-	0.018	-
Lysosomal lumen acidification	38	28	1.08	-	0.027	-
Cell dedifferentiation	47	31	1.10	-	0.03	-
Inflammatory response	1087	703	1.001	-	0.041	-
Cell processes regulated by 0.15 µg dieldrin/g food						
fatty acid metabolism	156	118	-	1.06	-	0.0075
vasculature development	20	15	-	1.03	-	0.016
lipid metabolism	470	345	-	1.02	-	0.034
artery remodeling	80	61	-	-1.09	-	0.043

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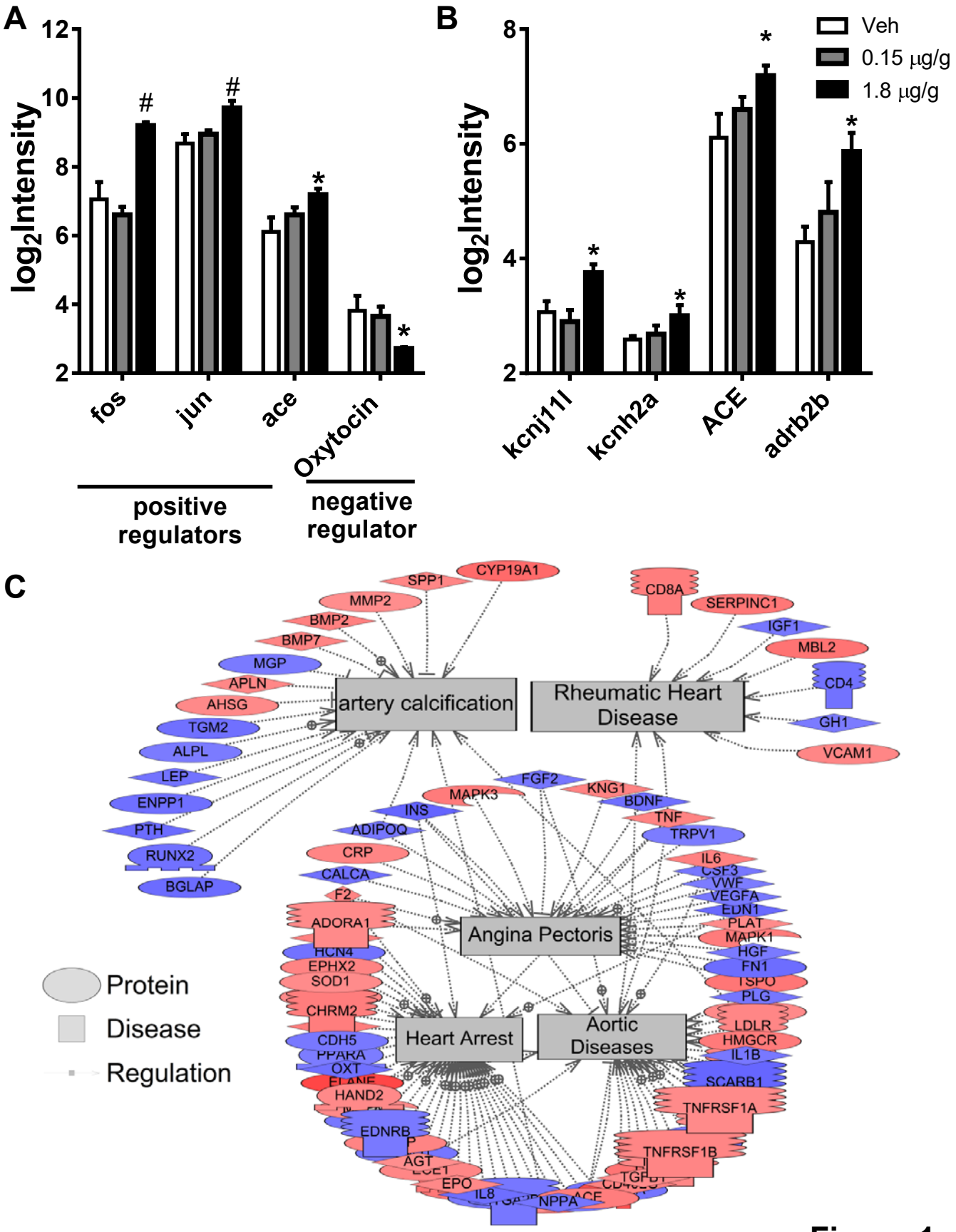

Figure 1

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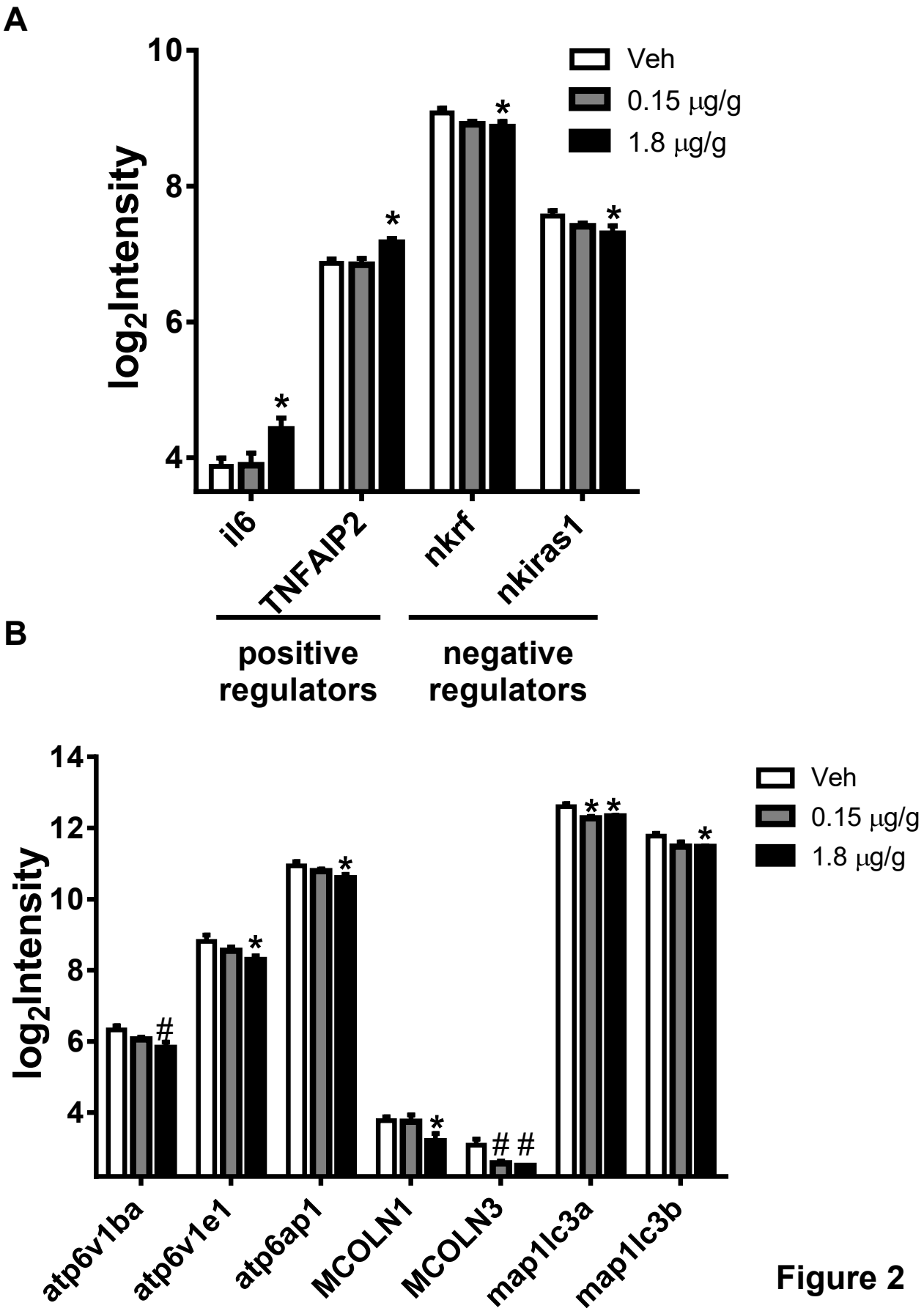

Figure 2

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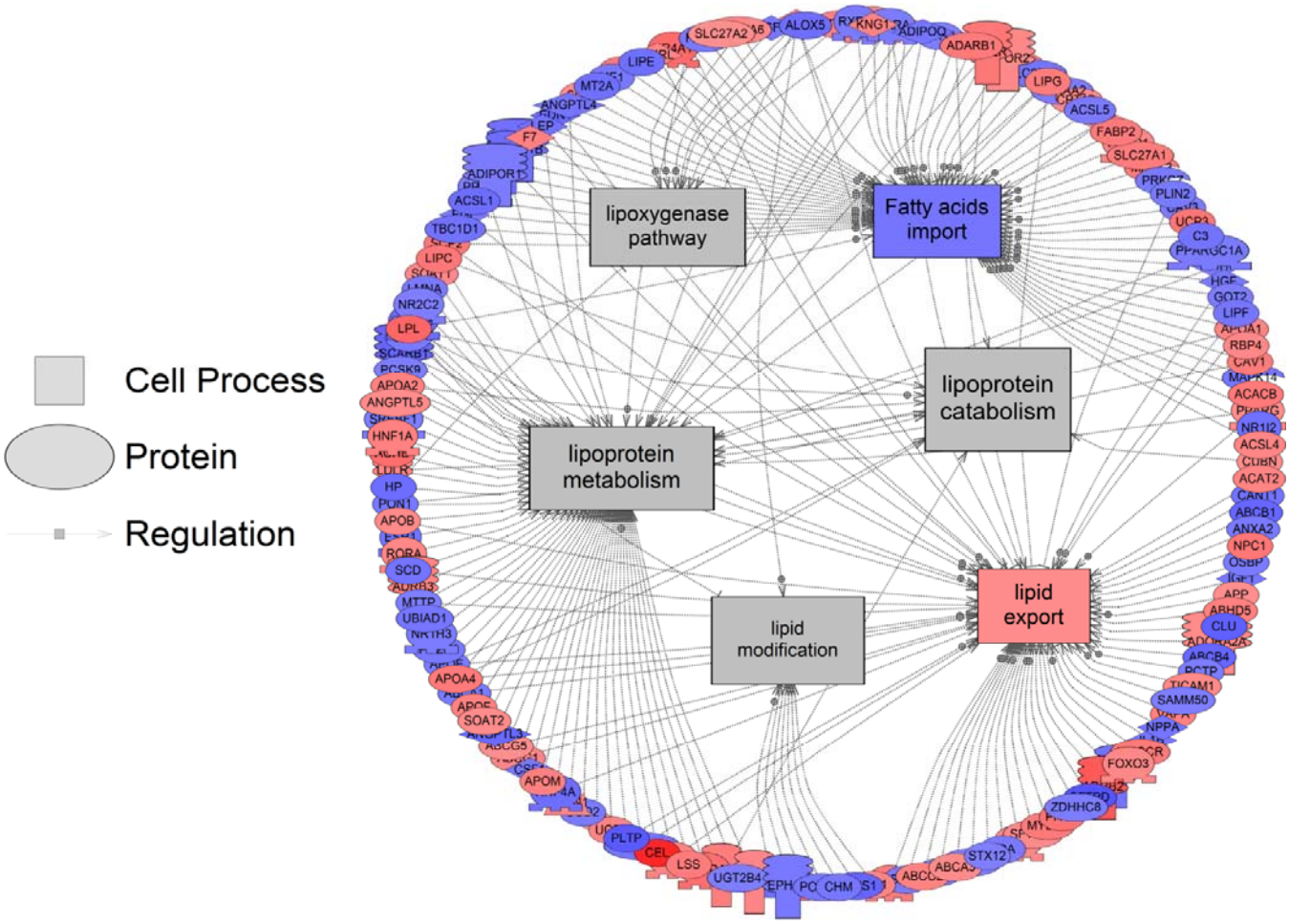

Figure 3

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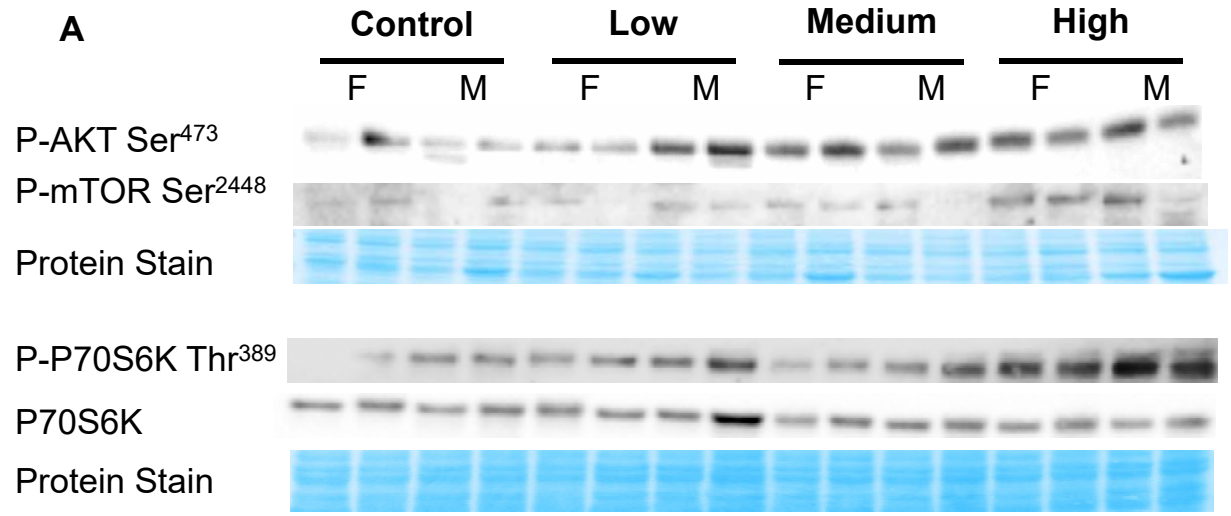

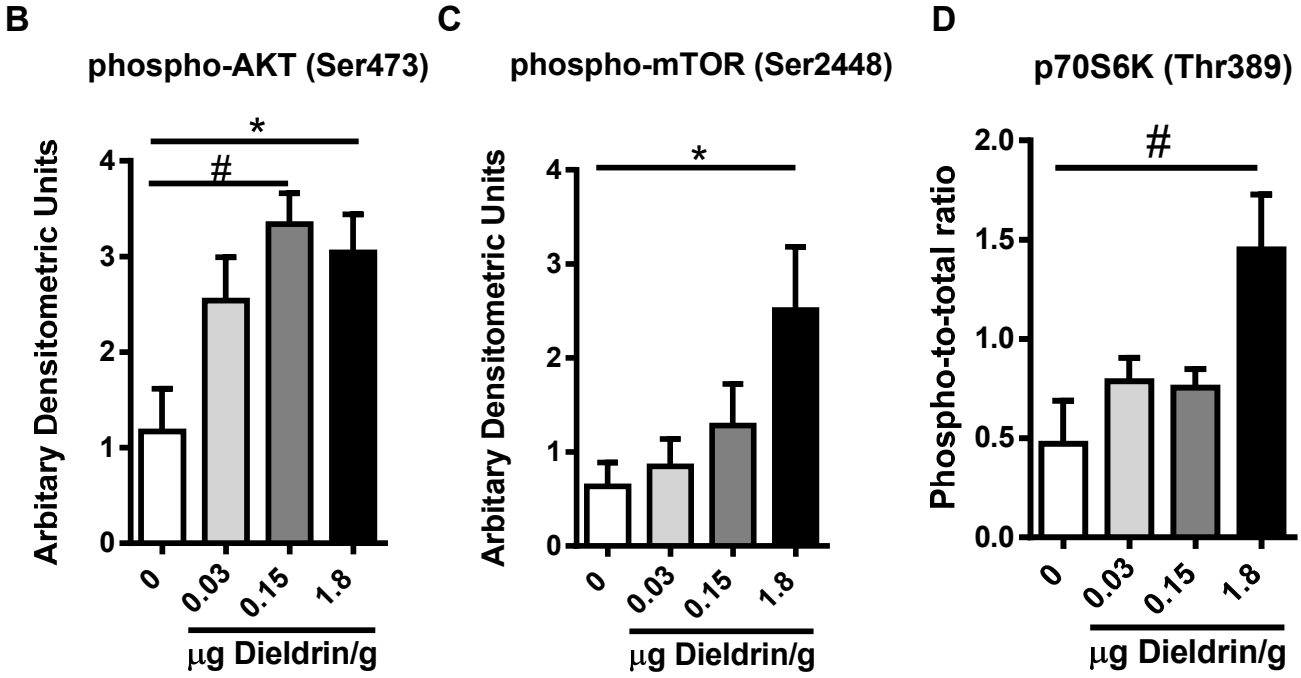

Figure 4

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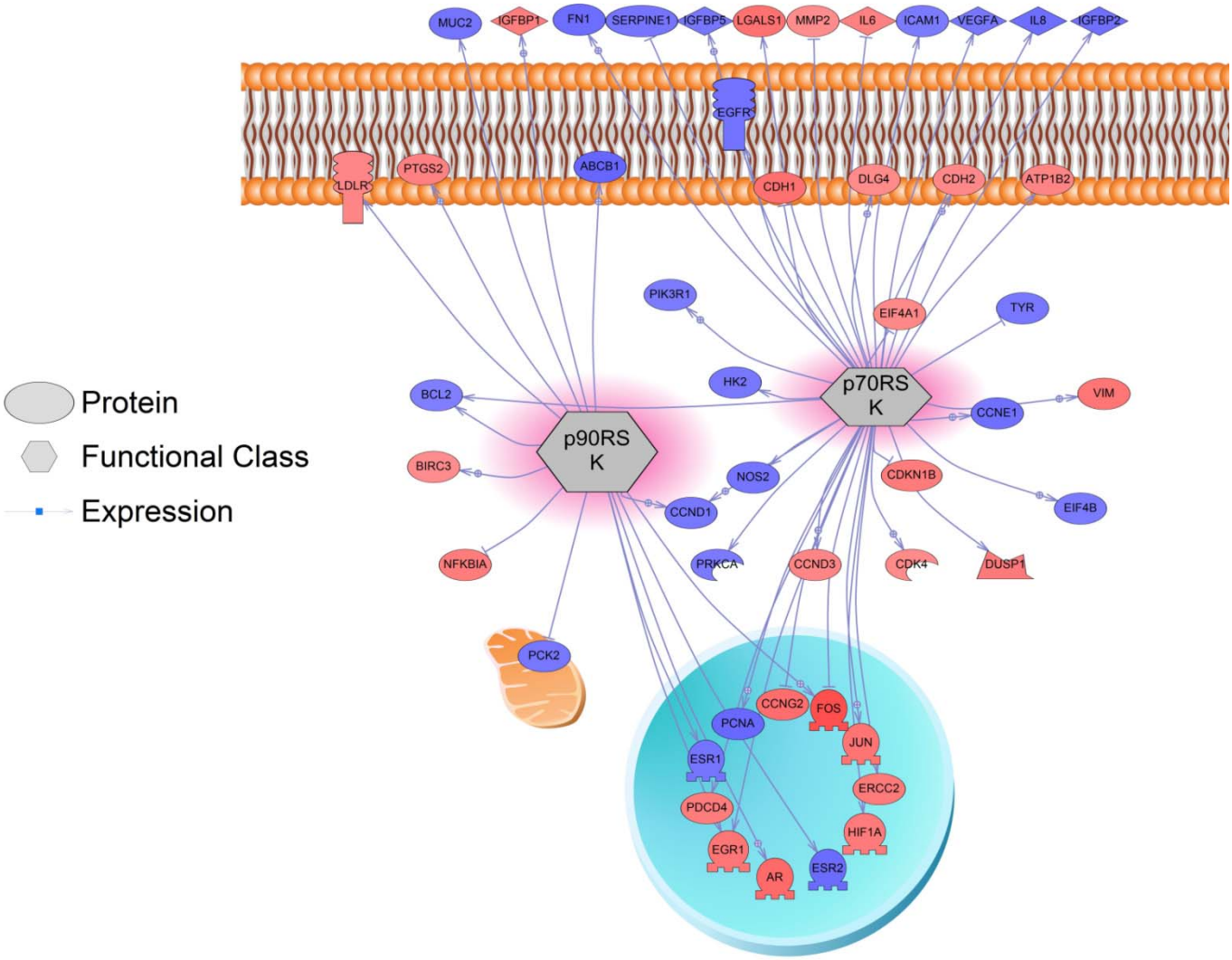

Figure 5

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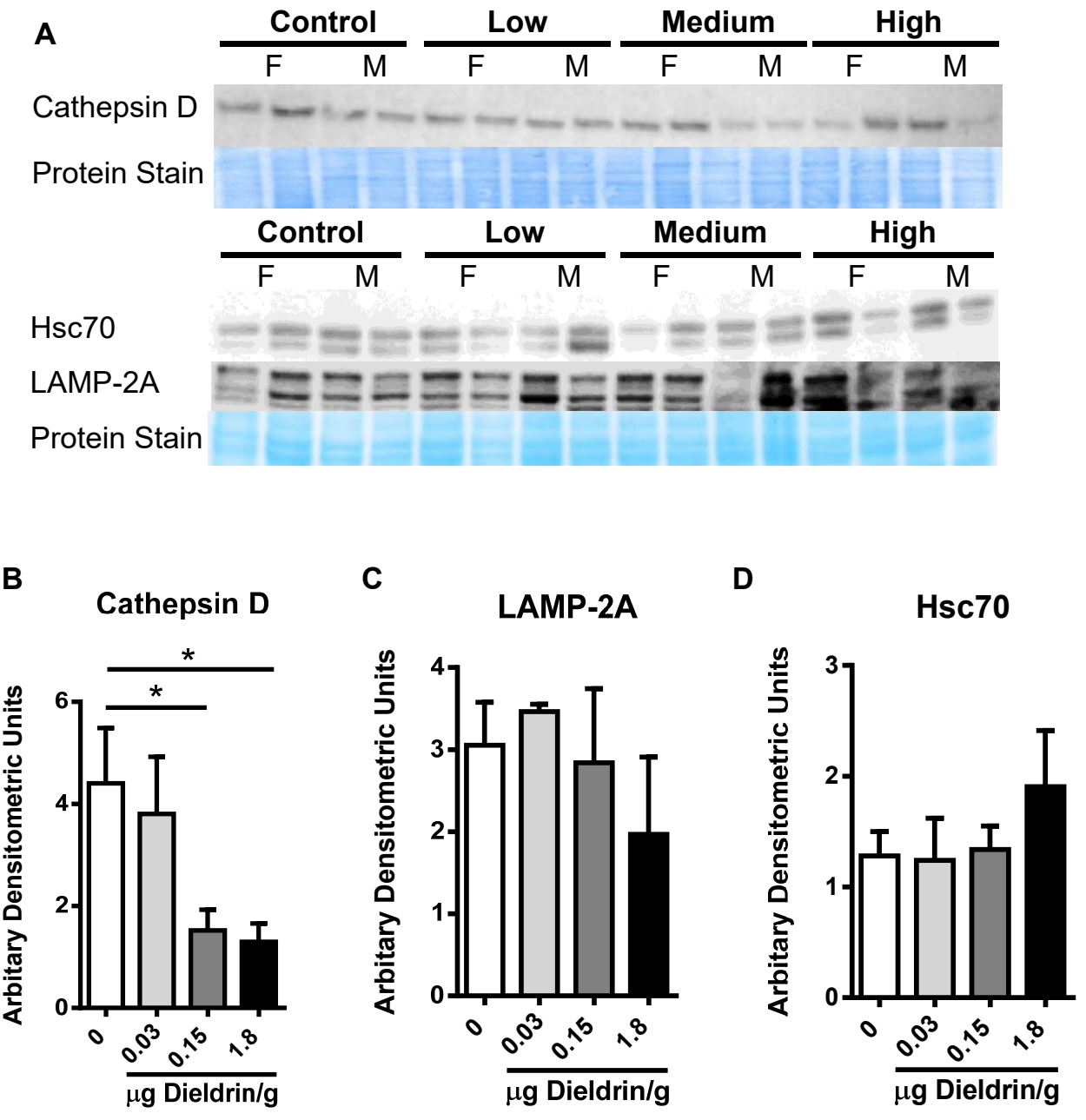

Figure 6