The expression of essential selenoproteins during zebrafish development requires SECIS binding protein 2-like.

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Abstract

The dietary requirement for selenium is based on its incorporation into selenoproteins, which contain the amino acid selenocysteine (Sec). The Sec insertion sequence (SECIS) is an RNA structure found in the 3' UTR of all selenoprotein mRNAs, and it is required to convert in-frame UGA codons from termination to Sec-incorporating codons. There are two proteins that bind to SECIS elements, but only one, SECIS binding protein 2 (Sbp2), has been shown to be required for Sec incorporation. The Sbp2 paralogue, SECIS binding protein 2-like (Secisbp2l) is conserved in all vertebrates and shares many features with Sbp2, but its function is unknown. Here we set out to determine the relative roles of Sbp2 and Secisbp2l by introducing CRISPR mutations in both genes in zebrafish. By monitoring selenoprotein synthesis with ⁷⁵Se labeling during embryogenesis, we found that *sbp2*^{-/-} embryos still make a select subset of selenoproteins but *secisbp2l*^{-/-} embryos retain the full complement. Abrogation of both genes completely prevents selenoprotein synthesis and juveniles die at 14 days post fertilization. Embryos lacking Sbp2 are sensitive to oxidative stress and express the stress marker *vtg1*. We propose a model where Secisbp2l is required to promote essential selenoprotein synthesis during stress.

Introduction

Selenium is an essential trace element that is incorporated as the amino acid selenocysteine (Sec) into ~20 - 50 vertebrate proteins known collectively as selenoproteins. This class of proteins serve a variety of disparate functions, for example resolving oxidative stress (the glutathione peroxidases) and hormone synthesis (iodothyronine deiodinases). Since Sec is encoded by in-frame UGA codons that would otherwise be interpreted as termination codons, a dedicated set of signals and factors are required to allow Sec incorporation. In every selenoprotein mRNA there is a stem-loop feature in the 3' UTR, the Sec insertion sequence (SECIS), that autonomously converts upstream in-frame UGA codons to specify Sec. In addition, a SECIS binding protein (Sbp2) binds specifically to the SECIS element and recruits the ternary complex consisting of the Sec-specific translation elongation factor (Eefsec), Sec-tRNA^{Sec} and GTP. These factors are known to be sufficient to allow Sec incorporation in a plant in vitro translation system, which is otherwise devoid of selenoprotein related factors (Gupta et al. 2013).

Although Sbp2 is known to be necessary and sufficient for Sec incorporation in vitro, all vertebrates possess a paralogous gene called *secisbp2l*, which also binds to all SECIS elements, but attempts to reveal Sec incorporation activity in vitro and in mammalian cells have failed to reveal a potential role for Secisbp2l (Donovan and Copeland 2012). Since Secisbp2l is preferentially expressed in epithelial cells (Kapushesky et al. 2010) it might be expected to play a role in responding to external stimuli. Additionally, Secisbp2l expression is strongly positively correlated with protection from lung adenocarcinoma, which underscores the importance of determining function (McKay et al. 2017).

Elimination of SBP2 in mice resulted in lethality during gastrulation (Seeher and Schweizer 2014), and subsequent studies showed that this block in development could be overcome by expressing a single selenoprotein (GPX4) with a Cys residue substituted for Sec (Ingold et al. 2018). Additionally the muscle-specific selenoprotein SELENON was reported to be required for normal calcium flux in zebrafish embryos. Despite these efforts, very little is known about the role of selenoproteins during development, so here we have used the zebrafish model system to determine the relative roles of Secisbp2l and Sbp2, thereby establishing a highly tractable system in which to study the mechanism of Sec incorporation and the consequences of selenoprotein deficiency in vivo. The largest selenoproteomes exist in bony fishes, and zebrafish has been reported to possess 38 selenoprotein genes, albeit with several duplications (Mariotti et al. 2012). Considering the accessibility of developmental biology in the zebrafish system and the relative paucity of literature about the role of selenoproteins during vertebrate development, a lot can be learned by leveraging the tractability of this system to answer key questions surrounding selenoprotein synthesis and function.

In this report we have used CRISPR/Cas9 methodology to generate zebrafish lacking either Sbp2 or Secisbp2l. Although no overt phenotypes were observed, we found that most but not all selenoprotein production was substantially reduced in the *sbp2*^{-/-} larvae, and they were significantly more sensitive to oxidative stress. *Secisbp2l*^{-/-} animals showed slightly reduced selenoprotein production, which was completely inhibited and led to death at 14 days post fertilization when both genes were ablated.

Methods

Zebrafish Husbandry

All fish were maintained in a flow-through system with a light/dark cycle of 14h/10h according to standard procedures (Mullins et al. 1994). Embryos and larvae (0 to 5) dpf were grown at 28°C in embryonic medium (0.005% Instant Ocean® and .1% Methylene Blue). We performed all experiments involving fish according to animal protocols that were approved by Rutgers, The State University of New Jersey.

Generation of Secisbp2l and Sbp2 Knockout Zebrafish

Ablation of the *secisbp2l* gene was performed by Knudra, now InVivo Biosystems (Eugene, OR). An *in-vitro* assembled CRISPR Cas9 RNP complex (consisting of a single guide RNA (Table 1), and recombinant Cas9) was microinjected into TU zebrafish embryos at the 1 cell stage. Injected embryos were shipped to Rutgers and raised to the juvenile stage prior to genotyping (see below). Ablation of the *sbp2* gene was performed in-house via microinjection into EK zebrafish embryos at the 1-cell stage using an *in-vitro* assembled CRISPR Cas9 RNP complex (consisting of a crRNA, tracrRNA, and recombinant Cas9). Alt-R® CRISPR-Cas9 crRNA (IDT) and Alt-R® CRISPR-Cas9 tracrRNA, ATTO™ 550 (IDT) were diluted to a final concentration of 3 µM each in IDTE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0), heated to 95°C for 5 minutes, and then cooled to room temperature. In a separate tube, Alt-R® S.p. Cas9 Nuclease V3 (IDT) was diluted to a final concentration of 1.5 µg/µL in Cas 9 working buffer (20 mM HEPES, 150 mM KCl, pH 7.5). Equal volumes of each solution were incubated together at

37°C for 10 minutes before being cooled to room temperature then injected into 1-cell embryos (Wierson et al. 2019).

Generation of Secisbp2l and Sbp2 Double Knockout Zebrafish

Ablation of both *sbp2* and *sbp2L* gene was performed via microinjection into *sbp2* Knockout zebrafish embryos at the 1-cell stage using an *in-vitro* assembled CRISPR Cas9 RNP complex (consisting of a crRNA, tracrRNA, and recombinant Cas9). Alt-R® CRISPR-Cas9 crRNA (IDT) and Alt-R® CRISPR-Cas9 tracrRNA, ATTO™ 550 (IDT) were diluted to a final concentration of 3 µM each in IDTE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0), heated to 95°C for 5 minutes, and then cooled to room temperature. In a separate tube, Alt-R® S.p. Cas9 Nuclease V3 (IDT) was diluted to a final concentration of 1.5 µg/µL in Cas 9 working buffer (20 mM HEPES, 150 mM KCl, pH 7.5). Equal volumes of each solution were incubated together at 37°C for 10 minutes before being cooled to room temperature then injected into 1-cell embryos (Wierson et al. 2019).

Genotyping

For genotyping, DNA was extracted from juvenile tail fragments in 1X PCR buffer (10mM Tris HCl pH 8.3, 50mMKCl, 0.3% IGEPAL, 0.3% Tween 20), which were lysed at 95°C for 10 minutes. Samples were cooled to 55°C and PCR grade Proteinase K (Roche) was added to a concentration of 1 ug/ul, and samples were incubated for 16 hrs at 55°C. Proteinase K was then heat inactivated for 10 minutes at 95°C and a fragment surrounding the target site was generated by PCR. PCR products were gel purified using the PureLink™ Quick Gel Extraction Kit (Invitrogen) and sequenced. F0 adults with detectable editing were crossed to wild type EK zebrafish adults. F1 generation fish that had frame-shift mutations were isolated and an allele

with a 26bp exonic insertion in the *secisbp2l* gene was chosen for further analysis, while a 5bp exonic deletion in the *sbp2* gene was chosen. F1 interbreeding created F2 fish homozygous knockout, heterozygous, and wild type siblings for each allele. F2 fish homozygous for the 26bp allele (*secisbp2l*^{-/-}) and homozygous wt (*secisbp2l*^{+/+}) for the *secisbp2l* gene were used in experiments. In the case of SBP2, F2 fish homozygous for the 5bp allele (*sbp2*^{-/-}) and homozygous wt (*sbp2*^{+/+}) for the *sbp2* gene were used for all experiments in this report. The primers used for genotyping single and double knockout animals are shown in Table 1.

RNA Extraction and qRT-PCR Analysis

Pools of at least 15 embryos were euthanized and placed in a 1.5 ml conical tube and all embryo medium was withdrawn. Embryos were then immediately flash frozen on dry ice. Samples were then thawed on ice and the manufacturer's protocol for solid sample TRIzol Reagent (Ambion) extraction was followed. To remove genomic DNA, RNA cleanup protocol of RNeasy Mini Kit (Qiagen) with DNase treatment was used. cDNA was generated from total RNA utilizing the Superscript IV Reverse Transcriptase (Thermo Fisher Scientific) following manufacturer's protocol using poly(A) priming and 200 ng of total RNA. 10 ng/rxn of cDNA was then used in qRT-PCR using PowerSYBR™ Green PCR Master Mix (Applied Biosystems) with QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific). The total reaction volume was 20 µl with 5 µl of 1:5 diluted RT reaction. Working concentration of the primers in the reaction was 0.25 µM. Thermal cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 1min. Melt curve analysis was performed for each sample to ensure a single amplification product. Samples were analyzed in triplicate for both the reference gene and the target gene. Quantitation was performed using the comparative $\Delta\Delta C_t$ method. We used

eef1a as the normalizer and the calibrator sample was from each wild type background. Primers in the acceptable efficiency range (90-110%) were determined using the standard curve method.

secisbp2l and sbp2 cDNA cloning

Coding sequence for *sbp2* was PCR amplified from 0-5 dpf pooled cDNA using the forward and reverse primers shown in Table 1 and then TA cloned into pcDNA3.1 V5/His Topo (Thermo Fisher Scientific). For *secisbp2l*, most of the predicted coding region was amplified as overlapping fragments from 0-5 dpf pooled cDNA. Specifically the sequence obtained corresponded to the start of exon 2 (22 nt downstream from the predicted start) to the stop codon. The 5' end of the cDNA was added as synthesized fragment based on a multiple sequence alignment with other fish sequences.

Metabolic labeling with ^{75}Se

Embryos were placed in multiwell plates at a density of 83 μl per embryo in labeling medium (embryo medium containing 0.5% DMSO (v/v) and 375 nM ^{75}Se [~ 500 Ci/g; University of Missouri Reactor]). Embryos were incubated 28°C for 24hrs. After incubation, embryos were transferred to wash vessels at a density of about 10 ml per embryo for 10 min. 4 wash cycles were sufficient to remove background ^{75}Se label. Embryos were immediately frozen on dry ice and stored at -80 °C until used for protein extraction.

Protein Extraction of Zebrafish Embryos

Frozen aliquots of 1-4 fish were thawed on ice and 15 to 30 μl of Extraction Buffer (63 mM Tris-HCl (pH6.8), 10% glycerol (v/v), 3.5% SDS (w/v) and 5.0% BME (v/v) was added to the embryos before the fish were thoroughly homogenized with a pestle attached to a power drill

run at approximately 700 RPM. The samples were kept on ice while 3 10-sec pulses were delivered, separated by 10 second resting intervals. Samples were briefly centrifuged then heated to 85°C for 10 min and vortexed at room temperature halfway through heating. Samples were centrifuged for 20 minutes at 14000g and the supernatant was isolated. Bromophenol blue was added to a concentration of 0.1% (w/v) to samples. Samples were then analyzed by SDS-PAGE and the gels dried and exposed to PhosphorImager screens (GE Healthcare). Total protein was monitored by Coomassie Blue Staining.

Immunoblotting

SDS-PAGE was performed on lysates and transferred to nitrocellulose via wet transfer (Bio-Rad). SECISBP2 Polyclonal antibody (12798-1-AP; ProteinTech) was used at a 1:1000 dilution, and Beta Tubulin Monoclonal antibody (66240-1-Ig; ProteinTech) at 1:20000 dilution. Horseradish peroxidase-conjugated anti-rabbit IgG secondary (65-6120; Invitrogen) was used at a dilution of 1:40000. Blots were developed using the SuperSignal West Femto kit (Pierce) or fluorescence imaging according to the manufacturer's protocol with Amersham Imager 600 (GE Healthcare).

Hydrogen Peroxide Treatment

For the toxicity assay, 0 dpf embryos were transferred to 10 cm petri dishes containing 40 ml of labeling media either with or without 2 mM H₂O₂ (Sigma-Aldrich) at a density of 1000ul of embryo medium/embryo and kept at 28°C. Toxicity was assessed every 24 hours under a light microscope, checking for the evidence of a heartbeat. Embryos were removed from the dish when heartbeat was not detected, and medium was changed every 24 hours from the time of initial treatment. For labeling during peroxide treatment, 2-4 dpf embryos were transferred to a

12-well plate containing 1 ml of labeling media with or without 0.5 mM H₂O₂ per well. After 24 hours of treatment, larvae were washed then flash frozen on dry ice and processed for SDS-PAGE and phosphorimage analysis as described above.

Quantitative Mass Spectrometry

4 dpf larval extracts were prepared (4 larvae per tube) as described above. Mass spectrometry was performed at the Biological Mass Spectrometry facility at Rutgers Robert Wood Johnson Medical School. The entire sample was loaded onto an SDS-PAGE gel and run just enough to enter the gel. The gel was stained with Coomassie blue and all stained material was recovered as a gel slice. The gel slices were incubated at 60°C for 30 min with 10 mM DTT. After cooling to room temperature, 20 mM iodoacetamide were added and kept in the dark for 1 hour to block free cysteine. The samples were digested by trypsin at 1:50 (w:w, trypsin:sample) and incubated at 37°C overnight. The digested peptides were extracted and dried under vacuum and washed with 50% acetonitrile to pH neutral. The digested peptides were labeled with Thermo TMTpro (Lot #: UI292951) followed by the manufacturer's protocol. Labeled samples were pooled at 1:1 ratio for a small volume and analyzed with LC-MS/MS to get normalization factor. The labeling efficiency is 97.8%. The labeled samples were then pooled at 1:1 ratio for all the channels. The pooled samples were dried and desalted with SPEC C18 (Varian, WAT054960). The desalted samples were fractionated using Agilent 1100 series. The samples were solubilized in 200 µl of 20 mM ammonium formate (pH10), and injected onto an Xbridge column (Waters, C18 3.5 µm 2.1X150 mm) using a linear gradient of 1%B/min from 2-45% of B (buffer A: 20 mM ammonium, pH 10, B: 20 mM ammonium in 90% acetonitrile, pH10). 1 min fractions were collected and dried.

Nano-LC-MSMS was performed using a Dionex rapid-separation liquid chromatography system interfaced with a Eclipse (Thermo Fisher Scientific).). Selected desalted fractions 28-45 were loaded onto a Acclaim PepMap 100 trap column (75 μ m x 2 cm, ThermoFisher) and washed with Buffer A (0.1% trifluoroacetic acid) for 5 min with a flow rate of 5 μ L/min. The trap was brought in-line with the nano analytical column (nanoEase, MZ peptide BEH C18, 130A, 1.7 μ m, 75 μ m x 20cm, Waters) with flow rate of 300 nL/min with a multi step gradient (4% to 15% buffer B [0.16% formic acid and 80% acetonitrile] in 20 min, then 15%–25% B in 40 min, followed by 25%–50% B in 30 min).

The scan sequence began with an MS1 spectrum (Orbitrap analysis, resolution 120,000, scan range from 350–1600 Th, automatic gain control (AGC) target 1E6, maximum injection time 100 ms). For SPS3, MSMS analysis consisted of collision-induced dissociation (CID), quadrupole ion trap analysis, automatic gain control (AGC) 2E4, NCE (normalized collision energy) 35, maximum injection time 55ms), and isolation window at 0.7. Following acquisition of each MS2 spectrum, we collected an MS3 spectrum in which 10 MS2 fragment ions are captured in the MS3 precursor population using isolation waveforms with multiple frequency notches. MS3 precursors were fragmented by HCD and analyzed using the Orbitrap (NCE 55, AGC 1.5E5, maximum injection time 150 ms, resolution was 50,000 at 400 Th scan range 100-500). The whole cycle is repeated for 3 sec before repeat from an MS1 spectrum. Dynamic exclusion of 1 repeat and duration of 60 sec was used to reduce the repeat sampling of peptides.

LC-MSMS data were analyzed with Proteome Discoverer 2.4 (ThermoFisher) with a sequence search engine against uniprot human and a database consisting of common lab contaminants. The MS mass tolerance was set at +/- 10 ppm, MSMS mass tolerance was set at +/- 0.4Da. TMTpro on K and N-terminus of peptides and carbamidomethyl on cysteine was set as static

modification. Methionine oxidation, protein N-terminal acetylation, N-terminal methionine loss or N-terminal methionine loss plus acetylation were set as dynamic modifications. Percolator was used for results validation. Concatenated reverse database was used for target-Decoy strategy. High confidence for protein and peptides were defined as $FDR < 0.01$, medium confidence was defined as $FDR < 0.05$.

For reporter ion quantification, reporter abundance was set to use signal/noise ratio (S/N) if all spectrum files have S/N values. Otherwise, intensities were used. Quan value was corrected for isotopic impurity of reporter ions. Co-isolation threshold was set at 50% . Average reporter S/N threshold was set at 10. SPS mass matches % threshold was set at 65%. Protein abundance of each channel was calculated using summed S/N of all unique+razor peptides. The abundance was further normalized to the summed abundance value for each channel over all peptides identified within a file.

Protein abundance ratio was calculated based on protein abundance. Pairwise comparison was done using t-test. P-value was further corrected by Benjamini-Hochberg method for false discovery rate. Volcano plots were generated by VolcanoseR (Goedhart and Luijsterburg 2020) and gene ontology was performed by the Molecular and Genomics Informatics Core at Rutgers New Jersey Medical School.

Results

sbp2 and *secisbp2l* genes

The RefSeq RNA entry for zebrafish *secisbp2l* has a 2721 nt coding region and is missing several segments relative to other fish species such as goldfish (*Carassius auratus*), which has a coding region of 3012 nt. Using total RNA purified from zebrafish embryos grown to 5 days post fertilization (dpf), we attempted to clone the cDNA corresponding to zebrafish *secisbp2l*, but we were not able to obtain any single products corresponding to the full length sequence. However, we were able to clone overlapping fragments to obtain a candidate full length sequence with a 3085 nt coding region that is 85% identical to the goldfish sequence (see methods). The case of *sbp2* is very similar where the RefSeq RNA entry lacked the N-terminal portion of the mRNA relative to other species (Figure 1). The only annotated sequence that contains a predicted sequence upstream of the conserved domains was also found in goldfish. However, the *secisbp2* gene in goldfish is annotated as LOC113080603 and the corresponding mRNAs are incorrectly annotated as *secisbp2l*. Interestingly, the N-terminal ~80 amino acids of the predicted goldfish Sbp2 are only found in the Clupeocephala supercohort of teleost fish, which includes the cyprinidae (zebrafish and goldfish). The N-terminal sequence that was previously found in both Sbp2 and Secisbp2l in most species (Donovan and Copeland 2009) is only present in Clupeocephala Secisbp2l but not in Sbp2. Again using total RNA purified from larvae grown to 5 days and primers corresponding to the regions annotated as the 5' and 3' regions of the goldfish sequence we cloned the cDNA corresponding to zebrafish *sbp2* which yielded a 2274 nt coding region that is 83% identical to the goldfish sequence. Thus, the coding region identified for goldfish appears to be correctly

annotated in terms of sequence but it is incorrectly named as *secisbp2l* instead of *secisbp2* (in this report we refer to *secisbp2* as “*sbp2*” so it is easily distinguished from “*secisbp2l*” in the text). Figure 1 shows a diagram of alignments between the cDNA clones we obtained at 5 dpf and the NCBI gene sequences, highlighting discrepancies between the gene annotation and the sequences obtained by RT-PCR.

Selenoprotein mRNA expression during zebrafish development

In order to assess the expression of selenoprotein mRNAs and those that are required for selenoprotein synthesis during zebrafish development, we mined existing RNA-seq data obtained by the Busch-Nentwich group at Wellcome Sanger Institute (White et al. 2017). Figure 2 shows a heatmap of selenoprotein mRNA expression as well as Sec incorporation factor mRNA expression across a non-linear range of early developmental time covering 0 - 120 hours post fertilization (hpf). Sec incorporation factor mRNA expression is generally low, particularly for *eefsec* (Sec-specific translation elongation factor) and *pstk* (the enzyme that phosphorylates the Ser intermediate in Sec synthesis). It is notable that both *sbp2* and *secisbp2l* are expressed at low levels across the entire time range with small peaks of expression at 2-4 hpf and again at 96 hpf, and these peaks are generally correlated with higher levels of selenoprotein mRNAs. In addition to the selenoprotein mRNAs that have very low expression across the entire range, there are two other classes of selenoprotein mRNA expression: 1) early high expression (0-10 hpf above 200 TPM), and 2) late high expressing (24-120 hpf above 200 TPM). It is interesting to note that in cases where the increase in selenoprotein mRNA expression is very high (e.g. *selenop*), there is no correspondingly significant increase in mRNAs encoding *secisbp2* and *eefsec*, which are directly required for inserting Sec during translation. This dataset establishes that selenoprotein mRNA expression is biased toward larval development from 1-5 dpf, most

notably through expression of *selenop* and *gpx4*. This data also highlights the fact that selenoprotein mRNA transcription and/or stability is highly regulated during this time period, highlighting the significance of temporal regulation that cannot be studied in vitro or in cells.

Gene disruption and genomic analysis

We sought to determine the relative contributions of *Sbp2* and *Secisbp2l* to selenoprotein production in vivo by ablating their expression in zebrafish. For *secisbp2l*, a single guide RNA (sgRNA) was designed to target the RNA binding domain at position 2325-2344 (encoding KLVSLT, which is KLVELT in human; Figure 1). For *sbp2*, we used separate tracr and crisper RNAs, the latter also targeting a conserved region in the RNA binding domain within exon 13 at position 1955 - 1977 (encoding VPVSL which is VPVLS in human). The 3' biased locations were selected in order to avoid the possibility of downstream translation initiation that may bypass indels in the N-terminal half of the genes. This is necessary because the C-terminal halves of *Sbp2* and *Secisbp2l* are functional (Donovan and Copeland 2012). Injected embryos were screened by genomic PCR and sequencing of the targeted area revealed two edited alleles for *secisbp2l* with 19 and 26 base pair (bp) insertions, both of which caused a frameshift and introduction of a premature termination codon at the position of insertion. We chose the 26 bp insertion allele for further analysis (Figure 3A). In the case of *sbp2*, we obtained both 1 bp and 5 bp deletions. We chose the 5 bp deletion allele for further analysis. Outcrossing F0 founders to wt fish generated F1 heterozygotes that were used for inbreeding to create stable homozygous lines for the 26 bp insertion for *secisbp2l* and 5bp deletion for *sbp2*. Edited alleles were confirmed through sequence analysis of genomic DNA (Figure 3B). Additionally, sequence analysis of RT-PCR products did not reveal any traces of wild-type sequence for either *sbp2* or *secisbp2l*. No discernable overt phenotypes were observed for either *sbp2*^{-/-} or *secisbp2l*^{-/-}. This

was an unexpected result in the case of *Sbp2* since mouse embryos that lack *Sbp2* expression die prior to gastrulation (Seeher et al. 2014).

Analysis of disrupted *Sbp2* expression by immunoblot

Having established that the *sbp2* and *secisbp2l* genes were disrupted, we sought to examine protein expression across development in the wild type and mutant lines. For *Sbp2* analysis we were able to use an affinity purified commercial antibody raised against a C-terminal portion of the human protein (aa 506 - 854) which is 79% identical in the conserved L7Ae RNA binding domain. Figure 4 shows immunoblot analyses across 5 days of development. While the predicted size of full length zebrafish *Sbp2* is only 85 kDa, we observed a high intensity band migrating at ~130 kDa that is not present in the *sbp2*^{-/-} strain. Aberrant migration was not unexpected since mammalian SBP2 has a predicted molecular weight of 95 kDa but endogenous and recombinant SBP2 migrates at ~120 kDa in SDS-PAGE (Copeland et al. 2000). At 5 dpf the *sbp2* signal is substantially reduced relative to the tubulin control. This was not expected because the transcriptomic analysis indicated that the peak of *sbp2* expression occurred at days 4 and 5 (see Figure 2). For *Secisbp2l* analysis we raised a polyclonal antibody against a C-terminal fragment of the predicted zebrafish protein (aa 462 - 811), but we were not able to detect a candidate protein.

Selenoprotein expression is differentially reduced in *sbp2*^{-/-} and *secisbp2*^{-/-} embryos.

In order to assess selenoprotein expression during early zebrafish development we used ⁷⁵Se-selenite metabolic labeling. Since mRNA expression data shows substantial selenoprotein mRNA expression from days 3-5, we focused on this time frame. To analyze selenoprotein expression in embryos that lack *Sbp2*, we screened 5 dpf larvae that resulted from a cross

between heterozygous *sbp2*^{+/-} animals so that we might observe the effects of different *sbp2* gene doses in a single experiment. Embryos were exposed to 375 nM ⁷⁵Se-selenite for 24 hours and lysates were analyzed by SDS-PAGE followed by phosphorimager analysis. As expected, Figure 5A shows a high degree of variability in the amount of ⁷⁵Se-selenite labeling in progeny as compared to the wild-type parental strain. The significant reduction of radioactive bands in some samples corresponds to mendelian inheritance of the edited *sbp2* allele (25%). To confirm that the loss of selenoprotein expression was a result of Sbp2 loss, we repeated the labeling on bona fide *sbp2*^{-/-} embryos with verified genotypes. Figure 5B confirms that the *sbp2*^{-/-} larvae show a substantial loss of ⁷⁵Se-labeled proteins of most molecular weights except the ~68 kDa species at all three time points and a 24 kDa band in day 3, the former of which likely corresponds to thioredoxin reductase. Overall, the loss of selenoprotein production was not uniform. The most notable change was the loss of a diffuse band at ~45 kDa and a pair of bands at ~22/20 kDa. These results suggest that Secisbp2l may be supporting selenoprotein synthesis, particularly the 68 and 25 kDa species, when Sbp2 is absent. In the case of *secisbp2l*^{-/-} embryos, no band-specific differences were noted when compared to wild-type embryos in the 3-5 dpf period (Figure 5C). There was, however, a statistically significant reduction of selenoprotein expression in days 3 and 4 (Figure 5C). These data establish that the lack of Sbp2 expression is not sufficient to eliminate selenoprotein production, the baseline level of which is not affected by the lack of Secisbp2l.

Interestingly, we noted a significant increase in a ~130 kDa Coomassie stained band in *sbp2*^{-/-} but not wt or *secisbp2l*^{-/-} larvae (Figure 5B, arrow). This band was most prominent in 3 dpf larvae and not evident at all in 5 dpf larvae. Mass spectrometric analysis of the excised band revealed that this protein is vitellogenin-1 (vtg1), which is an abundant phosphoprotein present in yolk. As a well-studied biomarker for various types of stress, it provides a clear

indication that the loss of *sbp2* but not *secisbp2l* initiates a stress pathway that ultimately leads to vtg1 induction. Since vtg1 is known to be induced by estrogens, it is likely that the loss of selenoprotein expression is either directly or indirectly affecting the estrogen synthetic pathway. This is consistent with a previously observed correlation between selenium status, selenoprotein production and estrogen levels in rats (Damdimopoulos et al. 2004).

Analysis of embryos lacking both Sbp2 and Secisbp2l.

In order to generate a line lacking both *sbp2* and *secisbp2l*, we targeted *secisbp2l* for CRISPR/Cas9 mutagenesis in the *sbp2*^{-/-} background. We injected 80 embryos alongside the same number of uninjected controls. After 72 hours we collected 6 for genomic DNA analysis, 24 for metabolic labeling with ⁷⁵Se-selenite and 30 each were collected to be raised to study their survivorship. Notably, only one of the injected F0 animals with the *sbp2*^{-/-} background survived past 14 days post fertilization, and all but one of the genotyped embryos showed evidence of editing at the target site. To assess the effect on selenoprotein production we subjected the injected and control embryos to metabolic labeling with ⁷⁵Se-selenite. Figure 5D shows Phosphorimage analysis from a subset of injected embryos, all but one of which did not have detectable selenoprotein expression. This result strongly supports the idea that *Secisbp2* and *Secisbp2l* work in concert to provide full selenoprotein incorporation. Together these data reveal that selenoprotein production may not be required for early development in fish but that survival is limited to an early juvenile stage.

Quantitative analysis of selenoprotein mRNAs and proteins

In mammalian cells, reduced selenoprotein expression due to limiting selenium or reduced *Sbp2* expression is associated with a coordinated but selective reduction in

selenoprotein mRNAs (Shetty and Copeland 2015). Several studies have established that this response is due to reduced mRNA stability, likely through the nonsense mediated decay pathway (Squires et al. 2007; Santesmasses et al. 2019; Weiss et al. 1997; Wingler et al. 1999; Sun et al. 2001). In order to examine the response of selenoprotein mRNA levels to selenoprotein reduction due to loss of Sbp2 or Secisbp2l, we performed quantitative RT-PCR at 4 dpf for 6 selenoprotein mRNAs (*selenow*, *selenop*, *gpx1a*, and *gpx4a*, *selenof*, *txnrd1* [note, the zebrafish gene is annotated as *txnrd3*]) relative to the control mRNA encoding *eef1a1*, which is known to be evenly expressed across 3-5 dpf (McCurley and Callard 2008). Figure 6 shows that the loss of Sbp2 resulted in an ~5-fold reduction of both *selenop* and *gpx1a* mRNAs and a ~2-fold reduction of both *gpx4a* and *selenow* but had a much smaller effect on *selenof*, *txnrd1* or *txnrd2*. In the case of *secisbp2l*^{-/-} the mRNAs that were sensitive to the loss of sbp2 were also sensitive to the loss of secisbp2l but to a much lower extent. We observed ~40% reductions of *selenop* and *gpx4a* mRNA, 20% reductions in *gpx1a* and *selenow* and either no reduction or increases in *txnrd1*, 2 and *selenof* mRNAs. These results establish that the zebrafish system faithfully replicates that observed in mice and mammalian cells with regard to the regulation of selenoprotein mRNA levels under conditions where selenoprotein synthesis is impaired (Fradejas-Villar et al. 2017), and they open up the possibility that secisbp2l is playing a role in regulating selenoprotein mRNA levels.

For quantitative analysis of selenoprotein levels, we examined the proteomes of *sbp2*^{-/-} and *secisbp2l*^{-/-} larvae by quantitative mass spectrometry. We chose to analyze larvae at 4 dpf, which corresponds to a time when larval selenoprotein expression is high. 4 replicate samples for each strain were lysed in SDS buffer and subjected to in-gel digestion followed by TMT labeling and Nano-LC-MS/MS. As expected, the *sbp2*^{-/-} proteome was marked by significant decreases in all detectable selenoproteins except for Txnrd1 (Figure 6B). It is also notable,

however, that the extent of down-regulation was not uniform with a nearly 3-fold difference between the low-expressing selenoprotein (Gpx1a) and the highest (Selenop and Selenoj). For Txnrd1, the lack of downregulation supports the idea that the 68 kDa band that is not affected by the loss of Sbp2 is Txnrd1, although it is also possible that what we observed in the mass spec data corresponds to a truncated Sec-free version of Txnrd1 which has been shown to persist in selenium deficient conditions (Legrain et al. 2014). In the case of *secisbp2*^{-/-} larvae we did not detect any reduction of selenoproteins by quantitative mass spectrometry (Figure 6C), although Gpx4 seems to be upregulated, which was not detectable during ⁷⁵Se labeling shown Figure 5. Both datasets contained a discrete set of other proteins that were either up or downregulated, which are the subjects of ongoing analysis. Note that Vtg1 was more than 10-fold higher in the *sbp2*^{-/-} larvae, which is consistent with the band identified in the stained gel in Figure 5B.

Larvae lacking Sbp2 but not Secisbp2l are sensitive to peroxide stress

Considering the role of selenoproteins in resolving oxidative stress, we sought to determine if the loss of either Secisbp2l or Sbp2 affected sensitivity to oxidative stress. To this end 0-5 dpf embryos were treated with 2 mM hydrogen peroxide (Figure 7A). Since we started the treatment at 0 dpf, this analysis includes the natural die-off of ~20% of the embryos from 0 to 1 dpf. This is expected as any unfertilized, or unhealthy embryos die during this time period. While no significant peroxide sensitivity was noted in *secisbp2*^{-/-} larvae or either of the wild-type controls, none of the *sbp2*^{-/-} larvae survived past 2 dpf. Interestingly we observed a slightly enhanced survival of *secisbp2*^{-/-} larvae resulting from a lower initial die-off. Overall these results confirm that the reduction of selenoprotein expression correlates with increased sensitivity to oxidative stress and establishes the *sbp2*^{-/-} line as a potential tool for studying hypersensitivity to stress.

Because substantial selenoprotein synthesis persists in larvae lacking Sbp2 but is not affected in larvae lacking Secisbp2l, we hypothesize that Secisbp2l is required for “backup” selenoprotein synthesis under stress conditions (e.g. loss of Sbp2). In order to directly test this hypothesis, we examined the effect of peroxide treatment on selenoprotein synthesis in wild-type and *secisbp2l*^{-/-} 3-5 dpf larvae. We treated larvae with a sublethal dose of peroxide (0.5 mM) concomitant with ⁷⁵Se-selenite labeling for 24 hours. Surprisingly, Figure 7B shows that selenoprotein expression was almost completely eliminated by peroxide treatment in the 3 and 4 dpf larvae but was only about 50% reduced at 5 dpf. These results reveal a developmentally specific response to peroxide that results in dramatic selenoprotein downregulation.

Discussion

We have utilized the zebrafish system to investigate the roles of the two SECIS binding proteins in promoting selenocysteine incorporation during development. Overall our findings have revealed that Sbp2 is the main driver for basal selenoprotein production but that its orthologue, Secisbp2l, supports selective selenoprotein expression under stress conditions (e.g. loss of Sbp2). Use of the zebrafish system has allowed us to demonstrate the disparate roles of the two SECIS binding proteins in the context of embryonic development.

Analysis of *sbp2* and *secisbp2l* genes has revealed that *sbp2* coding sequence in Teleost fish is unique relative to all other vertebrate species. The predicted N-terminal ~95 amino acids are unique in the Clupeocephala supercohort, and there are no conserved motifs that might hint at function. Thus, the N-terminal motifs that are conserved between Sbp2 and Secisbp2l (Donovan and Copeland 2009) are present only in the latter for these species. The

zebrafish system, then, is unique in providing an opportunity to determine the function for these conserved motifs.

Our analysis of *sbp2*^{-/-} fish was surprising in that zebrafish larvae are able to survive this condition while mouse embryos die early during gastrulation (Seeher et al. 2014). While our immunoblot analysis could not definitely show a complete lack of Sbp2 due to the presence of cross-reacting species, sequencing at the genomic and transcript level confirmed that wt *sbp2* is not detectable. As such, it is likely that the relatively low oxygen exposure of fish eliminates the requirement for a full complement of selenoproteins during early development. This point was emphasized by our finding that injecting anti-*secisbp2l* CRISPR sgRNA was sufficient to completely eliminate selenoprotein synthesis. This is the first demonstration that Sec incorporation is fully dependent on SECIS binding proteins since all prior work has focused solely on Sbp2. The fact that these embryos did not survive past day 14 pinpoints a surprisingly late time in development that is sensitive to the loss of selenoproteins. Although it is not possible to pinpoint which selenoproteins might be required for survival, considering the central role of GPX4 in responding to ferroptotic damage and its ability to allow survival of mouse embryos that lack other selenoproteins ([Ingold et al. 2018](#)), it is a good candidate for playing a similarly key role in fish.

We found that selenoprotein production was significantly but selectively downregulated in *sbp2*^{-/-} but not *secisbp2l*^{-/-} larvae. The primary exception to downregulation is a 68 kDa selenium-labeled protein that we predict corresponds to thioredoxin reductase (Txnrd1). This leads to the key mechanistic question of whether Secisbp2l preferentially binds certain selenoprotein mRNAs. Considering that ablating Secisbp2l led to a general reduction of all

selenoproteins, it is more likely that selective expression in the *sbp2*^{-/-} background is a result of the altered mRNA levels in that strain.

The fact that selenoproteins are still made in the absence of Sbp2 strongly implicates Secisbp2l as a factor that can “survive” selenoprotein production under conditions where Sbp2 may be limiting. Prior work in mammalian cells indicated that oxidative stress resulted in glutathionylation and altered subcellular localization of Sbp2 from the cytoplasmic to the nuclear compartment, thus leading to reduced selenoprotein synthesis (Papp et al. 2006). Although this effect seen in cultured cells may not be related to what we observed in zebrafish, it is consistent with the idea that the primary role of Secisbp2l in the context of Sec incorporation may be to shift selenoprotein synthesis to a survival mode during stress. The continued use of zebrafish will allow mechanistic evaluation of this potentially novel stress pathway. Specifically, the *sbp2*^{-/-} strain will serve as a valuable testbed for studying the specific roles of individual selenoproteins in preventing oxidative stress in vivo.

The caveat to the idea that Secisbp2l is important for stress response is that we found *secisbp2l*^{-/-} animals to be no more sensitive to peroxide stress than wild-type controls. This was in contrast to *sbp2*^{-/-} animals where the larvae did not survive past 2 dpf in the presence of peroxide. We also found that peroxide treatment almost completely eliminates selenoprotein expression in 3-4 dpf but not 5 dpf embryos. The resistance at 5 dpf serves as an important control in ruling out the possibility that peroxide is preventing selenium uptake. This was an unexpected result considering that other studies have demonstrated reduced selenoprotein expression in the presence of peroxide, but this dramatic temporally regulated response has not previously been reported. This result also raises a question about why only *sbp2*^{-/-} larvae were sensitive to 2 mM peroxide. If the sensitivity was due to the lack of selenoproteins and peroxide is eliminating all selenoprotein production, then both lines should have been sensitive. Further

dissection of the response to peroxide stress will be required to determine the basis for this striking regulation of selenoprotein expression.

Interestingly, the loss of Sbp2 resulted in substantial upregulation of vitellogenin (Vtg1) which has previously been noted to be upregulated in stress conditions, specifically as a target of environmental toxins that are estrogen receptor agonists ([Hultman et al. 2017](#)). This confirms the idea that the loss of Sbp2 causes a specific type of stress that is likely caused by the loss of one or more selenoproteins. Future work with these zebrafish lines will allow the identification of precisely what type of stress might lead to the condition where Secisbp2l driven Sec incorporation is required.

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Figure Legends

Figure 1. Diagram of the zebrafish *secisbp2l* and *sbp2* gene loci (NCBI). The results of BLAST analysis using the cloned cDNAs for both genes is shown in purple. The CRISPR/Cas9 target sites in the RNA binding domains are indicated with arrows (note reverse orientation of *secisbp2l*).

Figure 2. Heatmap of existing transcriptomic data (REF) highlighting selenoprotein and selenoprotein synthetic factor mRNA expression during zebrafish development.

Figure 3. Domain diagrams illustrating the relative positions of the Sec incorporation domain (SID), RNA binding domain (RBD) and the Glu-rich domain (ERD), and sequence analysis of *secisbp2l* and *sbp2* mutations generated by CRISPR/Cas9 illustrating the 26 bp insertion for *secisbp2l* and the 5 bp deletion in *sbp2*.

Figure 4. Immunoblot analysis of Sbp2 expression. Wild-type (wt) and *sbp2*^{-/-} (Δ) embryos at the indicated days post fertilization (dpf) were lysed and 2 embryo equivalents were loaded onto a 4-12% gradient SDS-PAGE gel. Immunoblot was probed with a polyclonal antibody raised against human SBP2.

Figure 5. Metabolic labeling reveals selenoprotein synthesis defects. A) SBP2^{+/-} heterozygous fish were mated and offspring were incubated with 375 nM ⁷⁵Se for 24 hr at 4 days post fertilization (dpf). 16 larvae were randomly chosen for SDS-PAGE and phosphorimaging analysis. B) *sbp2*^{-/-} and *secisbp2l*^{-/-} larvae at the developmental stage indicated were labeled and analyzed as described in A). The 68 kDa band that is not affected by the loss of Sbp2 is predicted to be Txnrd1 based on molecular weight. The marked increase in Vtg1 in 3-4 dpf

larvae is noted in the Coomassie-stained gel. C) Quantitation of the Δ Secisbp2L gel in B). 3 replicate gels were analyzed and the data is presented as the mean $\pm$ standard deviation. Significance was determined with a Welch's t-test with the p value indicated. D) *sbp2*^{-/-} embryos were injected with sgRNAs targeting the *secisbp2l* gene at the 1 cell stage. At 3 dpf larvae were incubated with 375 nM ⁷⁵Se for 24 hr and lysates from individual larvae were analyzed by phosphorimage analysis. Lane one is lysate from an uninjected control embryo.

Figure 6. qPCR reveals reduced selenoprotein mRNA levels in *sbp2*^{-/-} but not *secisbp2l*^{-/-} larvae.

A) Total RNA was extracted from 4 dpf larvae was analyzed by qRT-PCR using primers specific for the genes noted. The data shown is the average $\Delta\Delta$ CT ratios from triplicate analyses using *eef1a* as the control plus/minus standard deviation. B) Total protein lysates from wild-type and *sbp2*^{-/-} larvae were subjected to quantitative mass spectrometry. The data shown represent the average mutant/wt peptide count ratio from 4 replicate samples, plus/minus standard deviation. C) Same as in B) for the *secisbp2l*^{-/-} samples.

Figure 7. Mutant *sbp2*^{-/-} larvae and general selenoprotein production are sensitive to peroxide treatment. A-D) Wild-type (orange) or mutant (blue) larvae (n=110) were raised in embryo medium with or without 2 mM hydrogen peroxide for 5 days (water was changed daily). Survival was analyzed by the Kaplan-Meier method and P values were determined by the log-rank test. E) 3-5 dpf larvae were incubated with 375 nM ⁷⁵Se with or without 500 μ M hydrogen peroxide for 24 hours. Radiolabeled proteins were analyzed by phosphorimager (top panels) and total protein was determined by fluorescence imaging of TCE stained gels (bottom panel). F) Quantitative analysis of selenoprotein production in the presence or absence of peroxide. ⁷⁵Se incorporation was determined by quantitation of the phosphorimages shown in E. Data was

normalized to wild-type untreated samples and shown as the average of three experiments plus/minus standard deviation.

Table 1. List of oligonucleotide sequences

<i>secisbp2l</i> sgRNA	UAAGCUGGUGUCCCUGACGG
crRNA	UCCCGGUCAGCU
<i>secisbp2l</i> genotyping fwd	GGCTGTTCAATAAGCTGGTGTC
<i>secisbp2l</i> genotyping rev	AGATGACGCTGCAGAAGGAG
<i>sbp2</i> genotyping fwd	CTTAGGTGGTCTAGATGAGGC
<i>sbp2</i> genotyping rev	TCTTCCTGTAGATCCTCCTGCG
<i>sbp2</i> cloning fwd	ATGGAGAATCATTCAAAAAGAGCTC
<i>sbp2</i> cloning rev	ACTTCCTTCATCGTCCAGAAGCTG
<i>secisbp2l</i> cloning fwd	GAGGAAGGATGTAAAGCTCTCTGC
<i>secisbp2l</i> cloning rev	GATCTGCCGCTGGTTCAGTG

Figure 1, Kiledjian et al

SECISBP2L Chromosome 18 - NC_007129.7. (5,363,383..5,392,802)

SECISBP2 Chromosome 5, NC_007116.7 (66248078..66271641)

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Gene Name	0	1	2	3	4	5	6	8	10	16	19	24	30	36	48	72	96	120	hpf
gpx1a	3	7	20	23	28	32	26	32	53	51	56	57	60	57	47	71	94	88	
dio1	0	1	1	1	2	0	0	0	3	3	3	2	2	3	2	7	17	21	
dio2	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	1	1	1	
dio3b	0	0	0	0	0	0	0	0	1	2	4	5	5	4	5	14	11	10	
gpx1b	23	37	34	19	24	18	11	3	10	10	12	12	7	8	6	19	37	38	
gpx3	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	5	9	9	
gpx4a	8	8	10	9	11	20	25	70	441	400	558	706	729	364	414	203	491	502	
gpx4b	14	44	79	76	85	165	149	214	301	376	312	248	276	276	261	319	292	221	
gpx7	0	0	0	0	0	1	0	1	18	31	42	58	63	46	48	30	16	6	
gpx8	13	16	64	98	61	19	9	13	42	49	30	31	29	22	15	11	8	5	
msra	27	29	45	68	34	20	7	2	3	4	5	3	3	4	6	8	15	19	
msrb1a	10	13	13	12	18	24	19	20	9	5	8	9	10	6	7	10	13	14	
msrb1b	109	153	60	30	31	58	66	67	49	45	54	30	24	23	19	25	22	21	
selenof	344	341	232	223	290	359	308	299	276	284	244	161	131	160	114	104	71	42	
selenoh	9	47	71	71	76	89	91	96	113	114	100	85	72	60	46	36	32	22	
selenoj	0.5	3	8	8	10	14	12	7	18	26	29	21	17	17	17	22	24	26	
selenok	63	71	33	34	46	42	37	41	41	48	55	46	42	46	43	51	48	41	
selenol	0	0	0	1	0	1	0	0	2	3	3	6	8	6	9	13	14	18	
selenom	0.8	2	7	9	9	13	8	7	33	57	79	65	65	56	54	57	38	18	
selenon	3	3	10	8	4	7	13	31	59	47	36	28	31	35	32	21	12	7	
selenoo1	92	95	81	71	49	29	23	25	21	18	20	14	10	10	8	8	11	16	
selenop	0.2	0.3	0.7	0.8	0.7	0.9	5	44	133	58	205	559	577	220	602	953	956	805	
selenop2	0	0	0	0	1	2	2	13	95	54	85	110	100	35	66	47	76	61	
selenos	13	20	38	37	22	11	10	16	23	22	22	16	19	20	19	28	29	28	
selenos	11	17	30	31	18	8	8	11	17	15	15	13	14	14	14	21	21	22	
selenot1a	94	110	165	141	125	110	84	90	54	59	62	60	55	62	66	90	75	59	
selenot1b	130	109	80	80	87	93	82	53	51	52	47	43	30	33	34	40	39	31	
selenot2	19	27	11	23	30	29	27	37	106	127	128	88	96	107	81	91	72	55	
selenou	12	30	14	29	28	23	17	15	25	38	45	39	39	54	51	56	58	56	
selenow1	42	90	72	74	108	132	114	148	164	203	219	226	276	254	324	331	252	272	
selenow2a	160	157	95	123	148	170	157	173	186	222	239	137	169	234	177	132	113	81	
selenow2b	3	2	4	3	0.8	0.7	0.7	3	11	15	19	18	29	33	43	71	80	60	
selt1a	92	116	169	154	127	112	83	86	57	58	63	61	56	60	68	88	75	59	
selt1b	130	106	79	86	88	93	86	53	50	50	48	43	34	35	33	36	38	32	
selt2	19	27	12	23	27	28	26	36	103	119	117	84	88	103	77	84	68	51	
Sep15	301	307	209	208	270	308	285	242	232	230	209	133	113	138	92	83	63	36	
txnd1	391	329	390	330	100	62	55	54	82	82	95	97	86	88	60	63	91	65	
txnd2	0	2	3	4	1	1	0	0	1	0	1	1	1	1	1	2	1	1	
eef1a	2391	1765	1353	1316	3123	7249	10672	10924	8378	7812	7399	7009	6116	5151	4601	3644	4044	4541	
gapdh	20	48	136	244	370	235	165	104	72	55	145	497	786	827	1183	908	1139	1458	
eefsec	1	1	21	20	13	5	3	8	14	10	13	15	12	11	10	10	4	3	
ptk	2	1	1	2	5	3	3	3	3	3	4	1	2	6	1	1	1	1	
secisbp2	29	30	76	61	39	5	2	5	9	6	8	6	5	4	4	7	24	21	
secisbp2l	3	7	22	22	30	36	34	31	9	7	10	12	10	9	12	18	27	15	
sephs1	185	163	123	115	150	206	258	242	166	146	131	116	87	84	55	39	58	41	
sephs2	43	63	119	108	137	151	109	91	67	55	60	53	53	56	54	57	6	7	
sepscs	32	58	35	43	21	9	7	9	13	10	11	9	5	5	4	7	7	6	
trnau1apb	24	25	57	59	78	144	153	159	124	117	110	73	61	71	49	44	11	7	

Figure 3, Kiledjian et al

Figure 4, Kiledjian et al

Figure 6, Kiledjian et al

Figure 7, Kiledjian et al