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TECHNOLOGY REPORT

Development of Translating Ribosome Affinity Purification for Zebrafish

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Summary: The regulation of transcription and translation by specific cell types is essential to generate the cellular diversity that typifies complex multicellular organisms. Tagging and purification of ribosomal proteins has been shown to be an innovative and effective means of characterizing the ribosome bound transcriptome of highly specific cell populations in vivo. To test the feasibility of using translating ribosome affinity purification (TRAP) in zebrafish, we have generated both a ubiquitous TRAP line and a melanocyte-specific TRAP line using the native zebrafish *rpl10a* ribosomal protein. We have demonstrated the capacity to capture mRNA transcripts bound to ribosomes, and confirmed the expected enrichment of melanocyte specific genes and depletion of non-melanocyte genes when expressing the TRAP construct with a cell specific promoter. We have also generated a generic EGFP-*rpl10a* Tol2 plasmid construct (Tol2-zTRAP) that can be readily modified to target any additional cell populations with characterized promoters in zebrafish. genesis 51:187–192, 2013. © 2012 Wiley Periodicals, Inc.

Key words: TRAP; polysome; capture; danio

RESULTS AND DISCUSSION

Within complex biological systems one of the remaining challenges is assessing the suite of genes being expressed within a specific tissue or more importantly, within a uniquely specified cell population. One technique for doing this is to derive transgenic lines that express, under the control of a cell specific promoter, a tagged protein which permits the capture of mRNA from the targeted cells. In recent years, the development of translating ribosome affinity purification (TRAP) has allowed neuroscientists to begin to tease apart the genes and pathways unique to numerous cell populations comprising the murine brain by affinity purifying an enhanced green fluorescent protein (EGFP)-tagged ribosomal protein and affiliated mRNAs (Doyle *et al.*, 2008; Heiman *et al.,* 2008). One notable advantage of this approach is that more time-consuming purifications of a specific cell population is altogether avoided and replaced by the much simpler purification of ribosomes from a crude lysate. In its simplest form, this technique allows the opportunity to capture gene expression during key windows of development and observe changes in gene expression over time and across cell types. Combining this technique with mutant genetic backgrounds, temporary gene knockdowns, and pharmacological or behavioral perturbation allows the opportunity to delve deeper into genetic and regulatory networks of specific cell populations, potentially providing unique insights for pathway targeting and drug development (Dougherty *et al.*, 2012; Heiman *et al.*, 2008; Schmidt *et al.*, 2012; Warner-Schmidt *et al.*, 2012).

Here, we have adapted the TRAP approach for zebrafish (Fig. 1). Since reported murine TRAP constructs were generated by fusing EGFP to the mouse RPL10a protein (Doyle *et al.*, 2008; Heiman *et al.*, 2008), we predicted comparable fusions would be tolerated in the zebrafish. The zebrafish *rpl10a* ribosomal unit was amplified from a cDNA library and fused in frame to the C-terminal end of EGFP, including a short 13 amino acid linker used in previous murine constructs (Doyle *et al.*, 2008; Heiman *et al.*, 2008). The *Xenopus* elongation factor 1 (x*ef1*α) promoter was cloned upstream of the

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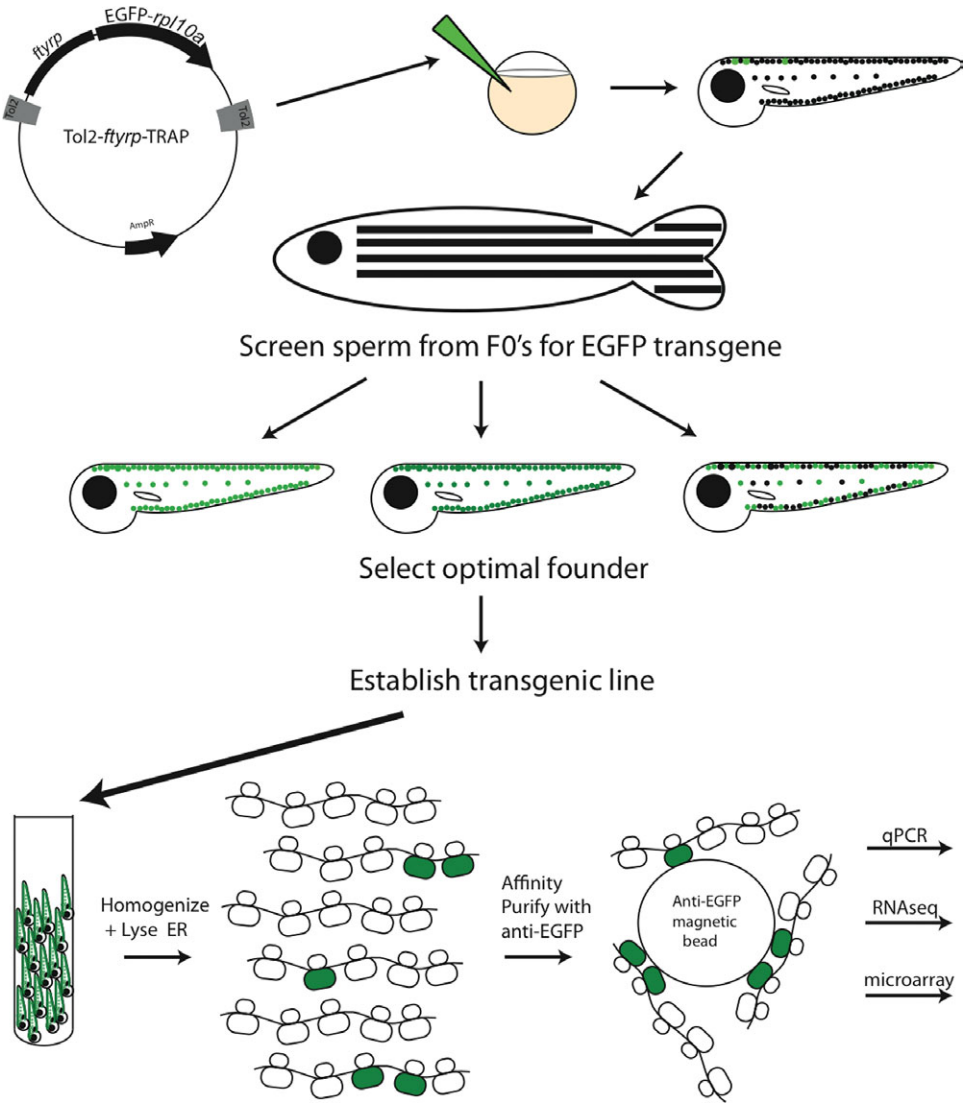

FIG. 1. Generating zebrafish transgenic lines and conducting TRAP. A promoter, here the melanocyte specific *ftyrp*, is cloned into the Tol2-zTRAP vector and co-injected with transposase mRNA into 1 cell embryos. While expression in larvae will confirm the promoter is sufficient to drive expression when the transposon integrates into the lineage being interrogated, all injected fish should be grown up to screen males for germline integrations of the Tol2 transposon. Multiple lines are screened for optimal expression and the strongest, most robustly expressing lines are chosen to establish stable transgenic lines. Hundreds of larval zebrafish are produced and homogenized to lyse the ER and release polysomes. Affinity purification with anti-EGFP beads selects only those ribosomes that were expressed in the cell population being screened. Purified RNA can then be used for qPCR, RNAseq, or microarray analysis.

TRAP construct in order to generate a ubiquitously expressed line for observing cellular localization and to test the ability to affinity purify the EGFP-*Rpl10a* fusion. To demonstrate cell-specific gene expression, the melanocyte specific *Takifugu rubribes* tyrosinase-related protein 1 (*ftyrp*) promoter was cloned upstream of the TRAP construct (Zou *et al.*, 2006). Finally, the x*ef1*α>TRAP or *ftyrp*>TRAP constructs were cloned into a vector containing flanking Tol2 transposable elements for integration into the zebrafish genome (Kawakami *et al.*, 2000).

Transgenic lines were generated (Fig. 1). Sperm from adult F0's was collected and screened for evidence of germline integration. Five putative founders, from which EGFP could be amplified from their sperm, were used to generate F1 offspring and form independent transgenic lines. F1 offspring with the most consistent and brightest EGFP expression were chosen to expand stable transgenic lines expressing each of the TRAP constructs.

The Tg(Xlae.ef1α:EGFP-rpl10a) line (referred to as x*ef1*α>TRAP) shows ubiquitous diffuse EGFP-Rpl10a

expression throughout the fish (Fig. 2a), and is clearly distinguishable from wild type controls (Fig. 2c). By contrast, the Tg(Trub.tyrp1:EGFP-rpl10a) line (referred to as *ftyrp*>TRAP) shows distinct expression in the stereotypical melanocyte stripes of larval zebrafish at 3dpf (arrows, Fig. 2b). Higher magnification of the dorsal stripe shows strong expression in all melanocytes of the *ftyrp*>TRAP line (Fig. 2b′). Previous mouse TRAP lines have shown the majority of EGFP-RPL10a protein to be cytosolic where the bulk of translation is occurring, with the remainder localized to the nucleolus where ribosomes are assembled. Confocal imaging of the zebrafish x*ef1*α>TRAP line shows a similar pattern of subcellular distribution (Fig. 2d). The majority of zebrafish EGFP-Rpl10a expression is cytosolic (Fig. 2d′) with the exception being strong nucleolar staining (arrows) that is absent in the rest of the nucleus (Fig. 2d″). To confirm melanocyte specific expression, the *ftyrp*>TRAP line was crossed to a line with strong melanocyte expression, Mü4192_30, in which an mCherry reporter integrated downstream of the native kita promoter (Fig. 2e) (Distel *et al.,* 2009). EGFP labeling from the *ftyrp*>TRAP (Fig. 2e′) co-localized with the melanocyte *kita*>mCherry expression (Fig. 2e″) and was not seen in additional cell types in this transgenic line.

We then tested the TRAP methodology with these lines. We homogenized parallel pools of *ftyrp*>TRAP, x*ef1*α>TRAP, and wild type fish. We harvested polysomes from these crude lysates (PreIP), as described in Materials and Methods, setting aside a small amount as a control, and conducted TRAP on the rest (Fig. 1). Initial experiments testing the ability of the TRAP protocol to capture the zebrafish EGFP-Rpl10a fusion showed successful capture of ribosomal RNA. The promoter driving ubiquitous expression captured 127.9 ng/μL, while the less abundant and restricted melanocyte expression captured 16.1 ng/μL RNA (Table 1). Despite comparable Pre-IP mRNA quantities isolated from the three groups, only 3.7 ng/μL mRNA was pulled down from the wild-type affinity purification. This low level of mRNA binding is likely a result of the residual stickiness of the affinity purification reagents used and is similar to what is seen in mouse (Dougherty *et al.,* 2010).

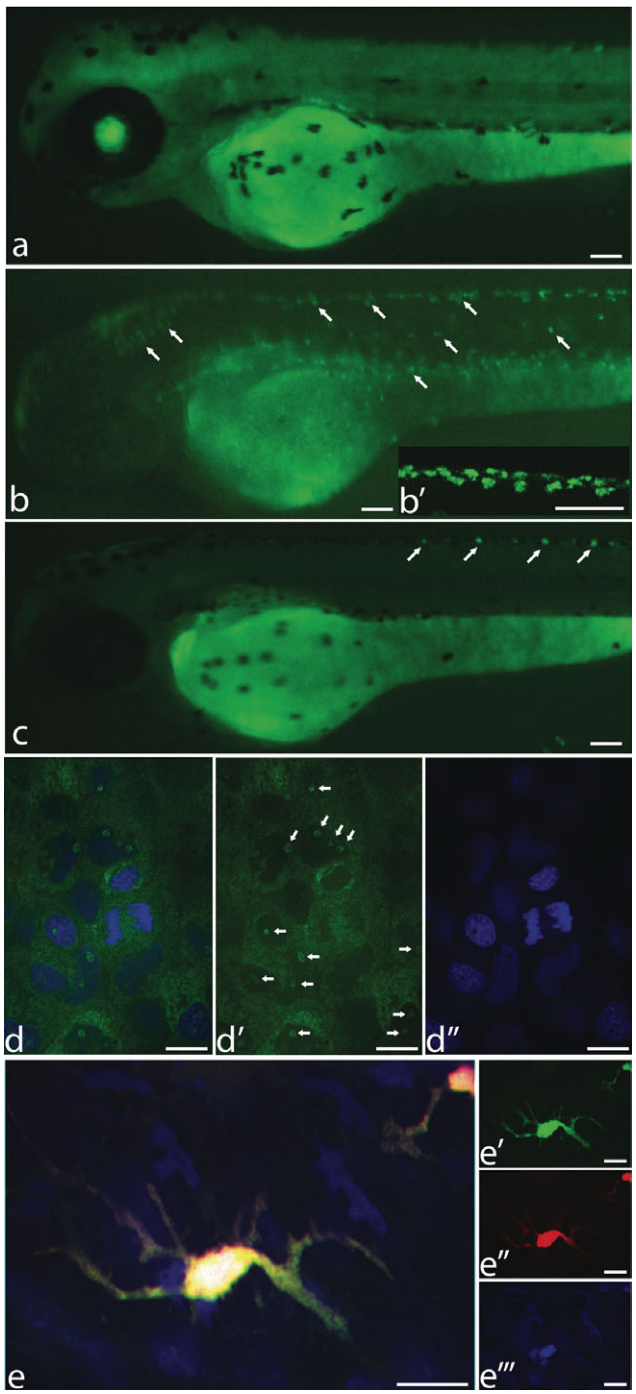

FIG. 2.

FIG. 2. Expression of the EGFP-Rpl10a protein in two stable transgenic TRAP lines. (**a**) The ubiquitously expressed x*ef1*α>TRAP line shows diffuse EGFP throughout the fish at 3dpf. (**b**) The *ftyrp*>TRAP line shows specific expression in melanocytes (arrows) constituting all of the larval stripes at 3dpf. *ftyrp*>TRAP embryos were treated with 200 μM PTU to block the synthesis of pigment in the melanocytes. (**b′**) Detail of melanocytes from the dorsal stripe expressing EGFP in the ftyrp>TRAP line. (**c**) Wild type control at 3dpf. The autofluorescent yolk and reflective iridiphores (arrows) in the dorsal stripe can be observed in all fish and do not reflect EGFP expression. (**d**) Merged confocal image (100× magnification) of epidermal cells ventral to the otic vesicle in the x*ef1*α>TRAP line at 30 hpf. (**d′**) EGFP channel showing predominantly cytosolic TRAP expression with punctate expression in the nucleolus (arrows). (**d″**) CFP channel showing nuclear 4′,6-diamino-2-phenylindole (DAPI) staining. (**e**) Merged confocal image (100× magnification) of a melanocyte in the *ftyrp*>TRAP line at 3dpf. (**e′**). EGFP channel showing *ftyrp*>TRAP expression. (**e″**) RFP channel showing mCherry reporter driven by the endogenous melanocyte *kita* promoter. (**e‴**) CFP channel showing nuclear DAPI staining. Scale bars: 100 μm (a–c); 10 μm (d, e).

Table 1
RNA Purification from TRAP Constructs

	Lysate (ng/μL)	Affinity purified (ng/μL)	28S:18S
Wild type	185.4	3.7	3.9
xef1α>TRAP	142.2	127.9	4.4
ftyrp>TRAP	274.1	16.1	4.0

Testing the integrity of the captured RNA with the Agilent Bioanalyzer revealed that both the 18S and 28S ribosomal subunits were captured by the EGFP affinity purification, indicating the efficient isolation of intact ribosomes (Fig. 3a,b). While previous TRAP purifications in adult mouse brain have revealed the expected 2:1 ratio of 28S:18S ribosomal subunits, our zebrafish TRAP purification has shown an average ratio of 3.8:1. At this time it is unclear whether this reflects normal regulation of translation during early development or if it is an unusual feature of zebrafish or this protocol.

Having shown capture of ribosomes, we next wanted to test whether the melanocyte specific TRAP line was enriched for known melanocyte transcripts and depleted of other non-melanocyte transcripts. Four genes essential for melanocyte differentiation and function, including microphthalmia-associated transcription factor a (*mitfa*, *P*-value 0.0043), tyrosinase (*tyr, P*-value 0.0015), tyrosinase related protein 1 (*tyrp1b*, *P*-value 0.0031), and dopachrome tautomerase (*dct*, *P*-value 0.0013), showed enrichment in the *ftyrp*>TRAP line (Fig. 3c). On the contrary, three genes unassociated with melanocytes were significantly depleted, including myosin light chain z which is highly expressed in muscle (*mylz2*, *P*-value 0.0011), hemoglobin alpha embryonic-3 which is expressed in blood (*hbae3, P*-value 0.0091), and trypsin which is expressed in the gut (*try*, *P*-value 0.0045) (Fig. 3c). In total, we conclude that in this *ftyrp*>TRAP line, EGFP-Rpl10a is specifically expressed in melanocytes and the qPCR analysis confirms that melanocyte specific genes are enriched in the resulting affinity purified mRNA samples.

The TRAP lines generated here will be useful for answering a variety of significant questions. The x*ef1α*>TRAP line allows for investigations into global changes in translation during early development. Alternatively, zebrafish are able to regenerate fins in their entirety upon amputation and the x*ef1α*>TRAP line could be combined with RNAseq to catalog the genes used in the blastema to coordinate and organize the regeneration process. The *ftyrp*>TRAP line can be used to ask whether melanocytes of different developmental origin have different transcriptional profiles.

To make this reagent broadly useful for the zebrafish community, a plasmid (Tol2-zTRAP) has been generated containing the EGFP-*rpl10a* fusion. A flexible multiple cloning site for promoter insertion sits upstream of the TRAP open reading frame, thereby requiring a single cloning step to begin generating other cell specific TRAP lines. Additionally, we are developing a 5× UAS responder for the TRAP construct that should permit TRAP analyses for previously developed GAL4 driver lines and further simplify the use of TRAP in zebrafish (Distel *et al.,* 2009).

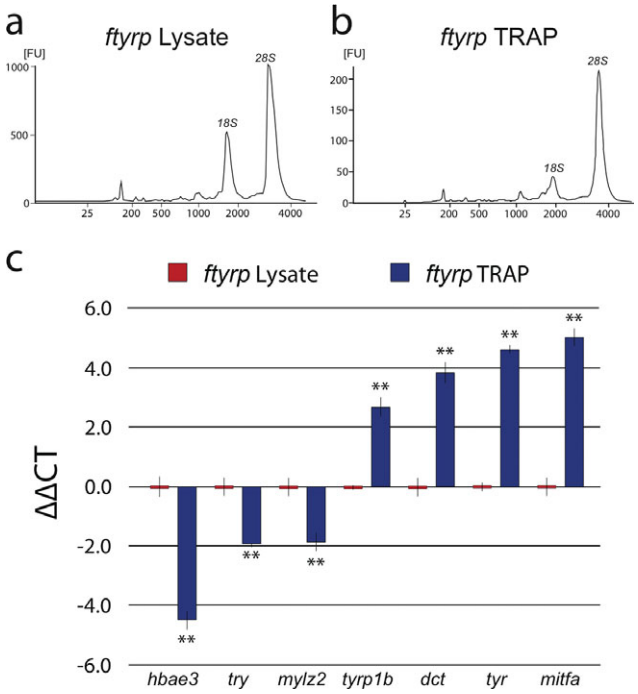

FIG. 3. Purification and enrichment of melanocyte specific genes with the *ftyrp*>TRAP line. (**a**) Agilent Bioanalyzer of the *ftyrp*>TRAP lysate showing an abundance of both small (18S) and large (28S) ribosomal subunits. (**b**) Agilent Bioanalyzer of the affinity purified *ftyrp*>TRAP lysate showing the capture of intact ribosomes. (**c**) qPCR results from three biological replicates comparing the relative abundance of four melanocyte specific genes (*mitfa, dct, tyr, tyrp1b*) and three non-melanocyte genes (*mylz2, hbae3, try*) in the *ftyrp*>TRAP line both before (lysate, red) and after TRAP purification (TRAP, blue). Δ*ΔCt* values are shown for each gene, with standard error bars from three TRAP experiments. A two-tailed *T*-test was used to determine statistical significance, with ** indicating a *P*-value < 0.01.

MATERIALS AND METHODS

Zebrafish TRAP Plasmid Construction

Zebrafish *rpl10a* was amplified from a cDNA library with primers 5′*RPL10A*Link: CGTTCGAATTCAGCAAGG TCTCGAGAGACACG and 3′*RPL10A*ClaI: GCATCGATCC TAGTAGAGGCGCTGTGGTT using Phusion High Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). The 668 bp amplicon was digested with EcoRI and ClaI restriction endonucleases (New England Biolabs, Ipswich, MA) and gel purified (Qiagen, Verlo, NL). Vector s296 con-

taining the murine EGFP-*Rpl10a* fusion was digested with AgeI and EcoRI restriction endonucleases. The 769 bp fragment was gel purified and contained the full EGFP open reading frame and an additional 39 bases comprising a linker coding 13 amino acids compatible with the murine RPL10A protein. The *ftyrp*>EGFP Tol2 vector was digested with AgeI and ClaI, gel purified, and the three fragments were ligated (New England Biolabs, Ipswich, MA) to generate the *ftyrp*>TRAP plasmid (Tryon *et al.,* 2011).

To generate the x*ef1*α>TRAP construct, primers X*EF1*α-Xho1: AGCTCGAGAAGTGTCGGCTTAAGGTCCA and X*EF1*α-AgeI: AGACCGGTCAGCTTCCATATGCGAC AAG were used to amplify the Xenopus *ef1*α promoter. The fragment was digested with AgeI and XhoI restriction endonucleases, gel purified, and ligated to the *ftyrp*>TRAP construct that had previously digested with PspXI (XhoI compatable) and AgeI. All constructs were sequenced to confirm accurate promoter and EGFP-*rpl10a* fusion open reading frames.

The Tol2-zTRAP plasmid was generated by cloning a 22 base pair multiple cloning site into the *ftyrp*>TRAP plasmid, after removing the *ftyrp* promoter with a double digest of AgeI and PspXI. Consequently, upstream of the EGFP-*rpl10a* open reading frame there are five unique sites for promoter insertion (5′ PspXI, NheI, SalI, BamHI, and AgeI 3′) allowing flexibility for directional cloning strategies. The Tol2-zTRAP plasmid will be freely available for distribution to others interested in using TRAP and submitted to addgene (www.addgene.org).

Transgenic Lines

Embryos were produced via in vitro fertilization and co-injected at the 1-cell stage with ∼5 pg of solution containing 10 ng/μL plasmid DNA and 5 ng/μL capped transposase mRNA (Tryon *et al.,* 2011). The *ftyrp*> TRAP line was produced on a temperature-sensitive mitfa (ts-mitfa/ts-mitfa) background in order to permit manipulation of melanocyte development by varying the temperature at which fish are reared (Johnson et al, 2011). The x*ef1*α>TRAP line was generated on a wild type SjA background. The x*ef1*α>TRAP and *ftyrp* >TRAP lines will be made available to the research community, either through the Johnson lab or the Zebrafish International Resource Center (ZIRC).

For TRAP experiments, *ftyrp*>TRAP fish were treated with 200 μM phenylthiocarbamide (PTU) at 1dpf to prevent melanocyte pigmentation. The dark pigment of melanocytes makes visualizing EGFP difficult and by blocking melanin synthesis we could confirm that only transgenic *ftyrp*>TRAP embryos would be homogenized when beginning the TRAP purification.

Confocal Imaging

Embryos were fixed for 15 min in a 4% paraformaledyhde solution in phosphate buffered saline (PBS). Following two 5 min rinses in PBS to remove fixative, embryos were stained for 10 min in a 1:1,000 dilution of 4′,6-diamino-2-phenylindole (DAPI) in PBS. Two more 5 min rinses in PBS were used to remove residual DAPI. Confocal imaging was carried out on a Perkin Elmer UltraView VoX confocal imaging system. Images in Figure 2d,2e were imaged with a ×100 oil objective using the Zeiss Axio Observer Z1 microscope and the Volocity 3D imaging analysis software.

Translating Ribosome Affinity Purification

For initial experiments testing the efficiency of affinity purification using the TRAP protocol on the x*ef1*α>TRAP, *ftyrp*>TRAP, and wild type lines, 130 zebrafish embryos were pooled for each sample. Subsequent testing for the enrichment and depletion of target genes in the *ftyrp*>TRAP line used 380 fish per sample and was conducted on three biological replicates. All polysome purifications and mRNA extractions were carried out as described (Heiman *et al.,* 2008) with a few modifications described in a forthcoming methods paper (M. Heiman, personal communication). Briefly, the zebrafish embryos were homogenized in extraction buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (pH 7.4), 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 100 μg/mL cycloheximide, rRNasin RNase inhibitors (Promega, Madison, WI), and Complete-EDTA-free protease inhibitors (Roche, Basel, CH), and cleared by centrifugation at 2,000*g*. IGEPAL CA-630 (NP-40; Sigma, St. Louis, MO) and DHPC (Avanti Polar Lipids, Alabaster, AL) were both added to the supernatant at a final concentration of 1% for each, and the mixture was cleared by centrifugation at 20,000*g*. Streptavidin MyOne T1 Dynabeads (Invitrogen, Grand Island, NY) were coated with Purified Biotinylated Recombinant Protein L (Pierce Thermoscientific, Rockford, IL) by incubating at room temperature for 35 min. The Protein L coated beads were washed five times with PBS containing 3% IgG, Protease free bovine serum albumin (BSA) (Jackson Immunoresearch, West Grove, PA) by separating the beads on magnetic rack, incubated with 100 μg monoclonal anti-EGFP antibodies (clones 19C8 and 19F7; see Heiman *et al.,* 2008) for minimum of 1 h. The polysomes were immunoprecipitated with the coated magnetic beads overnight at 4°C and washed with a high salt buffer containing 10 mM HEPES-KOH (pH 7.4), 350 mM KCl, 5 mM $MgCl_2$, 1% IGEPAL CA-630, 0.5 mM DTT, 100 μg/mL cycloheximide, and rRNasin RNase inhibitors (Promega, Madison, WI). Bound mRNA was extracted and DNase treated using Absolutely RNA Nanoprep kit (Agilent Technologies, Santa Clara, CA) as per manufacturer's instructions. RNA from cleared lysate was also purified as "Pre-IP" sample. RNA quantity was measured with a

Table 2
qPCR Primers

Primer name	Sequence (5′–3′)
β-actin1-e2f	ATCGCTGCCCTGGTCGTTGA
β-actin1-e2f	CCTCAGGGGCAACACGGAGC
dct-f	ACCTGTGACCAATGAGGAGATT
dct-r	TACAACACCAACACGATCAACA
hbae3-f1	AGCTGAGGAGATCGGCCGTGA
hbae3-r1	TTCTCGGACAGGGCCAGGCT
mitfa-f2	AAAGACCTGATGGCTTTCCA
mitfa-r2	TGGTGCCTTTATTCCACCTC
mylz2-e4f	CAAGGAAGCCAGCGGCCCAA
mylz2-e7r	GGGGAAGGCGGCCCACAGAT
try-e2f	GCCGCTCCTCTGGGAGACGAT
try-e3r	CCAGACGCACCTGGACACGG
tyr-f2	GTGTGCACGGATGAGCTG
tyr-r2	CTCGCGGAGGTTGTAACTTT
tyrp1b-e3f	TGCTTGGCGACCCGTCGTTT
tyrp1b-e5r	GGACGGGCCACGTTACCAGC

Nanodrop 1000 spectrophotometer and quality was assayed on an Agilent 2100 Bioanalyzer.

Quantitative RT-PCR

cDNA was synthesized from 25 ng of total RNA using the qScript cDNA Supermix (Quanta Bioscience, Gaithersburg, MD) according to manufacturer's protocol. About 2.5 ng of cDNA was used for each qPCR reaction and all samples were run in triplicate. qPCR was carried out in an Applied Biosystems ViiA™ 7 Real-Time PCR System using PerfeCTa® SYBR® Green Fast Mix (Quanta Biosciences, Gaithersburg, MD). PCR was conducted following standard cycling conditions (initial hold of 95°C for 20 s, then 40 cycles of 95°C for 1 s and 60 C for 20 s) and a continuous melt curve generated at 95 s for 15 s, 60 s for 1 min to determine product purity. All primers are listed in Table 2. Primers that did not yield product in at least two replicates prior to 35 cycles were excluded from further analysis. Samples where the standard deviation of CT was greater than 0.7 cycles were also excluded from further analysis. Data analysis was done by the comparative $\Delta\Delta Ct$ method to determine changes in gene expression. All data were normalized to β-actin as the endogenous control and lysate (Pre-IP) sample as the reference sample. All statistical analyses were performed in Microsoft Excel. A two-tailed paired *t*-test of ΔCt values from three biological replicates was used to calculate *P*-values.

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