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RESEARCH REPORT

TECHNIQUES AND RESOURCES

Translational profiling through biotinylation of tagged ribosomes in zebrafish

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ABSTRACT

Heterogeneity within a population of cells of the same type is a common theme in metazoan biology. Dissecting complex developmental and physiological processes crucially relies on our ability to probe the expression profile of these cell subpopulations. Current strategies rely on cell enrichment based on sequential or simultaneous use of multiple intersecting markers starting from a heterogeneous cell suspension. The extensive tissue manipulations required to generate single-cell suspensions, as well as the complexity of the required equipment, inherently complicate these approaches. Here, we propose an alternative methodology based on a genetically encoded system in the model organism *Danio rerio* (zebrafish). In transgenic fish, we take advantage of the combinatorial biotin transfer system, where polysome-associated mRNAs are selectively recovered from cells expressing both a tagged ribosomal subunit, Rpl10a, and the bacterial biotin ligase BirA. We have applied this technique to skeletal muscle development and identified new genes with interesting temporal expression patterns. Through this work we have thus developed additional tools for highly specific gene expression profiling.

KEY WORDS: TRAP, Gene expression profiling, Muscle development, BirA

INTRODUCTION

Cellular diversity is a defining feature of multicellular organisms. Growth and development requires the production of various cell types from undifferentiated progenitors that differ in fates and behaviors. Differential gene expression patterns maintain and refine this heterogeneity. Genome-wide assessment of cell type-specific expression patterns is thus central to our understanding of development, physiology and disease. The constraints associated with the currently available methods for cell type-specific RNA isolation calls for the implementation of novel strategies.

Most commonly used methods rely on cell-based purification strategies (Heiman et al., 2008). The target cell population is enriched from a heterogeneous cell suspension typically through fluorescence-activated cell sorting (FACS), although other methods of cell enrichment based on affinity purification (Wysocki and Sato, 1978) have also been employed. RNA is isolated from the purified cell population in a second step. A key asset of this methodology is that multiple markers can be evaluated, refining the cellular subtypes that can be isolated. However, because of the time required to isolate cell populations and the harsh dissociation methods often required to generate a single cell suspension, the cellular stress response must be considered.

To overcome these limitations, alternative strategies aimed at recovering the target RNA directly from intact complex tissues have been implemented. Genetically encoded phosphoribosyltransferase (UPRT) biosynthetically labels nascent RNA in the target cell type when provided with 4-thiouracil. Chemically modified RNA molecules can then be affinity purified, a technique called TU-tagging (Miller et al., 2009; Gay et al., 2013). Alternatively, mRNA-binding proteins can be affinity tagged in specific cells and used as anchors to co-purify associated mRNAs. Both poly(A)-binding proteins (Roy et al., 2002; Kunitomo et al., 2005) and ribosomal proteins (Heiman et al., 2008) have been successfully employed to measure cellular mRNAs. This latter technique, translating ribosome affinity purification (TRAP), the purification of labeled polysomes for subsequent analysis of actively translating mRNAs, has the additional advantage of assaying translating mRNAs, and not total cellular pools, which should more accurately represent protein expression. A common limitation of those methods compared with FACS is that they currently rely on a single genetically encoded transgene. For example, recent applications of TRAP in zebrafish use the *cmlc2* promoter (Fang et al., 2013) or the *ftyrp* promoter (Tryon et al., 2013) to drive expression of EGFP-Rpl10a.

Here, we describe a combinatorial version of the TRAP method where polysome-associated RNA is selectively recovered from cells with intersecting expression of two independently encoded transgenes. The first transgene controls the expression of an Avi (biotin ligase recognition peptide)-tagged Rpl10a ribosomal protein that serves as the substrate for the biotin transfer reaction controlled by a second transgene: the bacterial biotin ligase BirA. We have implemented this technology in zebrafish embryos, which for the study of vertebrate developmental genetics possess several advantages, including external fertilization, optical transparency, rapid organ development and facile transgenesis. Additionally, we have generated vectors for Tol2-based transgenesis of specific BirA and TRAP zebrafish lines and tested this system on skeletal muscle development.

RESULTS AND DISCUSSION

BirA activity in zebrafish embryos

We first sought to determine whether the biotin ligase system would be functional in zebrafish. We transiently expressed Avi-tagged EGFP and BirA in zebrafish embryos exposed to exogenous biotin and assessed the presence of biotinylated proteins with streptavidin-HRP (Fig. 1A) and streptavidin-Alexa Fluor 647 (supplementary material Fig. S1). We found that in addition to at least three endogenously biotinylated proteins (with approximate molecular

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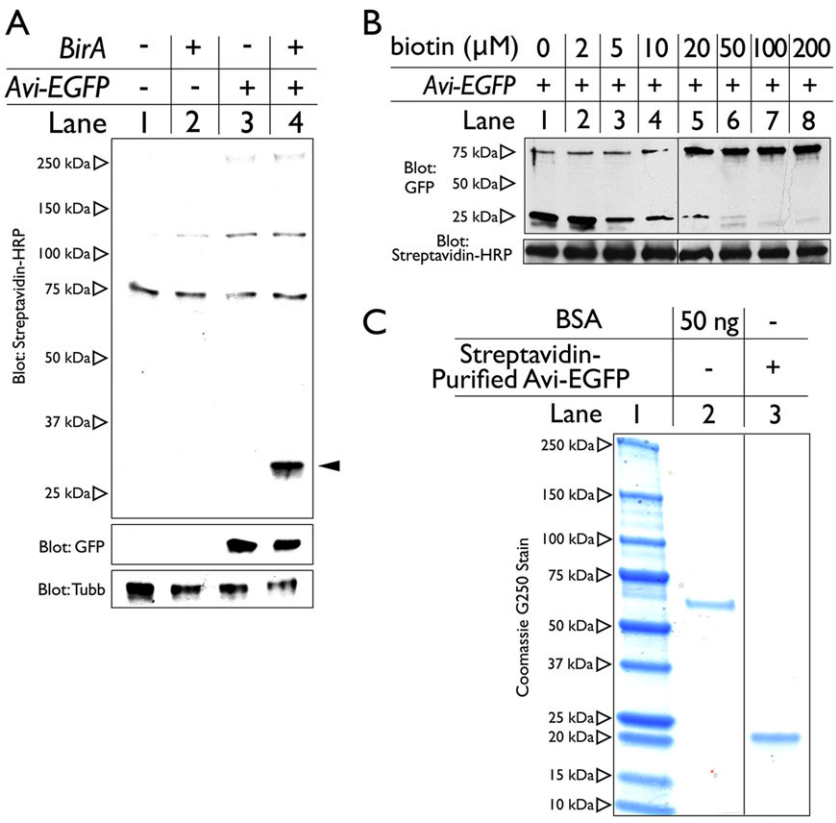

Fig. 1. Specific biotinylation of Avi-tagged EGFP in zebrafish embryos. (A) Avi-tagged EGFP (arrowhead) is selectively biotinylated when co-expressed with BirA, as shown by SDS-PAGE and streptavidin-HRP blotting (upper panel) of lysates from zebrafish embryos transiently expressing Avi-EGFP and BirA. The middle panel is an anti-GFP antibody blot confirming expression of Avi-EGFP. The bottom panel is an anti-β-tubulin (Tubb) antibody blot shown as a loading control. BirA and Avi-EGFP were transiently expressed by co-injection of 500 pg of *in vitro* transcribed mRNA into one-cell stage zebrafish embryos. Lysates for blotting analysis were generated from 24 hpf embryos. (B) Streptavidin gel shift assay (upper panel, anti-GFP antibody blot) performed on zebrafish embryos treated with indicated amounts of exogenous biotin following transient expression of Avi-EGFP and BirA. The lower panel is a streptavidin-HRP blot of the endogenously biotinylated 130 kDa protein shown as a loading control. Transient expression was performed as in A. (C) SDS-PAGE and Coomassie Blue G250 staining of streptavidin-agarose purified Avi-EGFP from lysates generated from 50 zebrafish embryos transiently expressing Avi-EGFP and BirA. Lane 2 contains 50 ng of BSA for comparison. Transient expression was performed by mRNA injection as in A. Thin lines in B,C mark positions where lanes from the same gel have been spliced together.

weights of 75, 130 and 260 kDa), a single streptavidin-reactive band consistent with the anticipated molecular weight of biotinylated EGFP could be readily detected only when Avi-tagged EGFP and the BirA ligase were co-expressed (arrowhead, Fig. 1A).

To confirm the identity of the streptavidin-reactive band, and to investigate the efficiency of the biotinylation process and determine if exogenous biotin is required, we performed a streptavidin shift assay on samples from embryos exposed to biotin ranging from 2 to 200 μM (Fig. 1B). In this assay, after co-expression of Avi-EGFP and BirA, zebrafish lysates were incubated with an excess of unlabeled streptavidin resulting in a ∼53 kDa shift in apparent molecular weight when biotinylated. GFP immunodetection in such lysates revealed that in the presence of 50 μM of exogenous biotin, Avi-tagged EGFP appeared to be completely biotinylated (Fig. 1B, lane 6). A streptavidin-HRP blot of the endogenously biotinylated 130 kDa protein is shown as a loading control.

To determine whether we could recover biotinylated proteins from zebrafish lysates, we transiently biotinylated Avi-EGFP *in vivo* and performed a pull down using streptavidin agarose from a lysate of 50 zebrafish embryos (Fig. 1C). The purified product was eluted, separated by SDS-PAGE, stained with Coomassie Blue G250 (lane 3) and compared with 50 ng of BSA (lane 2), indicating that ∼1 ng of Avi-EGFP protein could be purified per embryo. We therefore conclude that the BirA ligase is functional, at least transiently, in early zebrafish embryos, that ectopic expression of the bacterial enzyme results in no discernible developmental defects and that the Avi-tag may be used to purify fusion proteins.

Biotinylation of tagged ribosomes in zebrafish

Previous studies have shown that EGFP fused to the N terminus of the large subunit ribosomal protein L10a is compatible with its incorporation into functional polysomes in mammalian cells and in zebrafish (Heiman et al., 2008; Fang et al., 2013; Tryon et al., 2013). We evaluated whether an N-terminal Avi-tagged version of EGFP-Rpl10a would be similarly incorporated into functional ribosomes and would act as a suitable substrate for the biotin ligase BirA *in vivo* through transient expression assays similar to those described above.

Probing zebrafish lysates with streptavidin-HRP revealed a reactive band compatible with biotinylated Avi-tagged EGFP-Rpl10a when BirA and Avi-EGFP-Rpl10a were co-expressed (arrowhead, Fig. 2A) suggesting that the Avi-tag is accessible to the biotin ligase in the ribosomal fusion protein. To evaluate whether the biotinylated ribosomal subunit was incorporated into functional ribosomes, we performed streptavidin affinity purification of biotinylated proteins under native conditions. Under conditions where the TRAP is expected to function, i.e. when both the ligase and the tagged ribosomal protein are co-expressed, we could co-purify proteins with molecular weights corresponding to ribosomal proteins (Fig. 2B; see also supplementary material Fig. S3 and Table S1 for further validation) as well as ribosomal RNA (Fig. 2C), indicating that the biotinylated ribosomes can be incorporated into functional polysomes.

A binary genetically encoded ribosome biotinylation system in zebrafish

After demonstrating that transiently expressed Avi-EGFP-Rpl10a was biotinylated in zebrafish, we tested whether this system could be used with stable transgenic lines. We generated a line stably expressing BirA-EGFP driven by the *ubiquitin b* promoter, *Tg(ubb:birA-EGFP)*^{s972} (Fig. 3A, supplementary material Fig. S2), as well as a skeletal muscle-specific tagged ribosome line, *Tg(actc1b:Avi-EGFP-rpl10a)*^{s973} (Fig. 3A). Confocal imaging revealed that ribosomes were distributed throughout the multinucleated skeletal muscle cells, which appeared to be unaffected by transgene expression, and that localized fluorescence patterns in the

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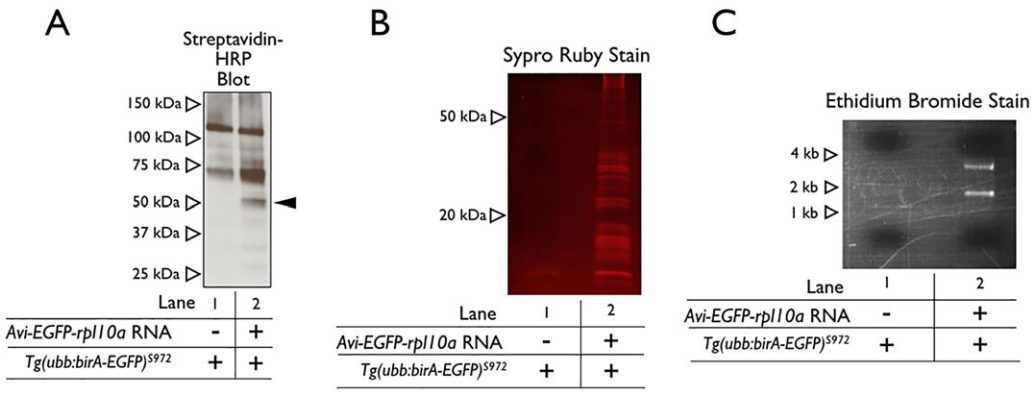

Fig. 2. Biotin ligase-acceptor peptide combinatorial TRAP in zebrafish. (A) Streptavidin-HRP blot of lysates generated from *Tg(ubb:birA-EGFP)*^{s972} embryos transiently expressing Avi-EGFP-Rpl10a (arrowhead). Transient expression was performed by injecting 300 pg of *in vitro* transcribed mRNA into one-cell stage embryos. Blots represent protein from 10 de-yolked embryos harvested at 24 hpf. (B) Sypro Ruby protein stain of eluates separated by SDS-PAGE following TRAP performed on *Tg(ubb:birA-EGFP)*^{s972} embryos transiently expressing Avi-EGFP-Rpl10a. Transient expression was performed as in A, and represents protein from 100 embryos. (C) Ethidium bromide stain of eluates separated electrophoretically following TRAP and Trizol extraction performed on *Tg(ubb:birA-EGFP)*^{s972} embryos transiently expressing Avi-EGFP-Rpl10a. Transient expression was performed as in A and represents RNA from 100 embryos.

nucleus appeared to be consistent with import to the nucleolus, indicating that Avi-tagged Rpl10a was being incorporated into ribosomes (Krüger et al., 2007). To determine the efficiency of biotinylation of skeletal muscle Avi-EGFP-Rpl10a when exposed to BirA expressed under control of the *ubb* promoter, we compared *Tg(ubb:birA-EGFP)*^{s972} or *Tg(actc1b:Avi-EGFP-rpl10a)*^{s973} crossed with wild-type fish with *Tg(ubb:birA-EGFP)*^{s972}crossed with *Tg(actc1b:Avi-EGFP-rpl10a)*^{s973} by streptavidin shift assay (Fig. 3B). Efficient biotinylation is indicated by the complete shift of Avi-tagged Rpl10a (arrowhead, Fig. 3B, lane 3). *Tg(ubb:birA-EGFP)*$^{s972/+}$ and *Tg(actc1b:Avi-EGFP-rpl10a)*$^{s973/+}$ were then crossed, and the offspring were harvested at 1.5 dpf and sorted into two pools based on the presence of skeletal muscle fluorescence. TRAP was then performed on the offspring of this cross (using the protocol described in supplementary material Fig. S5), and RNAs and proteins were extracted and subjected to electrophoretic separation (Fig. 3C). Protein gels were stained with Sypro Ruby and revealed that many proteins, generally smaller than 50 kDa, were specifically isolated by the TRAP (Fig. 3C, compare lane 1 with lane 2). Ethidium bromide stained agarose gels revealed bands characteristic of ribosomal RNA (Fig. 3C, compare lane 3 with lane 4). To confirm that the bands in Fig. 3C were ribosomal proteins, we performed immuno-blotting for Rpl7a (supplementary material Fig. S3) and mass spectrometric analysis and recovered at least one peptide from 27 out of 32 proteins that make up the small ribosomal subunit and 26 out of 47 proteins that make up the large subunit (Fig. 3D, supplementary material Table S1) (Nakao et al., 2004). Conspicuously absent from this list was Rpl10a itself. The lack of the tagged ribosomal protein is not surprising, however; the conditions under which the ribosomes were eluted (high salt) are not harsh enough to elute biotinylated proteins from the streptavidin column.

To test whether we could detect mRNAs using the combinatorial BirA and Avi-tagged Rpl10a system, we crossed *Tg(ubb:birA-EGFP)*^{s972} to *Tg(actc1b:Avi-EGFP-rpl10a)*^{s973} and performed TRAP on 48 hpf embryos. We then reverse transcribed RNA purified from samples that were eluted from the streptavidin column, as well as input samples. We then assayed by qPCR five non-skeletal muscle genes (*tie1*, *cmlc2*, *pax7a*, *pax7b* and *efnb3b*) and five genes known to be expressed in skeletal muscle (*myod1*, *myog*, *myf5*, *myf6* and *myl1*), and found that the relative enrichment of mRNA bound to the column was greater for the skeletal muscle-specific genes (Fig. 3E), indicating that this method is useful for separating mRNAs from cells that may be tightly associated with skeletal muscle, such as endothelial cells (*tie1*) and satellite cells (*pax7a* and *pax7b*).

Upon confirmation that we could biotinylate and purify translating ribosomes in skeletal muscle using the combinatorial TRAP, we set out to apply this approach to determine which genes are being actively translated in skeletal muscle during myoblast fusion. We crossed *Tg(ubb:birA-EGFP)*^{s972} with *Tg(actc1b:Avi-EGFP-rpl10a)*^{s973} and harvested embryos at four time points (21, 24, 27 and 34 hpf) surrounding the beginning of myoblast fusion in zebrafish (24 hpf) (Moore et al., 2007). Ribosome-associated mRNAs were purified and analyzed by microarray (Fig. 4). Fig. 4A depicts the relative ribosome association of individual mRNAs at 21 hpf on the *x*-axis plotted versus 34 hpf on the *y*-axis. As expected, most genes fall along the 45° line, i.e. their ribosome-association is not differentially regulated during this developmental window. However, a subset of genes was found to be either up- or downregulated between 21 and 34 hpf. Well-known skeletal muscle genes, such as *mylpfb*, *myoz1b*, *atp2a1*, *myhz2*, *ckmb*, *acta1b*, *tnnt3a*, *tnni2a.4* and *pvalb3* were upregulated (Fig. 4A, red circles; supplementary material Table S2) indicating that the TRAP was indeed assaying expression in developing skeletal muscle. Downregulated genes (Fig. 4A, blue circles; supplementary material Table S2) include *mespaa*, *msgn1*, *tbx16*, *ripply2*, *lft1*, *her1*, *her5*, *cdx4*, *wnt8a*, *tbx6* and *ved*, which are associated with mesoderm-related tissue specification and non-skeletal muscle cells (Sokol et al., 1991; Chapman et al., 1996; Muller et al., 1996; Griffin et al., 1998; Bisgrove et al., 1999; Bally-Cuif et al., 2000; Sawada et al., 2000; Yoon and Wold, 2000; Henry et al., 2002; Shimizu et al., 2002; Wang et al., 2005; Davidson and Zon, 2006; Morimoto et al., 2007), and are, as expected, expressed to a lesser extent in later skeletal muscle development.

To examine the changes over time in more detail, we plotted the relative ribosome association at 21, 24, 27 and 34 hpf for several interesting genes from the skeletal muscle TRAP (Fig. 4B, supplementary material Fig. S4). *tie1* encodes an endothelial cell-specific receptor tyrosine kinase (Lyons et al., 1998), and, as expected, is minimally expressed. *murca*, *murcb*, *rtn2a*, *rtn2b* and *tgm2a* were found to be upregulated throughout the time course, indicating a potential role in muscle development. To confirm that these mRNAs with novel regulation were skeletal muscle specific,

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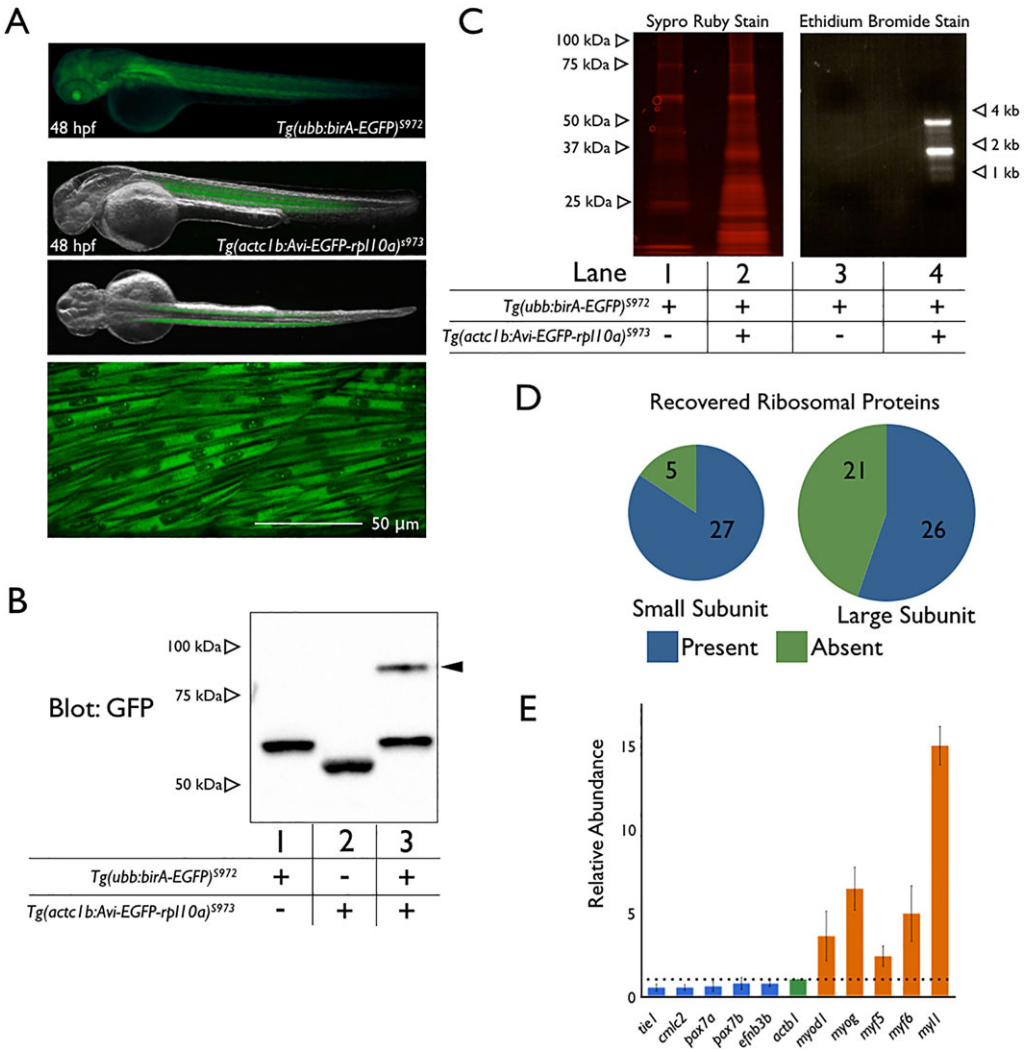

Fig. 3. Skeletal muscle-specific translating ribosome purification in zebrafish embryos. (A) Fluorescence image of Tg(ubb:birA-EGFP) expression (upper panel) at 48 hpf. Merged fluorescence and bright-field images of Tg(actc1b:Avi-EGFP-rpl10a) expression at 48 hpf in the middle two panels show lateral and dorsal views. The bottom panel is a confocal image of Tg(actc1b:Avi-EGFP-rpl10a) expression in skeletal muscle. Anterior is towards the left; dorsal towards the top. (B) Streptavidin shift assay with lysates from 15 embryos from *Tg(ubb:birA-EGFP)*^{s972} crossed with wild type (lane 1), from *Tg(actc1b:Avi-EGFP-rpl10a)*^{s973} crossed with wild type (lane 2), or from *Tg(ubb:birA-EGFP)*^{s972} crossed with *Tg(actc1b:Avi-EGFP-rpl10a)*^{s973} (lane 3). Arrowhead indicates Avi-EGFP-Rpl10a bound with free streptavidin. Protein from four larvae at 4 dpf was applied to each lane. (C) Eluates from the skeletal muscle TRAP performed on 200 embryos (36 hpf) from *Tg(ubb:birA-EGFP)*^{s972} crossed with *Tg(actc1b:Avi-EGFP-rpl10a)*^{s973}. Eluates were separated by SDS-PAGE and stained with Sypro Ruby protein stain (lane 1 is from sibling controls, whereas lane 2 is from embryos expressing both transgenes). Lanes 3 (sibling controls) and 4 (TRAP embryos) depict an ethidium bromide stained agarose gel following Trizol extraction. (D) Illustration of ribosomal proteins with at least one peptide recovered by mass spectrometry analysis of skeletal muscle TRAP eluates. The full list is provided in supplementary material Table S1. (E) qPCR of skeletal muscle and non-skeletal muscle genes from reverse transcribed mRNA purified by TRAP or from whole lysate (input) from 48 hpf embryos generated by crossing *Tg(ubb:birA-EGFP)*^{s972} to *Tg(actc1b:Avi-EGFP-rpl10a)*^{s973}. The data are shown as relative abundance of purified mRNA divided by total input mRNA (i.e. TRAP/input) normalized to *act1b*. Error bars represent s.e.m. from three independent experiments.

we performed *in situ* hybridizations and found that *murca*, *murcb*, *rtn2a*, *rtn2b* and *tgm2a* mRNAs were localized to the somites in 28 hpf zebrafish embryos (Fig. 4C).

Conclusions

We have applied a combinatorial biotin ligase-acceptor peptide TRAP to muscle development where biotin ligase expression was driven by the *ubiquitin* promoter and the *Avi-EGFP-rpl10a* gene was under the control of the skeletal muscle-specific *actc1b* promoter. Although the TRAP methodology is limited by how well promoters have been defined as well as the time required to generate the transgenic organisms, the development of the Tol2 transposase system has improved the efficiency of generating transgenic fish (Urasaki et al., 2006), mitigating some of the limitations associated with TRAP. The skeletal muscle TRAP described here provides a proof-of-principle test of the combinatorial biotin ligase and acceptor peptide-based TRAP during vertebrate embryogenesis. The combinatorial TRAP allows one to define a target cell population for analysis using two markers, as is possible with FACS, but retains the advantages associated with TRAP, such as minimal tissue manipulations, rapid cell lysis and assessment of ribosome-associated mRNAs and not total mRNA pools. Currently, we are generating additional stable TRAP and BirA lines to answer more detailed developmental questions. The increased specificity in gene expression analysis will aid in the discovery of novel biomarkers and targets for drug screening, suggest ways to

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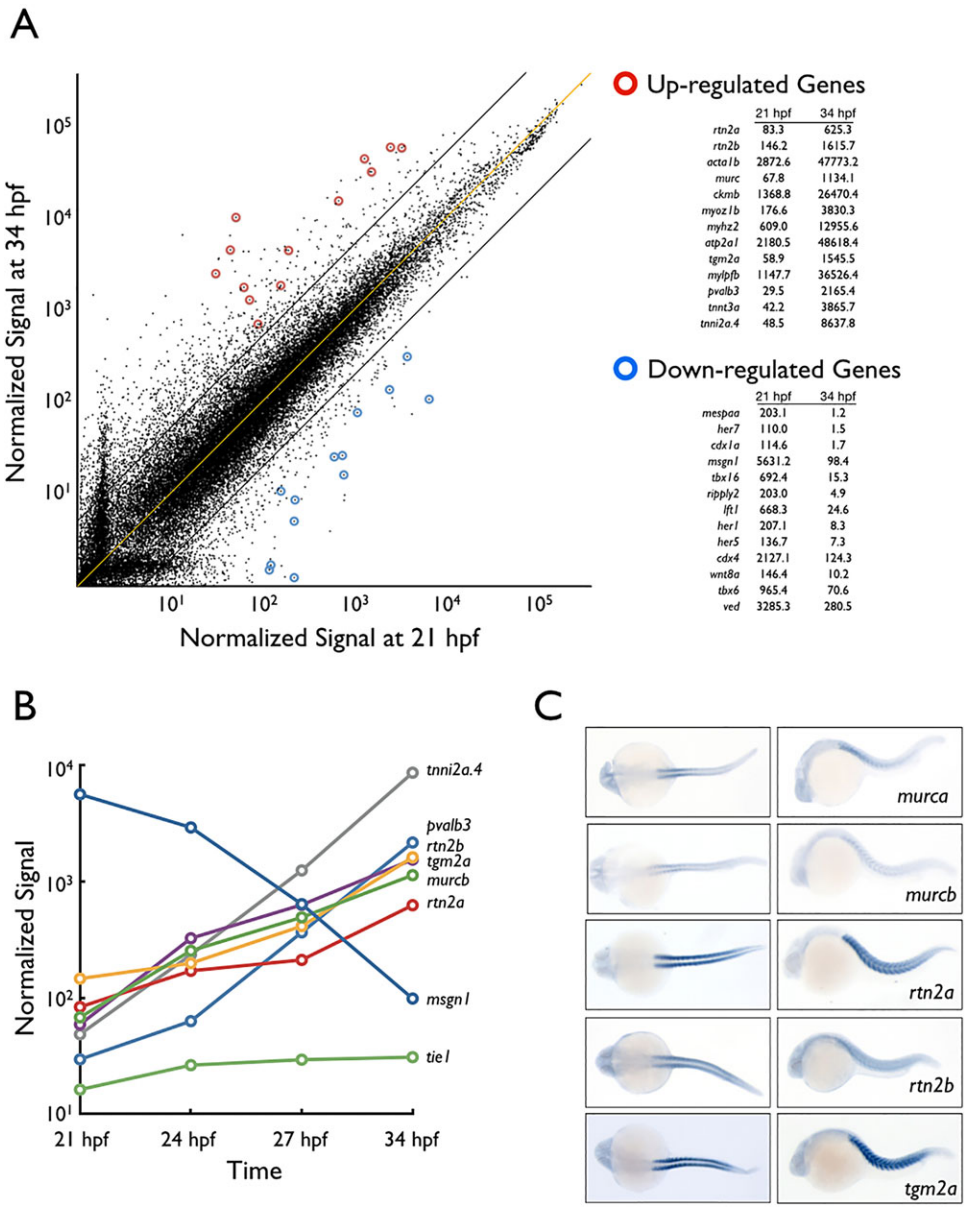

Fig. 4. Translational profiling of zebrafish skeletal muscle. (A) Expression profile of 21 hpf versus 34 hpf skeletal muscle-specific TRAP mRNA assayed by microarray hybridization. Dots represent normalized hybridization signals of individual genes plotted on a log scale. Blue circles indicate downregulation over time whereas red circles indicate upregulation. The yellow line represents no difference in expression between samples whereas the black lines depict a fivefold change. (B) Timecourse of relative expression (log scale, *y*-axis) of selected genes from A. *tie1* is an endothelial-specific gene and is minimally expressed. *mesogenin* (*msgn1*) is an example of a gene whose expression is downregulated during skeletal muscle development. (C) Whole-mount *in situ* hybridization at 28 hpf of selected upregulated mRNAs. Dorsal (left) and lateral (right) views.

challenge mutants generated by reverse genetic approaches, and further our understanding of cell-fate decisions, as well as cell behavior and function.

MATERIALS AND METHODS

DNA constructs, RNA synthesis and zebrafish lines

TRAP plasmids are based on the mini Tol2 transposase vectors (Urasaki et al., 2006) and are available at Addgene. See supplementary methods for details.

Antibodies, staining, blotting, gel shift assays and protein purification

Streptavidin shift assays were performed by lysing 10 embryos in Laemmli buffer, boiling for 5 minutes, then incubating with 30 µg streptavidin (Sigma) prior to SDS-PAGE and anti-GFP blotting. See supplementary methods for details.

TRAP

Embryos were collected in egg water supplemented with 50 µM biotin. Prior to harvesting at indicated time points, embryos were washed for 30 min in egg water to remove unincorporated biotin. Following dechorionation, embryos were washed in cold Ringer's solution, pelleted in 1.5 ml tubes and snap frozen on dry ice. Samples were lysed in 0.5 ml ice-cold polysome buffer (supplementary material Fig. S5). Lysates were cleared by centrifugation and applied to 100 µl of streptavidin-agarose (Pierce) followed by rotation at 4°C for 1 h. Beads were then pipetted into microspin columns (Bio-Rad) and washed twice with 1 ml wash buffer. RNAs and ribosomal proteins were eluted with 0.2 ml elution buffer and immediately added to 1 ml Trizol reagent (Invitrogen).

Mass spectrometry and protein identification

Peptides were analyzed by using nano-LC-ESI-MS/MS maXis Impact UHR-TOF (Bruker, Bremen, Germany) coupled with a 2D-LC Dionex UltiMate 3000 (Thermo). See supplementary methods for details.

RT-PCR and microarray profiling

Two-color microarray hybridization was performed using Zebrafish V3 44K gene expression arrays (Agilent Technologies) and the data have been deposited at NCBI (GEO GSE59355). See supplementary methods for details.

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Whole-mount *in situ* hybridization

Antisense probes were generated from cDNA using PCR primers listed in supplementary material Table S3. PCR products were cloned into pGEM-T Easy (Promega) and whole-mount *in situ* hybridizations were performed according to established protocols (Thisse and Thisse, 2008).

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Competing interests

The authors declare no competing financial interests.

Author contributions

B.V. developed the concept. M.P.H., S.R. and B.V. designed the experimental set up. M.P.H., M.D., M.R. and B.V. performed experiments and data analysis. M.P.H., D.Y.R.S. and B.V. prepared and edited the manuscript. D.Y.R.S. supervised the work.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.111849/-/DC1

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