

SUPPLEMENTAL INFORMATION

Ribosomal proteins Rpl22 and Rpl2211 control morphogenesis by regulating pre-mRNA splicing

Yong Zhang, Monique N. O'Leary, Suraj Peri, Minshi Wang, Jikun Zha, Simon Melov, Dietmar J. Kappes, Qing Feng, Jennifer Rhodes, Paul S. Amieux, David R. Morris, Brian K. Kennedy, David L. Wiest

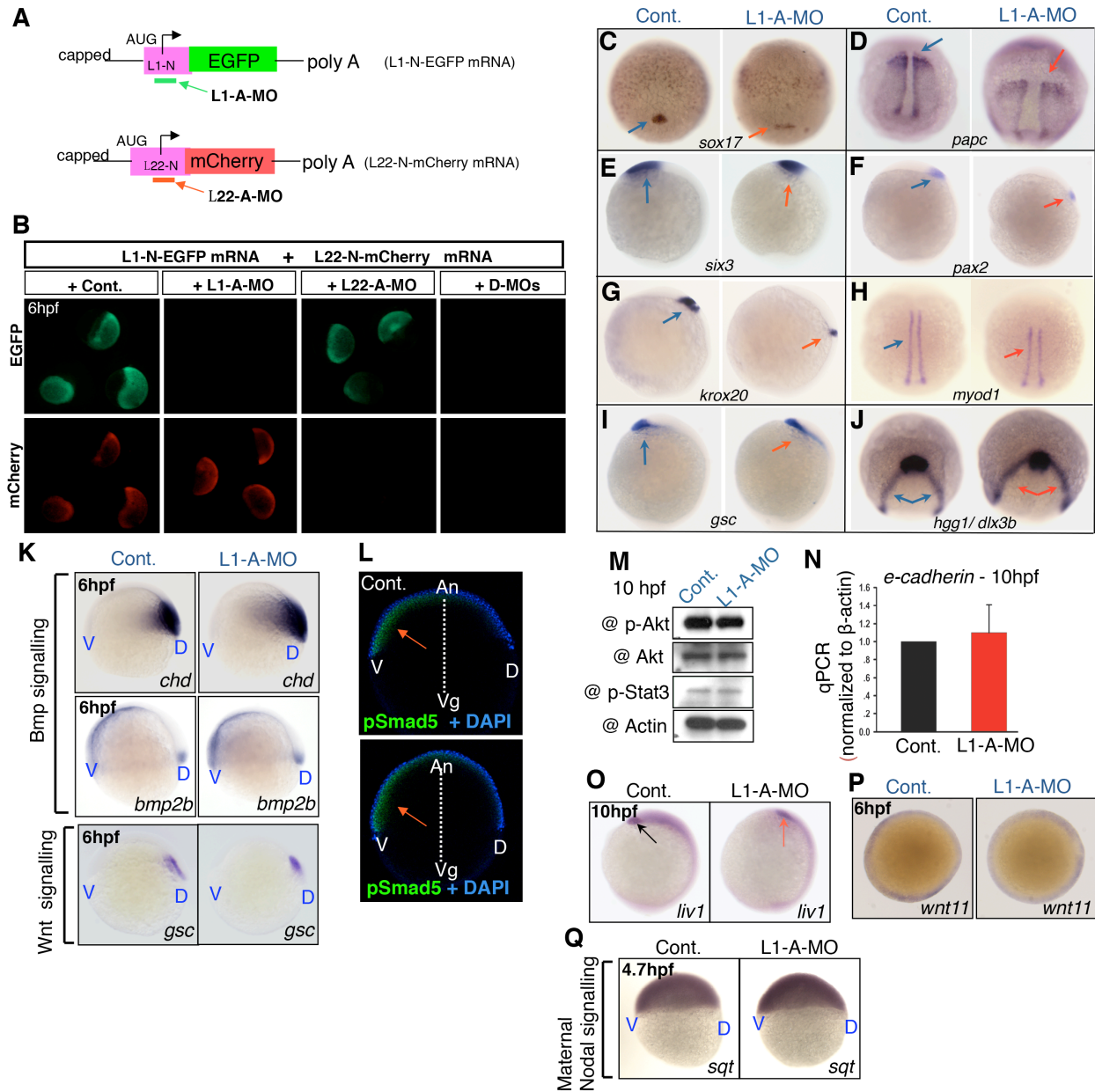


Figure S1, Related to Figure 1. Convergence and extension defects in Like1 morphants.

(A) Schematic of Like1-N-EGFP and L22-N-mCherry fluorescent reporter mRNAs containing the ATG-MO target sequence (lines under the pink boxes) fused in-frame with EGFP or mCherry. (B) Like1-ATG morpholino (L1-A-MO; 2ng), L22-ATG morpholino (L22-A-MO; 6ng) or both were co-injected with 100pg Like1-N-EGFP and 100pg L22-N-mCherry mRNAs. The fluorescence images of embryos were taken using either EGFP or mCherry filters under the fluorescent microscope at 6hpf and reveal that the Rpl22 and Like1 MO effectively repress the expression of their respective targets, but do not affect expression of their paralog. (C-J) Whole mount *in situ* hybridization (WISH) analysis of the expression of markers of developing tissues at 10hpf. (C) *sox17* marks endoderm. (D) *papc* marks paraxial mesoderm. (E) *six3*, marks forebrain. (F) *pax2* marks the mid-hind brain boundary. (G) *krox20* marks

the hindbrain. (H) *myod1* marks adaxial cells. (I) *gsc* marks anterior axial mesoderm. (J) *hgg1/dlx3b* marks the prechordal and neural plate. (K) WISH analysis at 6hpf of signaling pathways involved in early embryonic patterning and cell fate control, including *chd*, *bmp2b*, and *gsc*. D, dorsal; V, ventral. (L) Confocal section of phospho-Smad5 immunostaining (red arrows) and DAPI staining (blue) on 6hpf embryos. An, animal pole; Vg, vegetal pole. (M-P) Analysis of molecular effectors of gastrulation in Like1 morphants. Akt and Stat3 signaling were assessed by immunoblotting (M) in 10hpf embryonic protein lysates, *e-cadherin* by quantitative real-time PCR in 10hpf embryos (N), *liv1* expression by WISH analysis at 10hpf (O, lateral view) and *wnt11* expression by WISH at 6hpf (P, animal pole view). (Q) *Squint (sqt)* expression was examined by WISH at 30% epiboly (4.7hpf). All results are representative of three experiments performed.

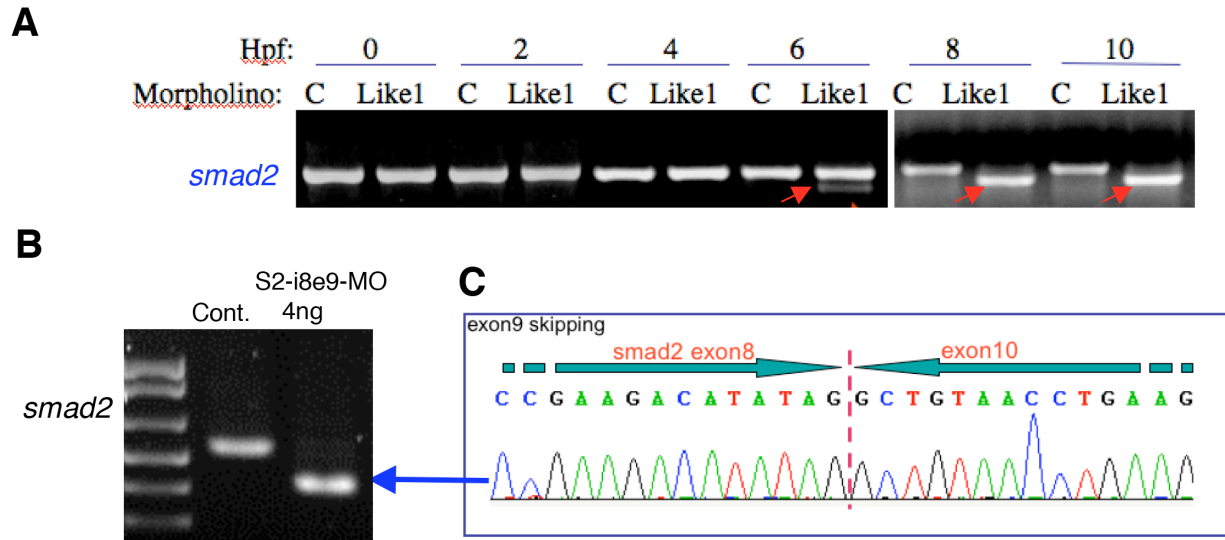


Figure S2, Related to Figure 2. The skipping of *smad2* exon9 begins after the onset of zygotic transcription.

(A) Time-course of the onset of *smad2* exon skipping in Like1 morphants. The extent of *smad2* mis-splicing was assessed by RT-PCR in control and Like1 morphant embryos at the indicated times. Mis-splicing becomes evident after the onset of zygotic transcription at 6hpf (red arrow), then becomes more pronounced at 8-10hpf (red arrows) in Like1 morphants. (B,C) MO-mediated induction of *smad2* exon9 skipping. The ability of the S2-i8e9-MO to induce *smad2* exon 9 skipping was verified by RT-PCR and sequencing in extracts from 10hpf embryos treated with 4ng S2-i8e9-MO (blue arrow). All results are representative of three experiments performed.

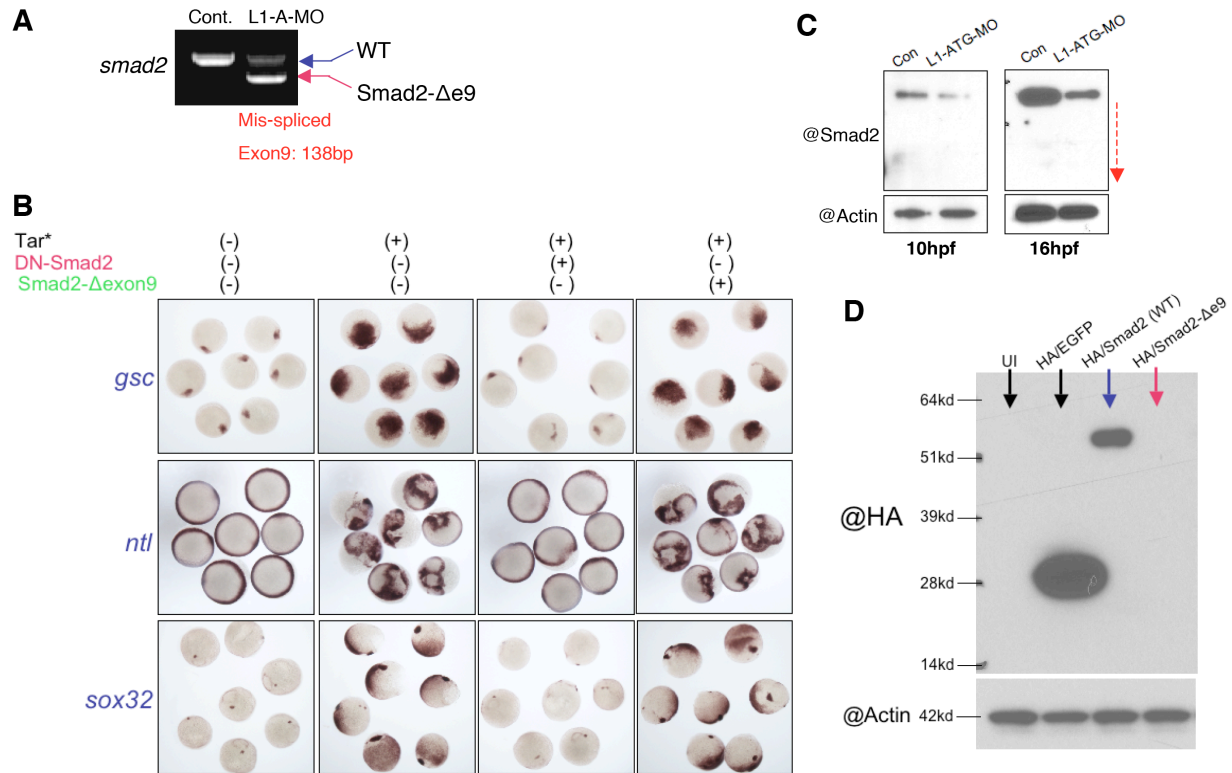


Figure S3, Related to Figure 3. Analysis of the function of the *smad2* splice variant lacking exon 9.

(A,B) Exon9 skipped *smad2* (*smad2Δe9*) does not function as a dominant negative mutant. (A) The ability of Like1 knockdown to induce *smad2* mis-splicing was verified by RT-PCR (red arrow). Elimination of exon9 removes 138bp but preserves the translational reading frame in the *smad2Δe9* variant. (B) 600pg of mRNA encoding the *smad2Δe9* variant was co-injected along with mRNA encoding Tar* mRNA (constitutively activated Alk4, 5pg) to determine if it inhibited ALK4 signaling, as assessed by WISH for markers of mesoderm (*gsc* and *ntl*) and endoderm (*sox32*) at 6hpf. mRNA encoding the *smad2Δe9* variant failed to inhibit the alterations in *gsc*, *ntl*, and *sox32* expression and localization induced by ALK4 signaling, while an equivalent amount of a dominant-negative *Smad2* mutant mRNA (DN-Smad2, S-A mutation) did inhibit these alterations. (C,D) The *smad2Δe9* splice variant fails to produce a stable, truncated protein. (C) Western blotting revealed that Like1 knockdown reduces total-Smad2 protein expression at 10hpf or 16hpf, but does not result in expression of a truncated form. (D) Injection of embryos with mRNA encoding HA-tagged intact *smad2* and the *smad2Δe9* splice variant revealed that despite retaining the translational reading frame, the *smad2Δe9* variant fails to produce a stable truncated protein. Embryos were analyzed by western blotting with anti-HA at 10hpf. All results are representative of three experiments performed.

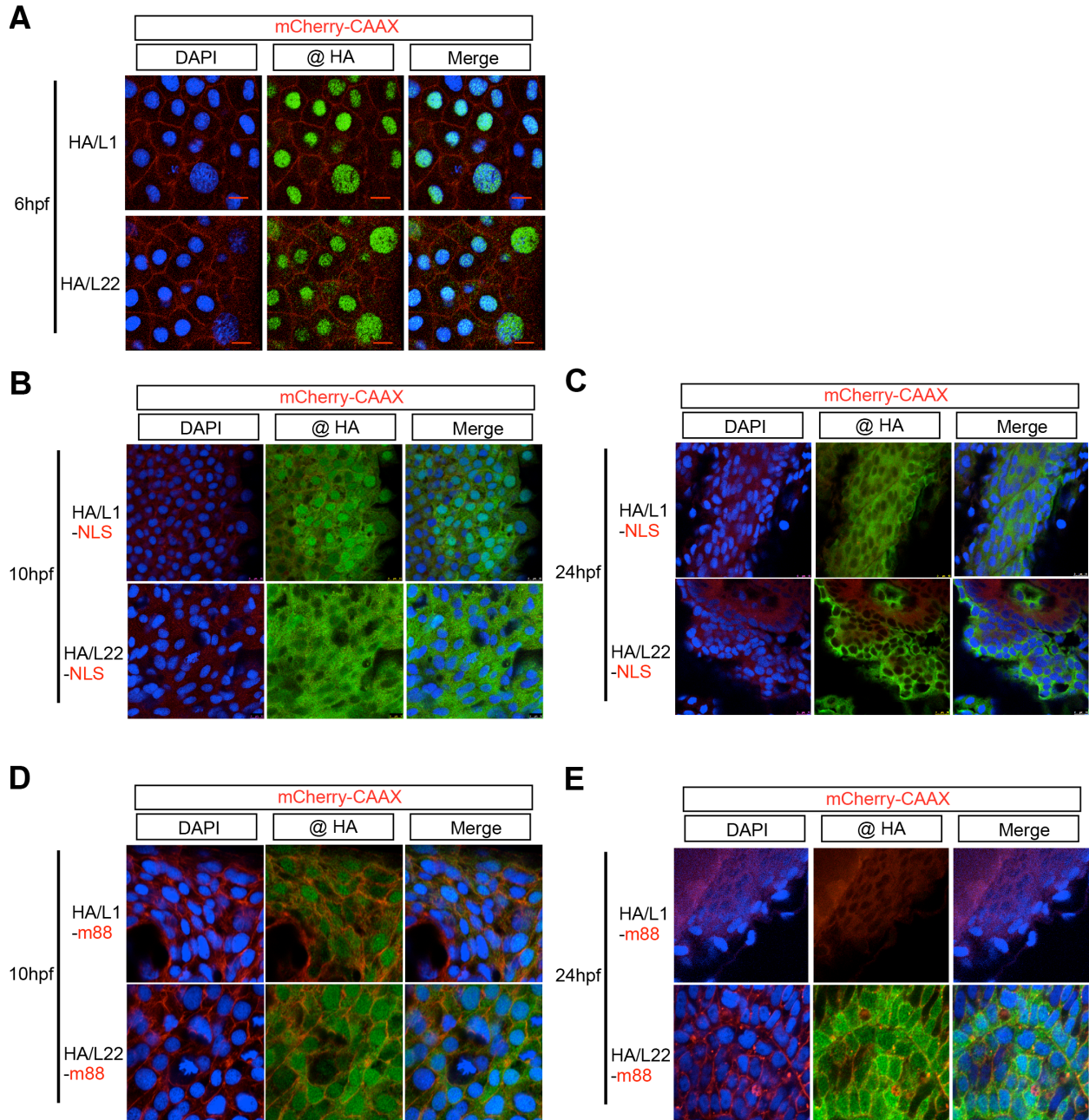


Figure S4, Related to Figure 4. Role of the NLS and RNA-binding motifs of Rpl22 and Like1 in controlling subcellular localization.

(A) The subcellular localization of Rpl22 and Like1 was assessed at the onset of gastrulation (6hpf). mRNA (100pg) encoding HA-zRpl22 (HA/L22) or HA/zLike1 (HA/L1) was co-injected with mCherry-CAAX (100pg) into 1-cell stage embryos and visualized by immunofluorescence using anti-HA antibody. DAPI marked nuclei; mCherry-CAAX marked the cell membrane; Scale bar =10 μ m. (B,C) Subcellular location of epitope-tagged NLS mutants of Rpl22 and Like1 at 10hpf (A) and 24hpf (B). 100pg of mRNA encoding HA-zRpl22-NLS and HA/zLike1-NLS were co-injected with mRNA encoding mCherry-CAAX into 1-cell stage embryos and visualized by HA antibody immunostaining. (D,E) Subcellular location of epitope-tagged RNA-binding mutants (m88) of zebrafish Rpl22 and

Like1 at 10hpf (D) and 24hpf (E). 100pg of mRNA encoding HA-zRpl22-m88 and HA/zLike1-m88 was co-injected with mCherry-CAAX mRNA into 1-cell embryos and visualized by anti-HA immunostaining. (B-E) All results are representative of three experiments performed.

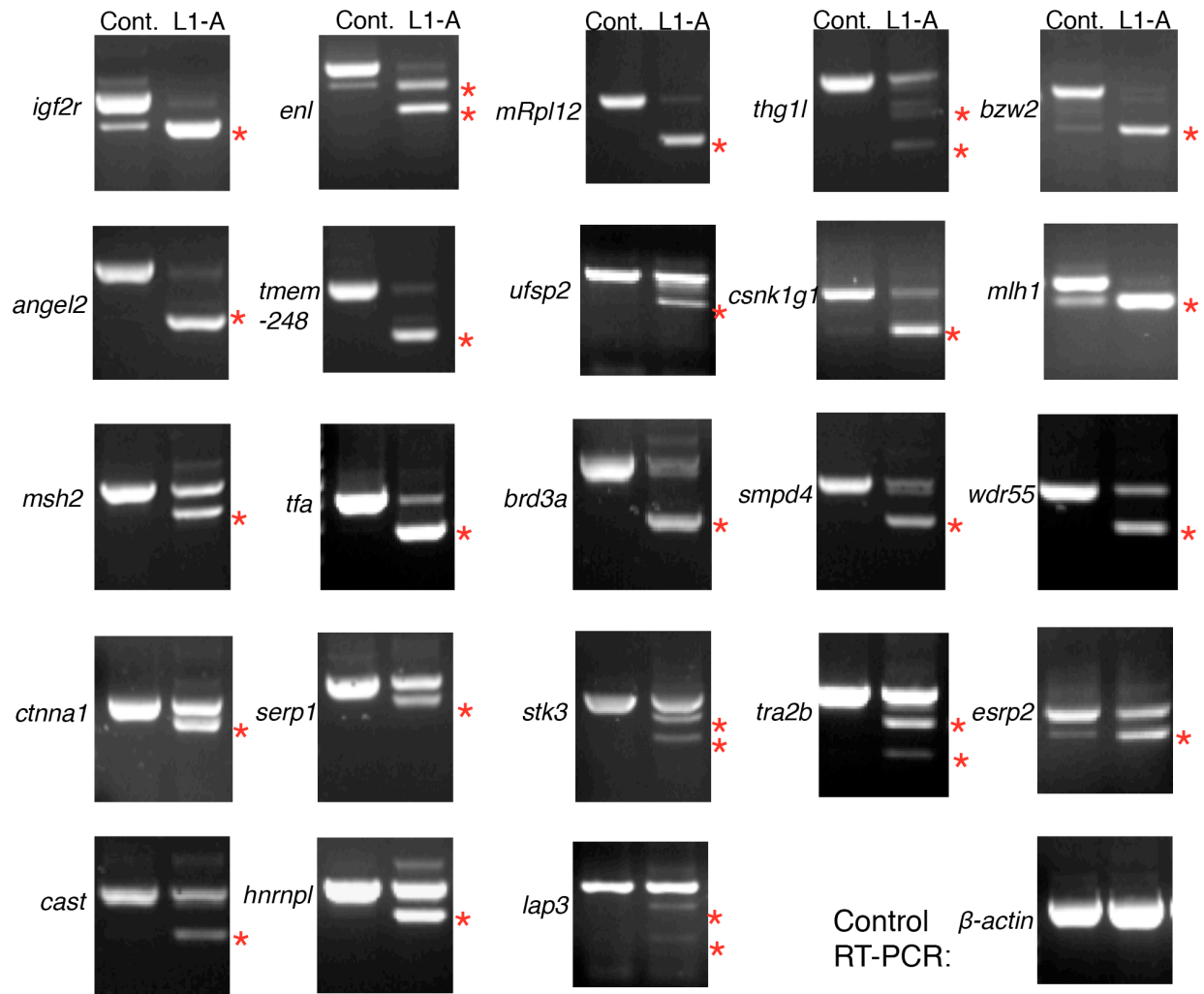
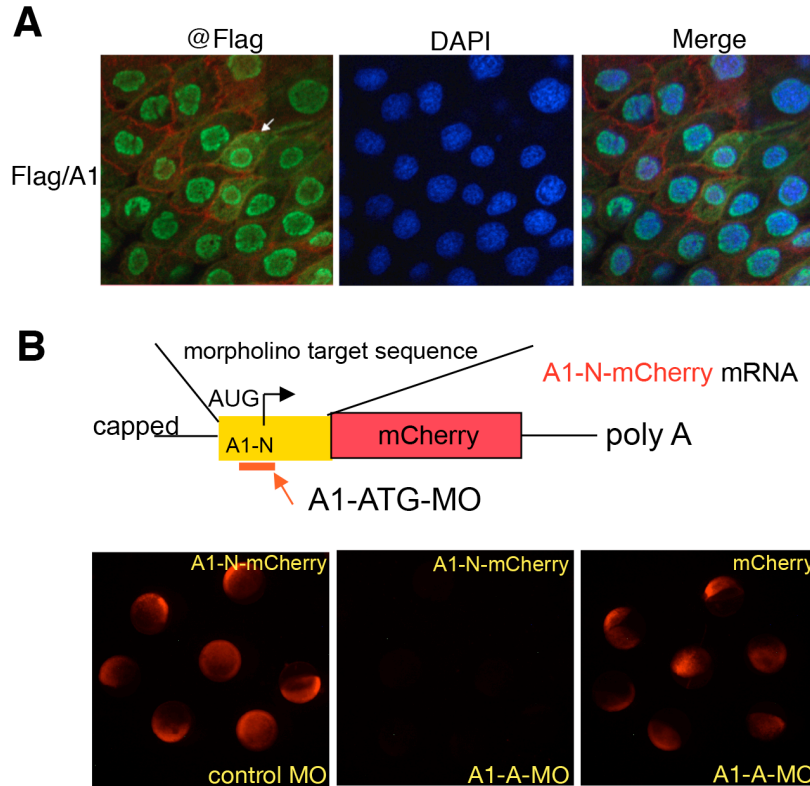


Figure S5, Related to Figure 6. Validation of mis-spliced mRNA targets identified in Like1 morphants by RNA-Seq.

Validation of mis-spliced Like1 targets revealed by RNA-Seq. Mis-splicing of mRNA targets was verified by RT-PCR in 10hpf Like1 morphants. *-indicates mis-spliced form. β -actin served as an RT-PCR and loading control. All results are representative of three experiments performed.



SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Morpholino Antisense Oligonucleotides (MO)

MOs (GeneTools) were designed against the ATG translational start sites for Like1 (AACAGTCTGCCTTTTCGGTGCCATC) and Rpl22 (CCGACAGTTTTGGCAGAAAGCCAGT). Sequences of other MO were as follows: zebrafish standard control morpholino (CCTCTTACCTCAGTTACAATTATA), *smad2* I8E9 splicing MO (TCGTCCTGATTCAAAACAAACAGCC), *hnrnpa1* start codon MO (GGCCCTCTTTGGACATCCTACCGAC). The MO were injected into 1-cell stage embryos at the indicated concentrations. The efficacy of each start site MO for translational silencing *in vivo* was tested by co-injection with mRNA encoding an EGFP or mCherry reporter bearing the MO-target sequence. The effectiveness of *smad2* I8E9 MO to induce mis-splicing was measured by RT-PCR.

Plasmids Construction, RNA Synthesis and Overexpression

N-terminal epitope tagged constructs were generated as follows. Full-length cDNA encoding epitope tagged HA-zRpl22 and HA-zLike1 were subcloned in the pCS2+. HA-tagged nuclear localization signal (NLS) mutants of zRpl22 (HA-zRpl22-NLS) and zLike1 (HA-zLike1-NLS) were generated by disrupting the N-terminal NLS motifs of Rpl22 (KKKK) and Like1 (KNKK) by mutating them to EEEE. RNA-binding mutant HA-tagged Rpl22 (HA-zRpl22-m88) and Like1 (HA-zLike1-m88) were generated by replacing the KKYLKK motifs with EEYLKE. These NLS and m88 mutants were constructed using the GeneTailor mutagenesis kit according to manufacturer's recommendations (Invitrogen, Carlsbad, CA). Additional cDNAs encoding the following HA or FLAG-tagged zebrafish cDNAs were cloned into pCS2+: wild-type and exon9-skipped *smad2* (*smad2*- Δ e9), and *hnrnpa1* (NM_200104.1). The epitope tags employed were not multimerized and all constructs were sequence-verified. The constructs encoding constitutively active TARAM-A (*Tar**) (Renucci et al., 1996), dominant negative zSmad2 (DN-zSmad2) (Jia et al., 2009), constitutively activated zSmad2 (Ca-zSmad2) (Dick et al., 2000), and membrane tagged mCherry-CAAX (Provost et al., 2007) were generously provided by zebrafish community. WISH probes and mRNAs were made from pCS2+ using the Ambion T3, T7 WISH probes kits and the mMessage mMachine kit (Ambion). The mRNAs were injected into 1-cell stage embryos at the indicated dosages.

Antibodies, Western blotting and Immunofluorescent staining

Zebrafish embryonic protein extraction and western blotting were carried out as described (Zhang et al., 2013). The following antibodies were already published to specifically detect zebrafish target proteins, and they were also validated and employed in this study: anti-phospho-Smad2 (Ser465/467, #3101, Cell Signaling), anti-total Smad2/3 antibody (#3101, Cell Signaling), anti-phospho-Smad1/5 (9511, Cell Signaling, specifically detects phospho-Smad5 during zebrafish early development), anti-phospho-STAT3 (Tyr705, D128-3, MBL international), anti-phospho-Akt (Ser473, #4058, Cell Signaling) and anti-Akt (#9272, Cell Signaling), anti-Actin (AC-40, Sigma), anti-HA tag (#3724, Cell Signaling), anti-Flag tag (#2368, Cell Signaling). For immunofluorescent staining, embryos at the indicated stages were fixed overnight in 4% paraformaldehyde at 4°C. After permeabilization with 0.1% Triton X-100 + 0.1% sodium citrate in PBS for 15 minute at room temperature (RT), embryos were blocked in 2% Goat

Serum, 2mg/ml BSA, 1% DMSO and 0.1% Triton in PBS, for 1 hour at RT. The embryos were then incubated overnight with rabbit anti-pSmad2 (#3101, Cell Signaling), and anti-HA (#3724, Cell Signaling) or anti-Flag antibody (#2368, Cell Signaling). Primary antibodies were followed with goat anti-rabbit Alexa 488 secondary antibody (1:1000; Molecular Probes, Life technologies) and antifade DAPI nuclear staining (Life technologies) before processing for confocal imaging. Embryos were embedded in 1% low-melting-point agarose for mounting. Images were acquired on Nikon C1 confocal laser scanning microscope equipped with a Plan Apo $\times 60$ oil objective.

Generation of *Rpl221*^{-/-} mice

An *Rpl221*-containing BAC clone was obtained from the BACPAC Resources Center (BPRC) and used as a template to amplify the left-arm (LA) and right-arm (RA) fragments (Primers: LA-Forward, GCGCGCTAGCTTCAGCTCGATCCTCTTGACAG; LA-Reverse, GCGGCCATATGGCGGACCTGGCCACTTAG; RA-Forward, GCGCGCTGCAGTTTCTTGGAATTCAGTGTCTAA G; RA-Reverse, GCGCGCTGCAGTGGCCATGAAATACACAATCTTAC). For the LA, a ~3.7kb fragment was generated, and NheI and NdeI sites on the 5' and the 3' ends, respectively were introduced to insert the LA into the Targeting Vector 4595 D8 (obtained from the lab of Richard Palmiter). For the RA, a ~2.7 kb fragment was generated and PstI sites introduced at both ends, allowing insertion into pBluescript II SK(+) (pBS-RA). One LoxP site was inserted into the NdeI site in the pBS-RA to create a Loxp site between the 3rd and 4th exons of *Rpl221*. The RA+Loxp fragment was cut out of pBS with Eco5 and SmaI and cloned into the PmeI site of the Targeting Vector containing the LA to complete the final targeting construct, which was then verified by sequencing and electroporated into R1 ES cells. Following G418 selection for neomycin resistance, plated cells were analyzed by PCR to verify correct targeting. ES cells were then injected into C57BL/6 blastocysts. High percentage male chimeras were mated to C57BL/6 females to generate heterozygous *Rpl221*^{Loxp/+} mice. To ubiquitously disrupt *Rpl221* expression, *Rpl221*^{Loxp/+} mice were mated to *Mox2-cre* mice to generate *Rpl221*^{Loxp/-} mice, which were subsequently bred to C57BL/6 mice to create *Rpl221*^{+/-} mice. *Rpl221*^{+/-} mice were interbred to generate embryos and mice to assess *Rpl221*^{-/-} viability. Genotyping analysis of mice was performed by a multiplex PCR on embryonic or tail DNA (F1, TCCACCTGGAGGTTTCATTTG; F2, GGCTCGACTGGGACGATAG; R, CGCACTGCCACATTT GTAAC), which amplifies 607 bp and 420 bp bands for wildtype and *Rpl221*^{-/-} alleles, respectively). All mice were maintained at an AAALAC-accredited animal facility either at the University of Washington or the Buck Institute for Research on Aging and all procedures were approved by the Institutional Animal Care and Use Committees (IACUC).

RT-PCR and Quantitative Real time PCR

Total RNA was isolated from embryos at the indicated stage using Trizol (Life technologies) and glycogen (Ambion), following the manufacturer's instructions. RNA was reverse transcribed using random hexamers and Superscript II Reverse Transcriptase (Life technologies). For regular RT-PCR, PCR products were separated on a 2% agarose gels. Quantitative real-time PCR was performed on an ABI 7500 real-time PCR system using 2 \times SYBR-Green Master mix (Qiagen). The relative expression values were normalized against *actb* by using the 2 ^{$\Delta\Delta C_t$} method.

For validation of RNA-Seq data by RT-PCR, exon-specific primer sets spanning the region exhibiting exon skipping were designed using primer-blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primer sequences will be provided upon request.

Co-immunoprecipitation (CoIP) of Rpl22 and Like1 with hnRNP-A1

Co-immunoprecipitation of Rpl22 and Like1 with hnRNP-A1 was performed on detergent extracts of zebrafish embryos using minor modifications to a well-established protocol (Little and Mullins, 2009). Briefly, HA or Flag tagged mRNAs were injected into 1-cell embryos. At the shield stage (6hpf), Approximately 200 dechorionated zebrafish embryos were collected and centrifuged at 800g at room temperature for 30s to completely remove egg water. After washing twice in PBS, embryos were transferred to lysis buffer (50mM Tris pH 7.5, 120mM NaCl, 1mM EDTA, 5% glycerol, 1% Triton X-100, and 0.15% Sodium Deoxycholate, containing 1mM PMSF and a protease inhibitor cocktail (Roche)). Embryos were manually disrupted in 1.5ml tubes with a pre-chilled pestle, using 4-5 strokes. Samples were gently rotated at 4°C for 30min, following which the lysates were clarified at 12000g for 5min at 4°C, and the supernatant was saved for immunoprecipitation. 1% of the total lysate was saved as the input. In the RNaseA treated group, embryonic lysate was pretreated with RNase A (0.5µg/µl, Sigma) for 10min at room temperature. After pre-clearing using Protein A Magnetic Beads (NEB), the embryonic lysate was incubated with anti-HA antibody (C29F4, #3724, Cell Signaling, 1:50) for 2h and then rotated gently overnight at 4°C with 25µl protein-A magnetic beads (NEB). The same amount of magnetic beads alone without HA antibody were used as a negative control. The bead-Ab-Ag complexes were washed 3 times in wash buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitors). To elute the samples, beads were transferred to clean tubes, resuspended in 2×SDS loading buffer (30µl), and incubated at 70°C for 5 minutes. The eluted samples were resolved by SDS-PAGE and blotted with the indicated antibodies (anti-Flag, #2368, Cell Signaling, 1:1000; anti-HA, 16B12, Covance, 1:1000).

Embryonic RNA-Protein Crosslinking and Immunoprecipitation (E-CLIP)

To develop the E-CLIP method for Rpl22/Like1 interaction with *smad2* mRNA in zebrafish embryos, we modified published protocols (Lu et al., 2013; Niranjanakumari et al., 2002; Ule et al., 2003). Briefly, mRNAs encoding HA-tagged Rpl22 and Like1 were injected into 1-cell embryos, following which ~500 zebrafish embryos that were dechorionated using pronase were collected at 10hpf and washed 3 times in egg water to remove residual pronase. Embryos were transferred to clean tubes and immediately cross-linked by adding 37% formaldehyde (Sigma) to 1.85% vol/vol final concentration, following which the embryos were gently mixed on a rocker platform for 15min at RT. Fixation was quenched by adding 1/20 volume of 2.5M glycine and shaking for 5min at RT. Embryos were centrifuged to remove the supernatant, washed 3 times in ice-cold PBS, and lysed in hypotonic lysis buffer (10mM Tris-HCl pH 7.5, 10mM NaCl, 0.5% NP40, containing protease inhibitors and SuperRNase inhibitor) by homogenization using a pre-chilled pestle. After incubation on ice for 15min, the nuclei from the embryos were pelleted at 3500 rpm for 5 min at 4 °C. The nuclei were resuspended in 1ml IP lysis buffer (50mM Tris-HCl, pH 7.4, 100mM NaCl, 1mM MgCl₂, 1% Triton-X100, and 0.1% SDS, supplemented with protease inhibitors and SuperRNase inhibitor) and lysed by sonication (Bioruptor sonication system). Following DNase treatment of the

nuclear extract for 15 min (Turbo DNase treatment, Ambion), RNA was partially trimmed by treatment with RNase T1 (Ambion) for 5min at 37°C as described (Lu et al., 2013). The nuclear extract was clarified by centrifugation at 22,000g for 30 min, pre-cleared with Protein A Magnetic Beads (NEB), and then incubated overnight along with specific HA antibody (C29F4, Cell Signaling) or control IgG (Abcam, ChIP grade) pre-bound to Protein A Magnetic Beads (NEB). Beads containing bound immune complexes were washed 3 times in IP lysis buffer, 2 times in high salt wash buffer (1M NaCl, 50mM Tris, 1% TritonX100, 0.1% SDS), and then resuspended in 100µl of elution buffer (50mM Tris HCl, pH7.0, 5mM EDTA, 10mM DTT, and 1% SDS, containing protease and RNase inhibitors). To reverse the crosslinking, samples were incubated at 70°C for 45 min with gentle shaking. The RNA was extracted from these samples using Trizol and glycogen precipitation protocol. RT-PCR and qPCR were carried out on the extracted RNA as described above.

SUPPLEMENTAL REFERENCES

- Dick, A., Mayr, T., Bauer, H., Meier, A., and Hammerschmidt, M. (2000). Cloning and characterization of zebrafish *smad2*, *smad3* and *smad4*. *Gene* *246*, 69-80.
- Jia, S., Wu, D., Xing, C., and Meng, A. (2009). *Smad2/3* activities are required for induction and patterning of the neuroectoderm in zebrafish. *Developmental biology* *333*, 273-284.
- Little, S.C., and Mullins, M.C. (2009). Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis. *Nature cell biology* *11*, 637-643.
- Lu, X., Goke, J., Sachs, F., Jacques, P.E., Liang, H., Feng, B., Bourque, G., Bubulya, P.A., and Ng, H.H. (2013). SON connects the splicing-regulatory network with pluripotency in human embryonic stem cells. *Nature cell biology* *15*, 1141-1152.
- Niranjanakumari, S., Lasda, E., Brazas, R., and Garcia-Blanco, M.A. (2002). Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo. *Methods* *26*, 182-190.
- Provost, E., Rhee, J., and Leach, S.D. (2007). Viral 2A peptides allow expression of multiple proteins from a single ORF in transgenic zebrafish embryos. *Genesis* *45*, 625-629.
- Renucci, A., Lemarchandel, V., and Rosa, F. (1996). An activated form of type I serine/threonine kinase receptor TARAM-A reveals a specific signalling pathway involved in fish head organiser formation. *Development* *122*, 3735-3743.
- Ule, J., Jensen, K.B., Ruggiu, M., Mele, A., Ule, A., and Darnell, R.B. (2003). CLIP identifies Nova-regulated RNA networks in the brain. *Science* *302*, 1212-1215.
- Zhang, Y., Duc, A.C., Rao, S., Sun, X.L., Bilbee, A.N., Rhodes, M., Li, Q., Kappes, D.J., Rhodes, J., and Wiest, D.L. (2013). Control of hematopoietic stem cell emergence by antagonistic functions of ribosomal protein paralogs. *Developmental cell* *24*, 411-425.