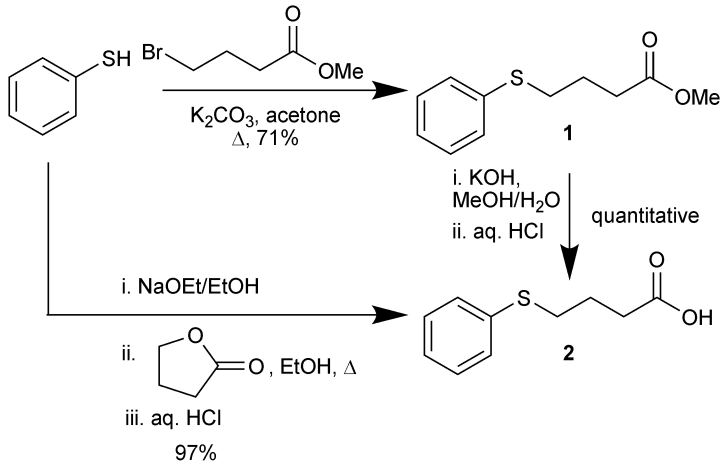
SUPPLEMENTAL METHODS

Synthesis of 4-(phenylthio)butanoic acid and methyl 4-(phenylthio)butanoate.



Methyl 4-(phenylthio)butanoate (**1**) was prepared from thiophenol, potassium carbonate, and methyl 4-bromobutyrate in refluxing acetone as described in the literature.[1] 4-(phenylthio)butanoic acid (**2**) was prepared either in quantitative yield by saponification of **1** with aqueous KOH in MeOH overnight at room temperature followed by acidification with aqueous HCl, or in 97% yield from reaction of the sodium salt of thiophenol and γ-butyrolactone in refluxing EtOH and subsequent acidification with aqueous HCl as described in the literature.[2] All compounds gave ^{1}H and ^{13}C NMR (400/100 MHz and/or 600/150 MHz), mass spectra (GC-EI-MS, LC-ESI-MS and high resolution MALDI-TOF-MS) and melting points consistent with the literature and their structures. All spectral and melting point data suggested >99% purity.[1-3]

Relative qPCR

Primer design. Primer sets were designed using NetPrimer and Beacon Designer (ver. 7.51) primer analysis software (PREMIER Biosoft). In each set, one primer was designed to span an exon boundary. In addition, at least one primer was confirmed to exhibit no significant cross-

homology when compared against the NCBI zebrafish RefSeq mRNA library by BLAST search. Primer melting temperatures were maintained between 60 and 64 ^{o}C as determined by NetPrimer. Each primer set was observed to generate a single amplicon of expected length following qPCR.

Primer sets. The reference gene primer sets have been previously described[4] with one modification. The *β-actin* (F) sequence was changed to: CGTGCTGTCTTCCCATCCA. This corrects a one-base discrepancy from the reported ENSDART accession number. Other primer sets included: *lhx1a* (F): TTCATACTATGGAGATTATCAAAGCG, *lhx1a* (R): GGTCCTGATGAGGGAACAAAAG, *pax2a* (F): GTCCCTGGAAGCGACTTTTC, *pax2a* (R): TTGACTGGGCTGCGATGG, *pax8* (F): GCTCCGCCGTCACTCCTC, *pax8* (R): TCTCCTGGTCACTGTCATCGTG, *ntla* (F): CGCAGCACTACCACCAATAACTAC, *ntla* (R): GAGCCTGATGGGGTGAGAGTC, *myod1* (F): TTCTGGAACATTACAGTGGAGACTC, *myod1* (R): GTGCGTCAGCATTTGGTGTG, *fli1a* (F): CGGAAAAGGCTCTCCAACAG, *fli1a* (R): TGCTGGTGGGTCCTAATATCTG.

cDNA synthesis. 1 μg RNA was heated to 75 ^{o}C for 5 min and then placed on ice. The following reagents were then added to a final volume of 29 μl: 1X Expand High-Fidelity PCR buffer without $MgCl_2$ (Roche), 3 mM $MgCl_2$, 500 μM dNTPs, 3.3 μM random hexamers, and 30 U Protector RNase Inhibitor (Roche). The mixture was preincubated to 42 ^{o}C for 5 min. 1 μl of 200 U/μl SuperScript II Reverse Transcriptase (RT) or RNase-free water was added for +RT or –RT reactions, respectively. Reactions were incubated at 42 ^{o}C for 1 h and then stopped by heating to 95 ^{o}C for 5 min. Reaction products were stored at -20 ^{o}C.

qPCR conditions. 25 μl reactions were prepared containing the following reagents: 12.5 μl 2X iQ SYBR Green Supermix (Bio-Rad), 5 μl 1 μM primer mix (1 μM each of forward and reverse primer), 5.5 μl RNase-free water, and 2 μl 1:10-diluted template (+RT or –RT product) or 2 μl RNase-free water (no template control). Each assay was performed in triplicate wells using an iQ5 Real-Time PCR Detection System (Bio-Rad). Thermal cycling was performed for 40 cycles, each consisting of 94 ^{o}C for 15 s, then 59 ^{o}C for 1 min. Following amplification, melt curve analysis was performed to assess non-specific amplification. Each primer set yielded a single peak, indicative of specific amplification. Reactions performed using –RT product or no template controls were observed to exhibit little or no amplification in comparison with their +RT counterparts.

Reference gene determination. Seven reference gene candidates [*Beta-actin* (*β-actin)*, *Beta 2 microglobulin*, *elongation factor 1 alpha*, *hypoxanthine guanine phosphoribosyl transferase 1*, *RNA polymerase subunit D*, *ribosomal protein L13a*, and *succinate dehydrogenase complex subunit A* (*SDHA*)][4] were screened to determine the gene(s) least affected by PTBA treatment. Relative qPCR experiments (n = 3, 180 embryos) were performed using trunk cDNA obtained from 10 somite embryos treated from 2 hpf with either 0.5% DMSO or 3 μM PTBA. The results were analyzed using NormFinder software (ver. 0.953) to determine the most stable reference gene or combination of genes.[5] The combination of *β-actin* and *SDHA* was observed to exhibit the most stability, and was therefore used for normalization of all qPCR data.

Data analysis. Relative gene expression was calculated using iQ5 software (ver. 2.0, Bio-Rad) to determine normalized expression levels (ΔΔCt method). For comparison of fold-differences,

the expression levels obtained from DMSO-treated controls were set to a value of 1.0. The amplification efficiency of each reaction was calculated using LinRegPCR software (ver. 11.4).[6] The mean efficiencies of each tested primer set fell between 91% and 100%.

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