MATERIALS AND METHODS

Zebrafish methods and genetic mapping

Zebrafish *(Danio rerio)* embryos were obtained and raised, and fish were maintained as described in (1, 2). The zebrafish fish husbandry and research protocols were reviewed and approved by the NIH ACUC. The *tars*^{y58} and *iars*^{y68} mutants were isolated in an ENU F3 genetic screen performed using the *Tg(fli1a:EGFP)*^{y1} transgenic zebrafish line (3), screening for vascular abnormalities at 3 days post-fertilization

The *y58* and *y68* mutations were mapped to LG 5 and LG 11 using bulk segregant analysis and a panel of 192 simple sequence length polymorphism Z markers (list available upon request). Additional polymorphic single sequence repeat (SSR) markers used for fine genetic mapping were obtained from the Sanger Zebrafish SSR search website (http://danio.mgh.harvard.edu/markers/ssr.html). A total of 1134 polymorphic embryos were used localize the *y58* mutation within a final map interval. SSR markers (*Table SI*.) derived from BACS BXOO5015 and BX276099 yielded a final interval with 2 recombinants on the left and 1 recombinant on the right. The only gene in the resulting interval was *threonyl tRNA synthetase (tars)*. The *y68* mutation was mapped using 1393 polymorphic embryos. The final map interval was denoted by 18 recombinants on the left derived from Zv6_scaffold1550 and 1 recombinant on the right derived from the BAC clone BX890614.10 (*Table SI.*).

The Thermoscript RT-PCR system (Invitrogen) was used to reverse transcribe total RNA extracted from 72 hours post-fertilization (hpf) wild-type (EK), *tars*^{y58}, and iars^{y68} embryos. Primer sequences encompassing the 5' CDS and 3' UTR of *tars* and *iars* can be found in *Table S1*. The PfuUltra II PCR system (Agilent Technologies) was used to amplify the respective genes, which were cloned into pCRII-TOPO (Invitrogen) and verified by sequencing. Sequence alignment and comparison was performed using the SeqMan program (DNASTAR, Inc.).

Mapping and sequencing of *tars*^{y58} and *iars*^{y68} mutants

The *y58* and *y68* mutations were mapped to LG 5 and LG 11 using bulk segregant analysis and a panel of 192 simple sequence length polymorphism Z markers (list available upon request). Additional polymorphic single sequence repeat (SSR) markers used for fine genetic mapping were obtained from the Sanger Zebrafish SSR search website (http://danio.mgh.harvard.edu/markers/ssr.html). A total of 1134 polymorphic embryos were used localize the *y58* mutation within a final map interval. SSR markers (*Table SI*.) derived from BACS BXOO5015 and BX276099 yielded a final interval with 2 recombinants on the left and 1 recombinant on the right. The only gene in the resulting interval was *threonyl tRNA synthetase (tars)*. The *y68* mutation was mapped using 1393 polymorphic embryos. The final map interval was denoted by 18 recombinants on the left derived from Zv6_scaffold1550 and 1 recombinant on the right derived from the BAC clone BX890614.10 (*Table SI.*).

The Thermoscript RT-PCR system (Invitrogen) was used to reverse transcribe total RNA extracted from 72 hours post-fertilization (hpf) wild-type (EK), *tars*^{y58}, and iars^{y68} embryos. Primer sequences encompassing the 5' CDS and 3' UTR of *tars* and *iars* can be found in *Table SI*. The PfuUltra II PCR system (Agilent Technologies) was used to amplify the respective genes, which were cloned into pCRII-TOPO (Invitrogen) and verified by sequencing. Sequence alignment and comparison was performed using the SeqMan program (DNASTAR, Inc.).

Morpholino injections

Morpholino antisense oligonucleotides were purchased from Gene Tools and injected into T*g(fli1:EGFP)*^{y1}, and *tars*^{y58} embryos as previously described (2). Morpholinos used in this study are as follows: *tars* translation blocking MO (translation start site underlined), 5'-

GATCAGTCACACTCTCATCCGCCAT- 3'; *iars* translation blocking MO, 5'-TGGACTCTGGCACTGCTTCCACCAT- 3'; *atf4* translation blocking MO, 5'-CAGCGTCCCCAACACACAGAGACAT - 3'. Morpholinos were injected at a dose of 2.5 ng.

Whole mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as previously described (4) with the following modifications. Fixed embryos, stained with BM Purple, (Roche) were cleared by an overnight incubation in 75% glycerol in PBST at 4° C. The *tars* riboprobe was synthesized using a linearized EagI-digested pENTR/D-TOPO (Invitrogen) clone containing the full-length open reading frame (pME-TARS) as a template for transcription. Riboprobes for *iars, atf4, atf6,* and *xbp1* were synthesized using PFUII – generated PCR products as templates for transcription. Primer sequences are listed in *Table SI*.

Expression constructs and DNA microinjections

All expression constructs, unless noted, were generated using Tol2kit components and previously described methods (5). DNA constructs were injected into the blastomere of one-cell stage zebrafish embryos as previously described (2).

A full-length *tars* cDNA clone was used as a template for PCR (*Table SI.*) to generate an entry clone with the pENTR/D-TOPO cloning kit (Invitrogen). The resultant vector was recombined in an LR clonase reaction to produce the βactin: *tars*-2AmCherry-polyA- Tol2CG2 construct. A mixture of 10 pg/nl of plasmid DNA and 100 pg/nl RNA transposase was injected into the blastomere of one-cell stage *tars*^{y58} zebrafish embryos.

An In-fusion clone containing the full-length *iars* CDS was used as a template for PCR (*Table SI.*) to generate an entry clone with the pENTR/D-TOPO cloning kit (Invitrogen). The resultant vector was recombined in an LR clonase reaction to produce the βactin: *iars*-2AmCherry-polyA-Tol2CG2 construct. A mixture of 15 pg/nl of plasmid DNA and 25 pg/nl RNA transposase was injected into the blastomere of one-cell stage *iars*^{y68} zebrafish embryos. The control construct, βactin: mCherry-polyA-Tol2CG2 was generated and injected under the same conditions and quantities described above.

Imaging methods

Phenotypic screening and quantification of zebrafish embryos was done using a Leica MZ16F stereomicroscope. Transmitted light images of zebrafish development and images of *in situ* hybridizations were obtained using an Olympus DP71 digital camera and a Leica MZ16F stereomicroscope. Fluorescent images were obtained using an Olympus FV1000 confocal microscope or a Leica SP5II confocal microscope using 20X water immersion objectives and Volocity image analysis software (PerkinElmer). Embryos imaged using confocal microscopes were anesthetized in tricaine solution as previously described (2) and embedded in 0.75% low melt agarose or 6% methylcellulose.

Phenotype quantification

Excessive angiogenesis phenotypes in the trunk region were quantified by counting the number of supernumerary trunk blood vessels. Supernumerary vessels were only counted if two blood vessel walls could be seen- filipodia were not counted. Branch points were recorded as intersections between supernumerary vessels and existing vessels or other supernumerary vessels.

Excessive angiogenesis phenotypes in the cranial region were quantified by taking confocal images of the dorsal head of each embryo examined. A maximum projection was exported into Photoshop (Adobe). The confocal stacks were analyzed z-section by z-section, and branch

points counted as described above. A student's t-test assuming unequal variances was used to test for significance between treatments. If p-values were less than 0.05, they are reported as significant.

Site-directed mutagenesis and amino acid adenylation assay

Site-directed mutagenesis was used to alter a residue in the active site of WT *tars* previously shown to be essential in the aminoacylation reaction (6). A histidine to alanine mutation at position 365 (H365A) was introduced by PCR *(Table SI)*, following the previously described method (7). Mutated plasmids were confirmed by sequencing. Open reading frames of WT and H365A *tars* were each amplified by PCR from cDNA clones and used for synthesis of the corresponding proteins and adenylation assays as previously described (8).

Open reading frames of WT and H365A *tars* were cloned into the vector pMAL-C2T (Del Campo et. al) and confirmed by sequencing. E. coli BL21 cells transformed with expression constructs were grown 0.5 L cultures in baffled flasks using LB auto-induction media (Studier). Cultures were grown for 6 hours at 37 degrees at which time the temperature was reduced to 25 degrees and grown for another 18-20 hours. Induction of the maltose binding protein (MBP)-TARS fusion protein was confirmed by SDS-PAGE of boiled cells. Cells were pelleted by centrifugation, resuspended in ~30 mL of 25 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA, 1 mM DTT and stored at -80 degrees. Cells were disrupted by sonication and debris pelleted by centrifugation. Nucleic acids were removed by the addition of 0.1% (v/v) polyethylinemine (pH 8.0) and centrifugation. Resulting lysates were run over High-Flow Amylose Resin (New England Biolabs) at a flow rate of 0.5 ml/min. Maltose-binding protein-TARS fusion protein was eluted with 10 mM maltose in loading buffer (25 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA). Elution fractions were poolethe d and ~4 mg of Tobacco Etch Virus (TEV) protease was added to cleave the fusion protein, while simultaneously being dialyzed overnight against 25 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT. TARS was separated from cleaved MBP and TEV protease by cation exchange chromatography. TARS was eluted from the column using a 100mM-1M NaCl salt gradient, pooled, and stored on ice.

Adenylation assays were performed as previously described (PMID:18241792) for monitoring ATP consumption. Briefly, purified enzymes (estimated final concentration 2 uM) were added to 20 uL reactions containing 100 mM HEPES pH 7.5, 10 mM MgCl2, 100 mM KCl, 1 mM DTT, 10 uM ATP, 1000 CPM gamma-32P-ATP, 2.5 mM L-threonine (or L-tyrosine), and 0.5 U pyrophosphotase. 4 uL aliquots were removed and quenched in 10 ul 10% TCA at varying time points. 2 uL of the quenched sample was spotted on pre-developed polyethylenimine TLC plates and developed in 3M urea, 750 mM KH2PO4 (pH 3.5). TLC plates were dried and imaged using a phosphoimager.

RT-qPCR

Total RNA was isolated from zebrafish embryos using TRIzol reagent. RNA was treated using the DNA-free DNase kit (Ambion). TaqMan® probes for *vegfaa* (Dr03435728_m1), *eef1a1|1* (Dr03432748_m1), *atf6* (Dr03137967_m1), *xbp1* (Dr03433279_g1), and *atf4* (AIS08ZU) were ordered from Life Technologies™. cDNA was synthesized using the High-Capacity RNA-to-cDNA kit and QPCR was performed with the above TaqMan® probes and TaqMan® Gene Expression Master Mix on a CFX96 Touch Real-Time PCR Detection System (Life Technologies, Bio-Rad). Results were analyzed using the ddCT method.

Western Blot

41 y58 mutants and control sibling embryos were lysed at 2dpf in either RIPA buffer, for whole cell lysates, or Buffer A (10mM HEPES, 1.5mM MgCl2, 10mM KCl, 0.5mM DTT, 0.05% NP40), for nuclear/cytoplasmic fractionation, supplemented with Halt™ Protease and Phosphotase

Inhibitor Cocktail. To collect the cytoplasmic fraction, we collected the supernatant of Buffer A embryo lysate centrifuged at 3000rpm for 10 min. To collect the nuclear fraction, we Dounce homogenized the pellet in Buffer B (5mM HEPES, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 26% glycerol) supplemented with Halt™ Protease and Phosphotase Inhibitor Cocktail. The homogenate was incubated on ice for 30 minutes and then centrifuged for 20min at 24,000g. The supernatants from Buffer A and Buffer B, in addition to the RIPA lysates, were boiled with an equal volume of Laemmli Sample Buffer. Samples were run on 10% polyacrylamide gels, transferred onto PVDF membranes, and then blocked in 5% BSA. Nuclear lysates were used to determine Rel-A abundance in the nucleus. The following antibodies were used: a-Tubulin (Sigma T6199), p38 (Abcam ab170099), Phospho-p38 (Cell Signaling 9211), Akt (Cell Signaling 9272), Phospho-Akt (Cell Signaling 9271), HDAC1 (Abcam ab41407), Rel-A (AnaSpec 55482). All Westerns were performed in triplicate, and quantification was determined by normalizing the proteins to a loading control (HDAC1 for nuclear lysates, a-tubulin for whole cell lysates). For p38 and Akt, the ratio between the housekeeper normalized phosphorylated and total protein values was determined.

Supplemental Table I

Primer sequences

Primer	5' to 3' sequence	Accession/Version	Position
y58_zC66I11_55235_55865-F	GAAGGGTGTTTTCGGCTAGA	BX005015	55482
y58_zC66I11_55235_55865-R	TGAAAAACCCCTGAAAAGGA		55631
y58_zC204C21_482_1101-F	TGGCATAGTGCATAACAGAGC	BX276099	745
y58_zC204C21_482_1101-R	TGCAAAAATAAAAACAGGAACG		801
y68-zv6scaff1550.1-4.2F	CACCATGCACTCATTCCAGA	zv6scaffold1550.1	92773
Y68-zv6scaff1550.1-4.1R	CGTAATGAAGCCCTCCACTG		92984
y68-zv6_zK46N5-1.4F	GCAGGACATTACAGCCGTTT	BX890614.10	36410
y68-zv6_zK46N5-1.4R	TCCTCATGTCTCCTTGTGTGA		36534
TARS-ORF-F	ATGGCGGATGAGAGTGTGACTGATC	NM_001122786.1	77
TARS-ORF-R	CATCAGGATCACTTCCAGACA		2556
IARS-47-5'-F	ATGGTGGAAGCAGTGCCAGA	NM_199896.1	47
IARS-2.7-5'-R	AAGGCTCCTTTCAGCCGTTT		2753
IARS-3'-F	ATGGATAAATGGATCCAGTCTTTCACACAG	NM_199896.1	2120
IARS-3'-R	GTTTTCAGTTACGCTTCTGCTGTTGTTG		3843
pENTR/TARS-F	CACCTGTACTAAACTGTAAGTTATTGGATCG	NM_001122786.1	14
pENTR/TARS-R	CATCAGGATCACTTCCAGACA		2556
IARS-ribo.-F	ATGGTGGAAGCAGTGCCAGAGTCCATCCACTTCCC	NM_199896.1	47
IARS-ribo._T7-R	TAATACGACTCACTATAGGGACGTACCAGTTAGTG		2236
ATF4-ribo.-F	ATGTCTCTGTGTGTTGGGGACGCTGGAGCCCTGCT	BC067714.1	111
ATF4-ribo._T7-R	TAATACGACTCACTATAGGGCTAGCGTTTGCTCTTGCGCT		1111
ATF6-ribo.-F	GATTTAATGAATCCTTCGATGAGTAGTAAGGAAGT	NM_001110519.1	22
ATF6-ribo._T7-R	TAATACGACTCACTATAGGGTATCCCCCCTATCCTGCGGG		1831
XBP1-ribo.-F	ATGGTCGTAGTTACAGCAGGGACCGGAGGAGCCCA	NM_131874.1	113
XBP1-ribo._T7-R	TAATACGACTCACTATAGGGTCAGTTCATTAAGGGCTTCC		885
pENTR/IARS-F	CACCATGGTGGAAGCAGTGCCAGAG	NM_199896.1	47
pENTR/IARS-R	CGCTTCTGCTGTTGTTGGAAGAACC		3835
H365A_TARS-F	CAACAAGATGATGCTGCCATCTTCTGCTCGATG	NM_001122786.1	1436
H365A_TARS-R	CATCGAGCAGAAGATGGCAGCATCATCTTGTTG		1436

REFERENCES

1 Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn. 1995;**203**:253-310.
2 Westerfield M. The zebrafish book. The zebrafish book (Universiy of Oregon Press, Eugene, ed). 1995:1-8.
3 Lawson ND, Weinstein BM. In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev Biol. 2002;**248**:307-318.
4 Hauptmann G, Gerster T. Two-color whole-mount in situ hybridization to vertebrate and Drosophila embryos. Trends Genet. 1994;**10**:266.
5 Kwan KM, Fujimoto E, Grabher C, et al. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. Dev Dyn. 2007;**236**:3088-3099.
6 Sankaranarayanan R, Dock-Bregeon AC, Romby P, et al. The structure of threonyl-tRNA synthetase-tRNA(Thr) complex enlightens its repressor activity and reveals an essential zinc ion in the active site. Cell. 1999;**97**:371-381.
7 Fisher CL, Pei GK. Modification of a PCR-based site-directed mutagenesis method. Biotechniques. 1997;**23**:570-571.
8 Francklyn CS, First EA, Perona JJ, Hou YM. Methods for kinetic and thermodynamic analysis of aminoacyl-tRNA synthetases. Methods. 2008;**44**:100-118.