Supplementary Information

Modulation of Tcf3 repressor complex composition regulates *cdx4* expression in zebrafish

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Figure S1. Lnx proteins bind directly to E4f1

(*A*) 293T cells were co-transfected with indicated plasmids including E4f1 tagged with three copies of the T7 epitope, and HA-ubiquitin. After 48 h, IP and blotting were performed with the indicated antibodies. Note that all tested Lnx2b constructs co-precipitated with E4f1, but only wild type Lnx2b induced poly-ubiquitin chain outgrowth. Lnx2b Mu, stabilized Lnx2b containing two point mutations (H63A, C66A) in the RING domain; Lnx2b ΔN, N-terminal RING domain deleted mutant (Ro and Dawid, 2009).

(*B*) Human LNX1 and LNX2 co-precipitated with human E4F1 and induced polyubiquitylation of E4F1. Polyubiquitylated E4F1 did not undergo proteasomal destruction; the same is true of zebrafish E4f1 in (*A*).

(*C*) Direct interaction between Lnx2b and E4f1 was confirmed by GST pull-down with recombinant proteins purified from bacteria. GST alone failed to pull down S-tagged Lnx2b. Arrow indicates the GST-tagged E4f1 (MW, ~106 kD).

Figure S2. Multiple alignments of vertebrate E4f1 proteins

(*A*) Alignment was carried out using the ClustalW multiple alignment algorithm. The GenBank Accession Numbers are: human E4F1 (NM_004424), mouse E4f1 (NM_007893), rat E4f1 (XM_001055139), zebrafish E4f1 (NM_001025513).

(*B*) A phylogenetic tree of vertebrate E4f1 proteins constructed with TREEVIEW. The remaining GenBank Accession Numbers are: Bovine (XM_594119), Chick (XM_414860), *Xenopus tropicalis* (NM_001078698), *Xenopus laevis* (NM_001135223).

Figure S3. The spatio-temporal expression pattern of *e4f1* in zebrafish embryos

(*A-C*) *e4f1* transcripts are maternally expressed and ubiquitously distributed from 2-cell to germ ring stage (GR). (*D,E*) *e4f1* mRNA was slightly enriched in putative notochord at 80% epiboly stage. (*F,G*) *e4f1* was ubiquitously expressed but slightly enriched in Kupffer's vesicle at the tailbud stage (TB). (*H,I*) *e4f1* expression became stronger in the notochord at the 5-somite stage. (*J*) Notochord enriched *e4f1* expression ceased from the 15-somite stage onward. (*K*) At 24 hpf, *e4f1* expression was confined to the anterior region. (*L*) Expression levels of *e4f1* transcripts at different developmental stages were analyzed by RT–PCR. *e4f1* is expressed maternally and zygotically, and its expression is maintained at least until day 5. β-actin was used as loading control.

(*A-D*) Lateral views. (*C,D*) Dorsal to the right. (*E*) Dorsal view. (*F,H,J,K*) Lateral view, anterior to the left. (*G*) Posterior view, dorsal is up. (*I*) Dorsal view, anterior is up. Scale bar, 200 μm.

Figure S4. Depletion of E4f1 compromised *cdx4* expression

(*A*) Uninjected control embryo. (*B*) 100 pg *e4f1* mRNA injected embryo. (*C*) 5 ng of *e4f1* MO injected embryo. In situ staining with *cdx4* riboprobe was carefully monitored and stopped before the signal became saturated. (*D*) 5 ng of *e4f1* MO or 100 pg of *e4f1* mRNA was injected at the one to two-cell stage, and embryos were harvested at 6 hpf for RT-qPCR analysis.

(*A-C*) Embryonic shield stage (6 hpf). Lateral view. Dorsal is right. Scale bar, 200 μm.

Figure S5. E4f1 cooperates with Wnt signaling to induce primitive erythropoiesis via *cdx4*

Injected reagents are listed above panels, probes shown at bottom right. (*A-F*), *gata1* expression pattern was examined at the 8-somite stage. (*A*) Control embryo (Ctr); *gata1* expression is detected in posterior lateral plate mesoderm. (*B*) *cdx4* morphant (2 ng *cdx4* MO) showed strong reduction of *gata1* expression. (*C*) Embryo injected with 12.5 pg of *dkk* mRNA; *gata1* expression was reduced. (*D*) 5 ng *e4f1* MO injected embryo; *gata1* expression level was slightly compromised. (*E*) *gata1* expression decreased dramatically

in embryos injected with 12.5 pg of *dkk* mRNA and 5 ng *e4f1* MO. (*F*) The compromised *gata1* expression was rescued by co-injection of *cdx4* mRNA (5 pg). (*G-L*) *pax2.1* expression at the 8-somite stage in a region close to the *gata1* domain was relatively well maintained in embryos treated as in (*B-F*).

(*A-L*) Dorsal views, anterior is up. The percentage of embryos is shown in each panel. The parenthesis indicated the number of embryos analyzed. Scale bar, 200 μm.

Figure S6. *lnx2b* SP MO specificity, *lnx2b* genomic sequence, Tcf3 binding

(*A*) RT-PCR of *lnx2b* morphants injected with different concentration of *lnx2b* splice donor blocker (SP MO). Injection of 10 ng SP-MO caused 80% reduction of *lnx2b* transcript. The amount of *lnx2b* PCR products was measured using ImageJ. SP MO targeting region is shown in (*B*). (*B***)** Genomic structure of *lnx2b* and Tcf3 binding. Position of splice MO is shown on map of the *lnx2b* gene. There are five relatively well conserved LEF/TCF consensus sites in the *lnx2b* regulatory region (arrowheads). ChIP assay was carried out using embryos at the 10-somite stage and anti-Tcf3a antibody. Chromatin fragments encompassing LEF/TCF binding element (1) were precipitated by anti-Tcf3a antibody while region (2) from exon 10 was not. IgG was used as additional negative control.

Figure S7. Expression of *lnx2b* is enhanced by Wnt signaling and inhibited by Cdx1a/Cdx4. (*A-M*) Tail bud stage embryos. Treatment is shown at bottom left, in situ probes at bottom right. (*A-F*) Wnt signal is critical for *lnx2b* expression in the caudal region, except in Kupffer's vesicle. For concentrations of MOs and *dkk1* mRNA see Figure 2. (*G,H*) *cdx4* expression is up-regulated by activation of Wnt signaling by treatment with 0.2 M LiCl for 40 min at mid gastrula. (*I-M*) *lnx2b* expression is up-regulated by LiCl treatment and by inhibition of *cdx1a/cdx4* expression. (*L,M*) MOs for *cdx1a* (2 ng) and *cdx4* (2 ng) were co-injected into one-cell embryos. The number of embryos analyzed is given at the upper right.

(*A,C,E,G,H,I-M*) Lateral view. Dorsal is right. (*B,D,F*) Posterior view. Dorsal is up. Scale bar, 200 μm.

Figure S8. Tcf3 binds to LEF/TCF consensus elements in the *cdx4* regulatory region.

Amplicons a, b and c are identified in Figure 4A. Gel electrophoresis of the PCR products obtained by ChIP assay with chromatin from *cdx4-egfp* transgenic embryos after IP with anti-Tcf3a antibody or rabbit IgG as control.

Figure S9. Mutagenesis of Tcf3 consensus sites in the *cdx4* reporter.

(*A*) Schematic drawings of reporter constructs (a-d). Black arrowheads stand for the LEF/TCF consensus sites. Red oval shapes represent mutated LEF/TCF binding consensus (-756 to -749, AACAAAGT → AACAcctT; -411 to -404, ATCAAAGT → ATCAccaT; -44 to -37, CCTTTGCC → CaggTGCC). Chevrons indicate deletion of LEF/TCF binding elements. (*B*) The activity of the different luciferase reporter constructs (a-d in *A*) in embryos at 6.5 hpf after co-injection with *tcf3a* mRNA (100 pg).

Figure S10. *cdx4* expression in Tcf3 deficient embryos does not change in response to manipulation of E4f1 levels.

(*A*) Wild type embryo. (*B*) *tcf3b* MO (2 ng) injected *MZ hdl* embryo. (*C*) 100 pg of *e4f1* mRNA was co-injected with 2 ng of *tcf3b* MO into the *MZ hdl* embryo. (*D*) *e4f1* MO (5 ng) was co-injected with *tcf3b* MO (2 ng) into the *MZ hdl* embryo. Note the altered *e4f1* expression did not significantly influence the elevated *cdx4* expression level in the Tcf3 depleted embryos. The number of embryos analyzed is depicted in the upper right corner.

(*A-D*) Embryonic shield stage. Lateral view. Dorsal is right. Scale bar, 200 μm.

Figure S11. E4F1 association domain mapping of Tcf3a. Upper panel shows schematic diagrams of Tcf3a deletion constructs. Lower panel shows co-IP of Myc-tagged Tcf3a constructs with Flag-E4F1 after transfection into 293T cells. Note that the Groucho binding domain and the HMG DNA-binding domain of Tcf3a are required for co-precipitation with E4F1.

Supplementary materials and methods

RT-PCR. Total RNA was isolated using Trizol (Invitrogen), and 3 μg of RNA was used for RT-PCR. Zebrafish *β-actin* was used as loading control. RT-qPCR was performed with LightCycler 480 (Roche).

axin2 forward primer, 5'-TAACTCAAAACGGATGTCCAATG-3';

axin2 reverse primer, 5'-AGGCTTGCAGAGGCGTGCTGGTC-3';

cdx4 forward primer; 5'-GTCCTCTAGCGGTAAGGGTCAC-3';

cdx4 reverse primer; 5'-GAAACTCCGTACCTGTAGTTCTC-3';

e4f1 forward primer, 5'-CCGTACCACTGCAGCTTCTGTGACAAGAGC-3';

e4f1 reverse primer, 5'-TGCTGCACTACTTTCATGATGTGACTGTCC-3';

lef1 forward primer, 5'-CCAGACATTCCCAATTTCTATC-3';

lef1 reverse primer, 5'-GTAAAGCTGCATATGGAGCTGC-3';

lnx2b forward primer, 5'-GCATGCAACGTTGTGAGTTGCAACCTCATC-3';

lnx2b reverse primer, 5'-GTCGCCTCAGCCACAAGACTGCACCTC-3'.

tbx6 forward primer, 5'-TCATATCAGCCTTCACAAACCC-3';

tbx6 reverse primer, 5'-TCCCTGAGGGTGTGGAGGCTCTG-3';

β-actin forward primer, 5′-GAGGAGCACCCCGTCCTGCTCAC-3′;

β-actin reverse primer, 5′-GATGGCTGGAACAGGGCCTCTG-3′;

GST Pull-down assay. Purified GST or GST-E4f1 was incubated with S-tagged Lnx2b (Ro and Dawid, 2009) and Glutathione Sepharose 4 Fast Flow (GE Heathcare Life Science) for 2 h at 4°C with gentle agitation. Mixtures were centrifuged, and pellets were washed four times with ice-cold buffer (150 mM NaCl, 0.02% NaN_3, Complete protease inhibitor cocktail, 10 mM Tris-Cl, pH 7.2, 0.5% NP-40, 1 mM DTT). Bound proteins were eluted by adding 30 μl of gel loading buffer and boiling for 5 min, separated by

SDS-PAGE (12%), transferred to PVDF membrane, and detected by immunoblotting using anti-S·tag antibody (Novagen). After stripping the bound S·tag antibody using Restore Western Blot Stripping Buffer (Thermo), the same blot was subjected to immunoblotting with anti-GST antibody.

Internal deletion constructs. Internal deletion constructs of LEF/TCF consensus sites in the *cdx4* promoter/1st intron and in the Tcf3a coding region were generated using a PCR-based method. For example, Tcf3a-ΔG/H was generated using four different primers.

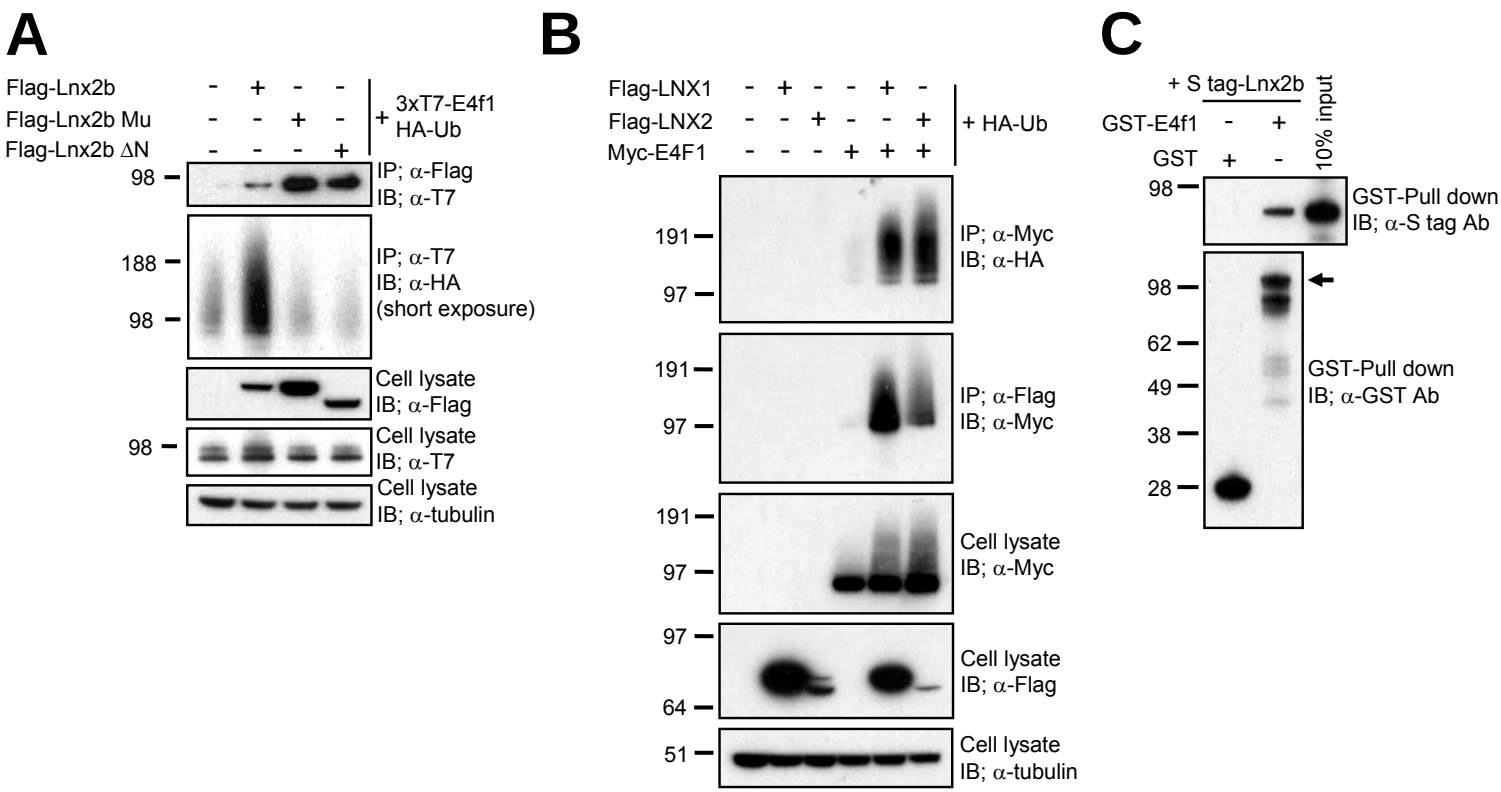
P1, 5'-GATCGAATTCCAACATGCCTCAGTTAAACGGAGGAG-3';

P2, 5'-CTTTCCGTAGTTGTCTCTTGGGATCATAAGAAACG-3';

P3, 5-CGTTTCTTATGATCCCAAGAGACAACTACGGAAAG-3' and

P4, 5'-GATCCTCGAGTCACTCCACAGATTTGGTGACGAGG-3'. Primary PCR reactions were carried out with two different sets of primers (P1 + P2 and P3 +P4) using PfuUltra DNA polymerase (Stratagene). After purification, the mixture was used as template for a second PCR reaction with P1 and P4 primers.

Supplementary Information Fig. S1



Supplementary Information Fig. S2

A

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Mouse     1 MEGAMAVRVTAAHTAEAGAEAGREAGEGGVAA-AAALSSGGFLGLPAPFSEEDEDDVHRCGRCQVEFTALEDFVQHKIQKTCHRAPQEALPTTPAATALLDQEVVPTAAEGGPDEPITVA
Human     1 MEGAMAVRVTAAHTAEAQAEAGREAGEGAVAAVAAALAPSGFLGLPAPFSEEDEDDVHRCGRCQAEFTALEDFVQHKIQKACQRAPPEALPATPATTALLGQEVVPAAP--GPEEPITVA
Zebrafish 1 ----------------------MTEENNNTAATVTDQTEHSNIITIQTTLGDEDED-IHKCGKCLAEFSALDAFIQHKLSRSCKRTQDPQT------------NVVPNEG-----------
            :   : : :              * .:. .*:::     ..:: : :.:.:**** :*:**:* .**:**: *:***:.::*:*:            ::  :***         : :

Rat         HIVVEATSLAEDISHAPDLVGSGHIKEVIVAAEAEPGDVEMAEAPGSPNHQELGLLGEGEQAHVKLLVNKEGRYVCMLCHKTFKTGSILKAHMVTHSSRKDHECKLCGASFRTKGSLIRH
Mouse       HIVVEATSLAEDISHAPDLVGSGHIKEVIVAAEAEPGDGEMAEAPGSPNHQELGLLGEGEQAHVKLLVNKEGRYVCMLCHKTFKTGSILKAHMVTHSSRKDHECKLCGASFRTKGSLIRH
Human       HIVVEAASLAADISHASDLVGGGHIKEVIVAAEAELGDGEMAEAPGSPHQQGLGLAGEGEQAQVKLLVNKDGRYVCALCHKTFKTGSILKAHMVTHSSRKDHECKLCGASFRTKGSLIRH
Zebrafish   -VSVEVRSSENECSSDETATANGEKQDAKVASGDKKRSRSTSEDESSS------------PSKVVWKLNTEGRYVCDICAKTFKTTNILRTHMFTHSDQKNFVCEMCETAFRTKGSLIRH
             : **. *   : *     ...*. ::. **:  :  . . :*  .*.    : :      ::*   :*.:***** :* ***** .**::**.***.:*:. *::* ::**********

Rat         HRRHTDERPYKCAKCGKSFRESGALTRHLKSLTPCTEKIRFSISKDTAVGKEEVPAGS—SASTVGTVTSSVAGDPMETSPVIHLVTDAKGTVIHEVHVQMQELPLGMKALTPESPDSEE
Mouse       HRRHTDERPYKCAKCGKSFRESGALTRHLKSLTPCTEKIRFSISKDTAVGKEEVPAGS—SASTVGTVTSSVAGDPMETSPVIHLVTDAKGTVIHEVHVQMQELPLGMKALTPESPDSEE
Human       HRRHTDERPYKCSKCGKSFRESGALTRHLKSLTPCTEKIRFSVSKDVVVSKEDARAGSGAGAAGLGTATSSVTGEPIETSPVIHLVTDAKGTVIHEVHVQMQELSLGMKALAPEPPVSQE
Zebrafish   KRRHTDERPYRCNQCGLAFRESGALTRHLKSLTPCTEKIRYSQCKEILVSKD----------GIRKEVQPSSPEPEKEQIPVVRVVEAGQEIIQIEVVEEVQQVAS--QPQTATVVEADN
            :*********:* :** :**********************:* .*:  *.*:          .    . .* .    *  **:::*  .:  :  **  ::*::.  ::. :.    :::

Rat         LPCSSE-NSRENLLHQAMQNSGIVLERVAGEESALEPAPPSGSSPQCLGDGSPELPLLKVEQIET-VASEAATVPRTHPCPQCSETFPTAATLEAHKRGHIAPRPFTCTQCGKAFPKAYL
Mouse       LPCSSE-NSRENLLHQAMQNSGIVLERVAGEESALEPAPPSGSSPQCLGDGSPELPLLKVEQIETQVASEAATVPRTHPCPQCSETFPTAATLEAHKRGHIAPRPFTCTQCGKAFPKAYL
Human       LPCSSE-GSRENLLHQAMQNSGIVLERAAGEEGALEPAPAAGSSPQPLAVAAPQLPVLEVQPLETQVASEASAVPRTHPCPQCSETFPTAATLEAHKRGHTGPRPFACAQCGKAFPKAYL
Zebrafish   LICQAIINSGIALETEATEAAGQVEAQSP---KAVLGAPETETRITEIQVTEECVETLAEETQDSSVKEVEPVQSKLYKCPHCERMFKTLNYLRVHVKGHVGYKPFKCLTCQKEFLTGYV
            * *.:  .*   *  :* : :* *  : .    *:  ** : :    :     :  *  :  :: * .  .. .: : **:*.. * *   *..* :** . :** *  * * * ..*:

Rat         LKKHQEVHVHERRFRCGDCGKLYKTIAHVRGHRRVHSDERPFPCPQCGKRYKTKNAQQVHFRTHLEEKPHVCQFCSRGFREKGSLVRHVRHHTGEKPFKCYKCGRGFAEHGTLNRHLRTK
Mouse       LKKHQEVHVHERRFRCGDCGKLYKTIAHVRGHRRVHSDERPFPCPQCGKRYKTKNAQQVHFRTHLEEKPHVCQFCSRGFREKGSLVRHVRHHTGEKPFKCYKCGRGFAEHGTLNRHLRTK
Human       LKKHQEVHVRERRFRCGDCGKLYKTIAHVRGHRRVHSDERPYPCPKCGKRYKTKNAQQVHFRTHLEEKPHVCQFCSRGFREKGSLVRHVRHHTGEKPFKCYKCGRGFAEHGTLNRHLRTK
Zebrafish   LKKHMETHVSERRYKCGECGKQFKAIGHVREHMRAHSDERPYHCSFCDKSYKTKNALQVHHRTHADDKPYVCQHCSRGFREKSALVRHIRHHTGEKPFKCSKCGRGFAEHGTLNRHLRAK
            **** *.** ***::**:*** :*:*.*** * *.******: *. *.* ****** ***.*** ::**:***.********.:****:*********** **********:*

Rat         GGC---LLEVEELLVSEE----SPSAAATVLAEDPHTVLVEFSSVVADTQEYIIEATADDTETSEATEIIEGTQTEVDSHIMKVVQQIVHQAGAGHQIIVQNVTMDQETALGSEATAADT
Mouse       GGC---LLEVEELLVSEE---- PSAAATVLAEDPHTVLVQFSSVVADTQEYIIEATADDTETSEATEIIEGTQTEVR-------------------VWVR--------------------
Human       GGC---LLEVEELLVSED----SPAAATTVLTEDPHTVLVEFSSVVADTQEYIIEATADDAETSEATEIIEGTQTEVDSHIMKVVQQIVHQASAGHQIIVQNVTMDEETALGPEAAAADT
Zebrafish   GGCFAQLKDCEHVVNSEEQEVTTESVSTTIVSDDPHAVLVEFSSVVADTQEYIIGAPAEEAAQGEEVAIIQDNQQQMDSHIMKVVQQIVSQSHGGHQIIVRNVTADETPGIS---DSGDT
            ***   * : *.:: **:    : :.::*::::***:***:************* *.*:::  .* . **:..* ::   :: ::  ::        : *: :       :

Rat         ITIATPESLTEQVAMTLASAISEGTVLTAR-AGPNSTEQATVTMVSSEDIEILEHGGELVIASPEGQLEVQTVIV- 782
Mouse       -----------------DGRITG-------------------------------------LLGSPSG---------- 684
Human       ITIATPESLTEQVAMTLASAISEGTVLAAR-AGTSGTEQATVTMVSSEDIEILEHAGELVIASPEGQLEVQTVIV- 784
Zebrafish   ITIATPESLTEQVAMTLANAISDGTILTTTTEDTDETSHTTVTMVTAENVETIEQEEQYVIASPE-EVEIQTVVVV 719
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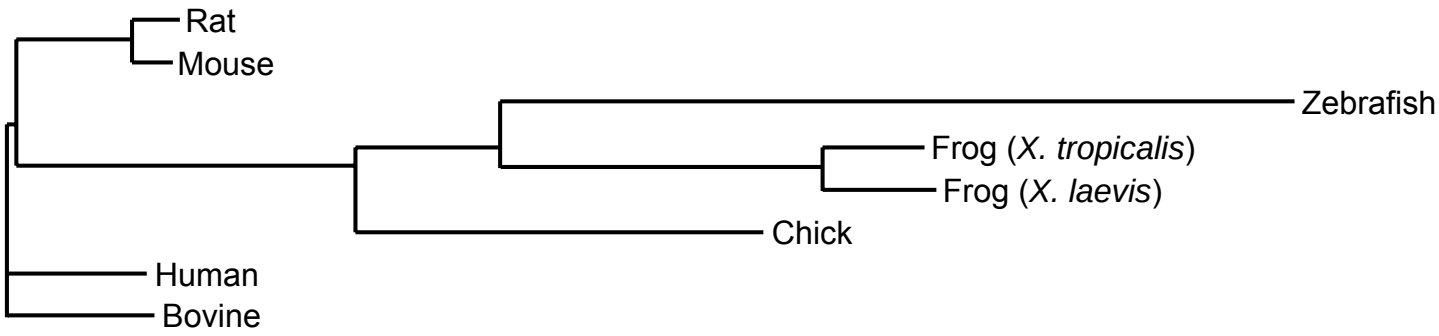

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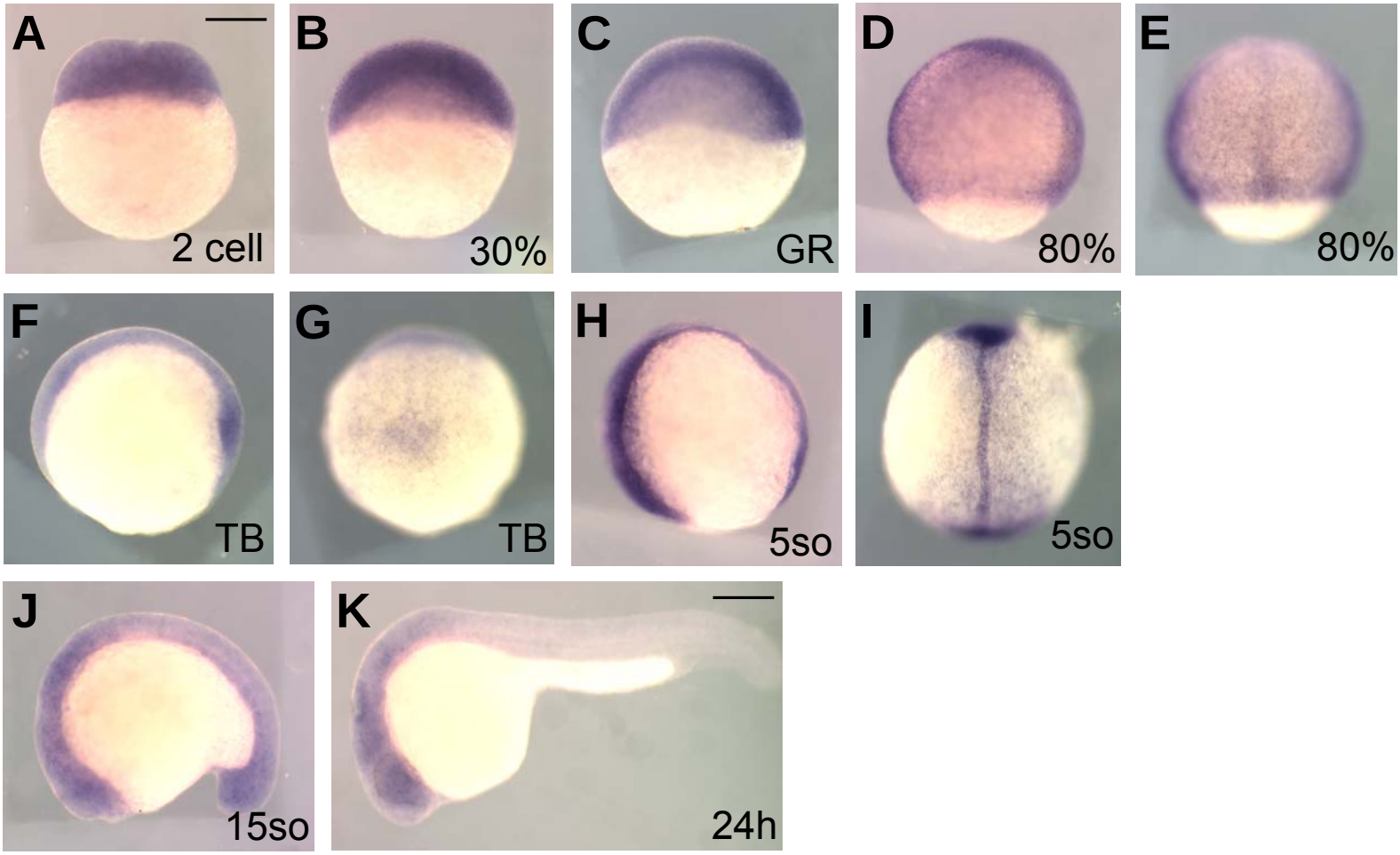

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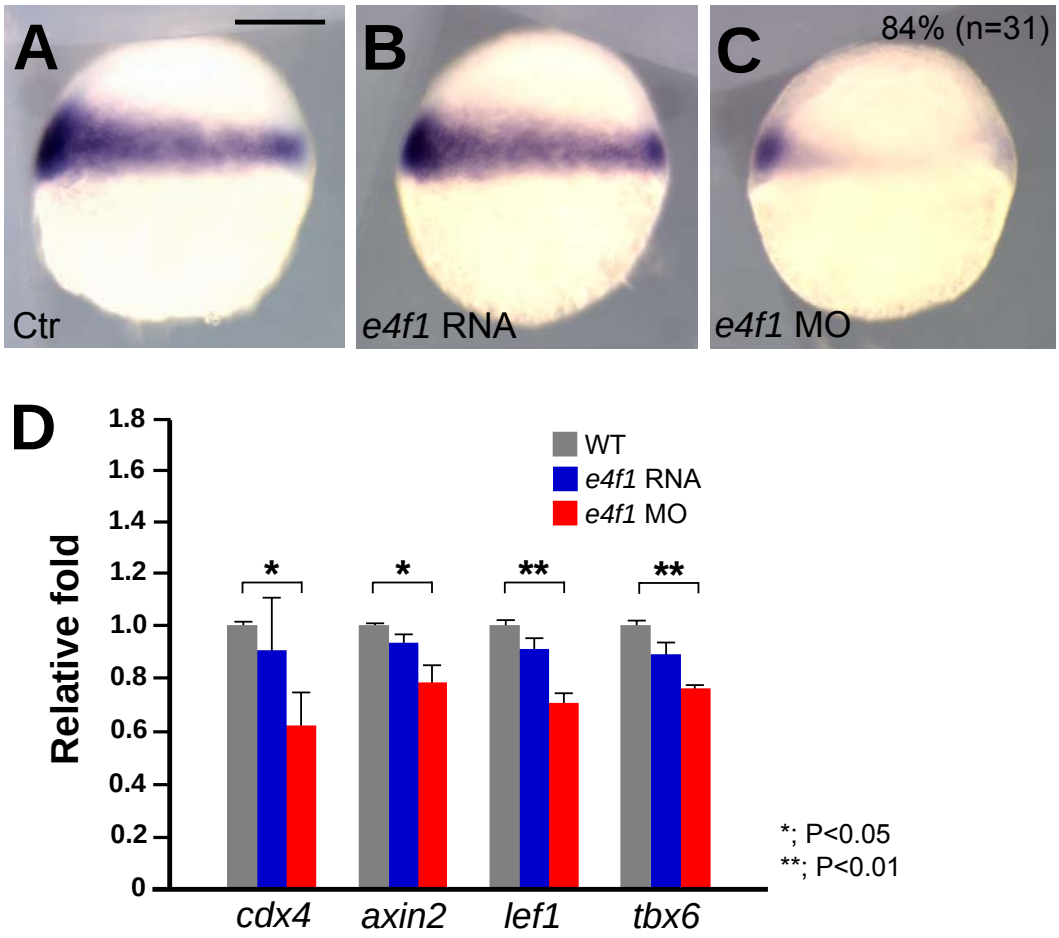

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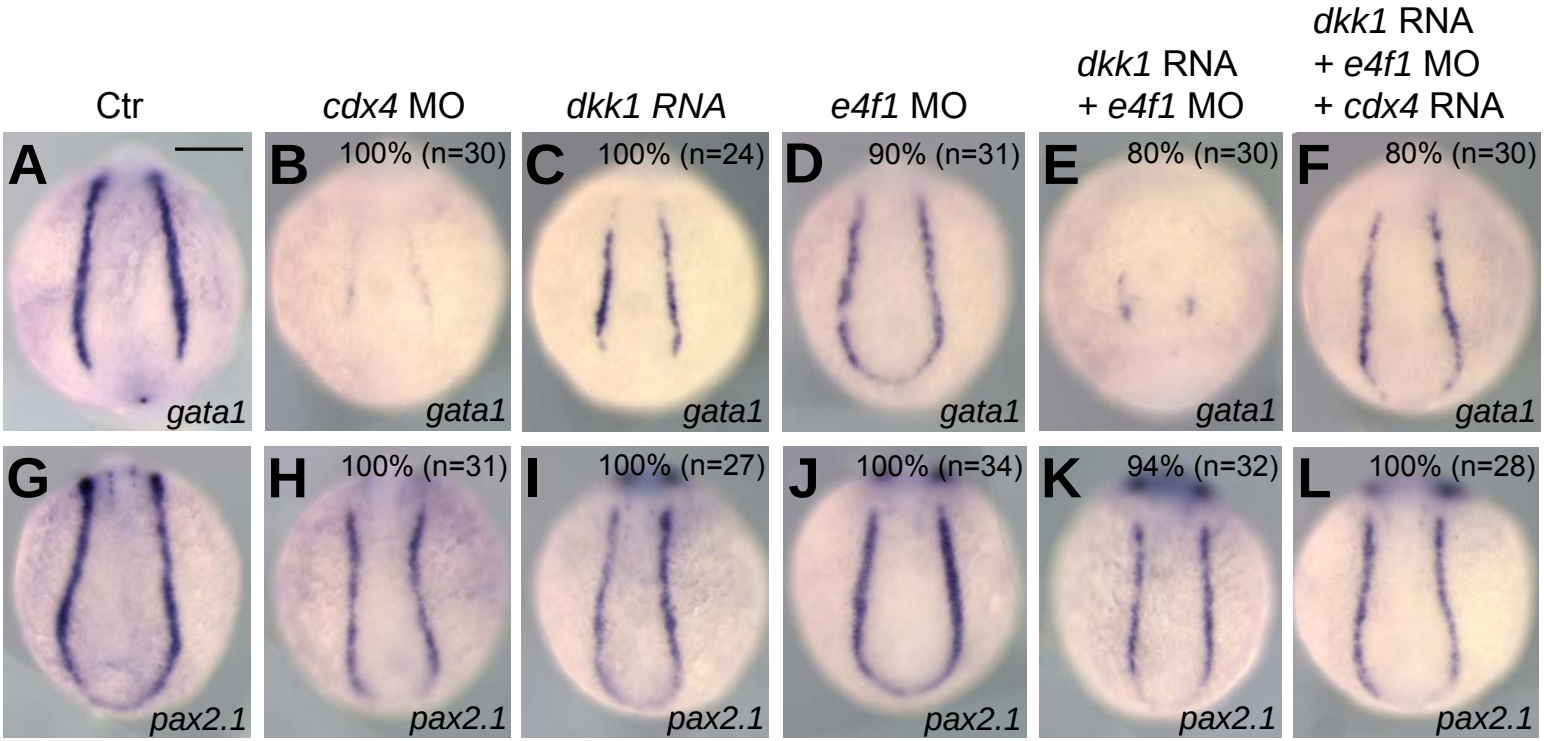

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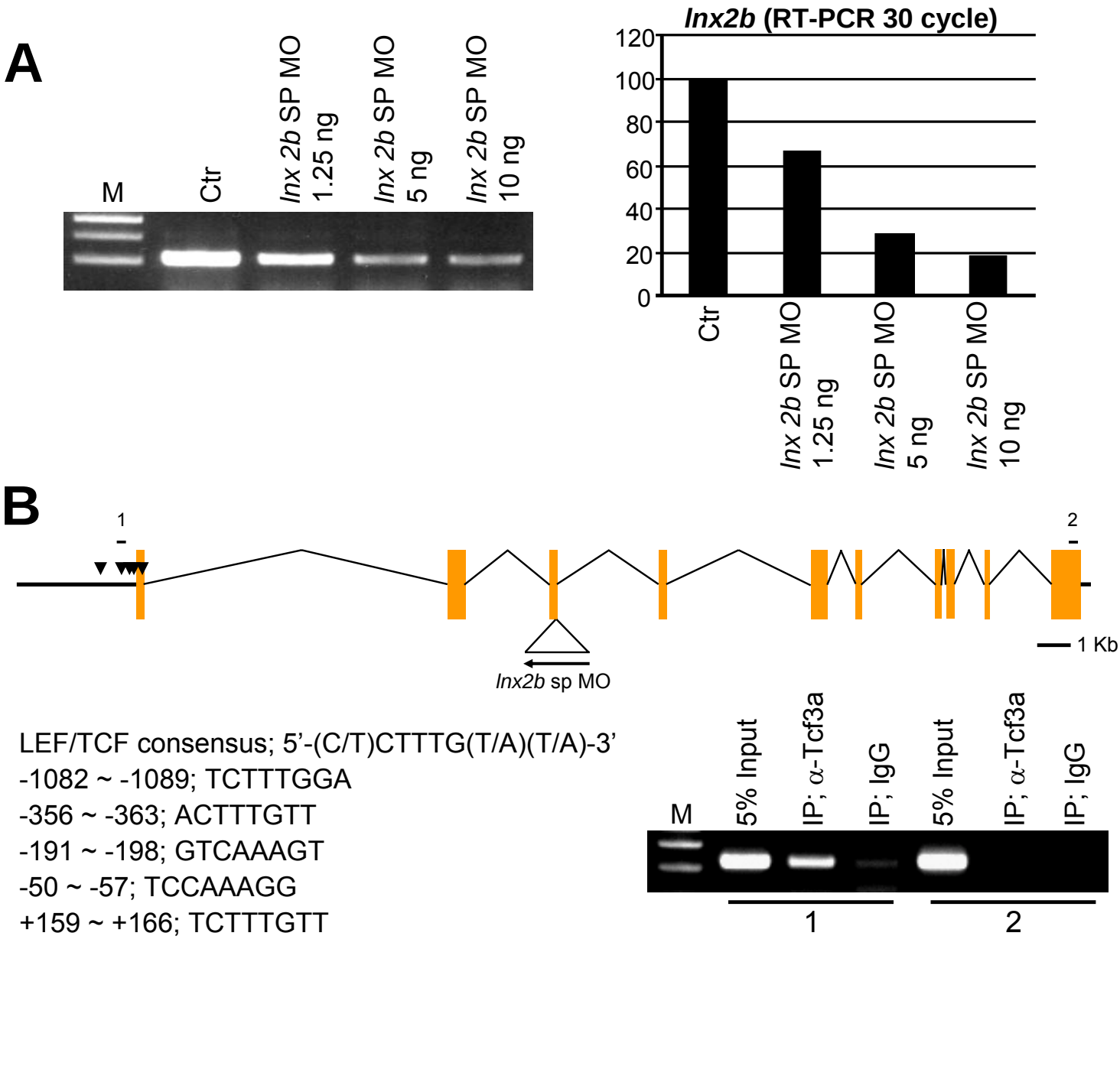

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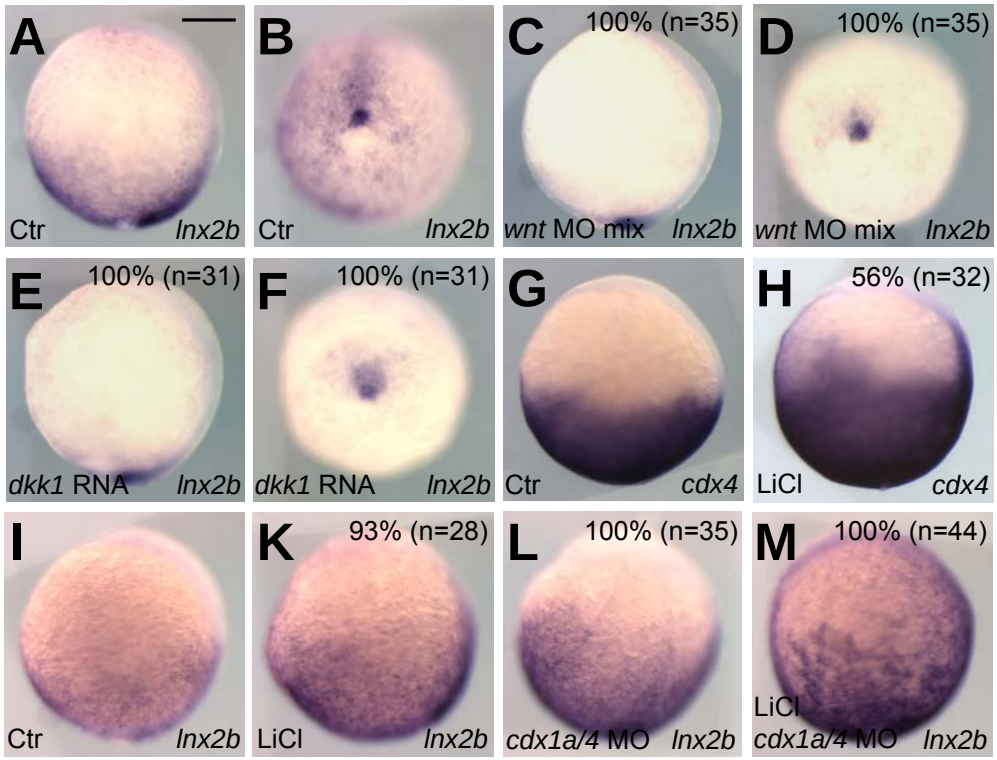

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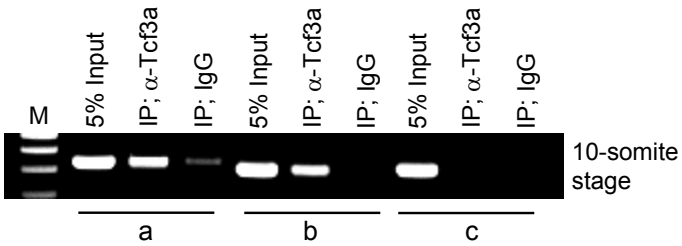

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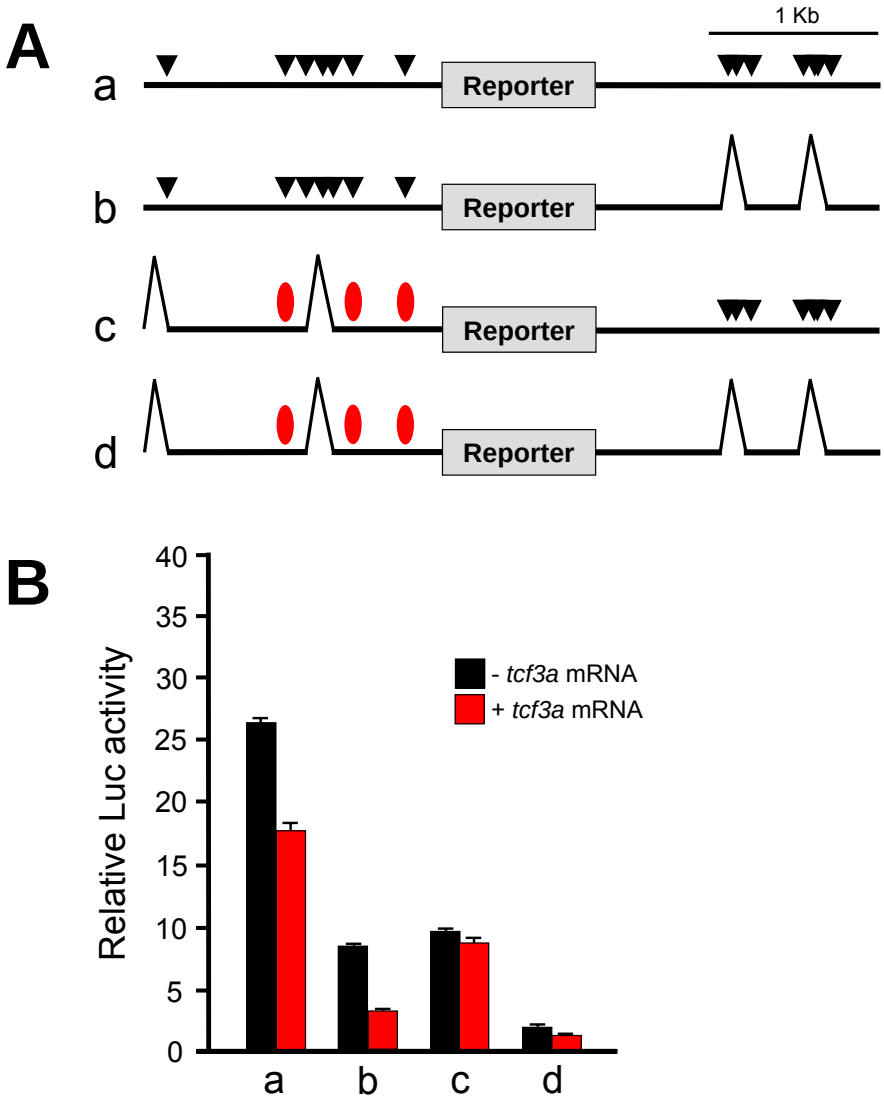

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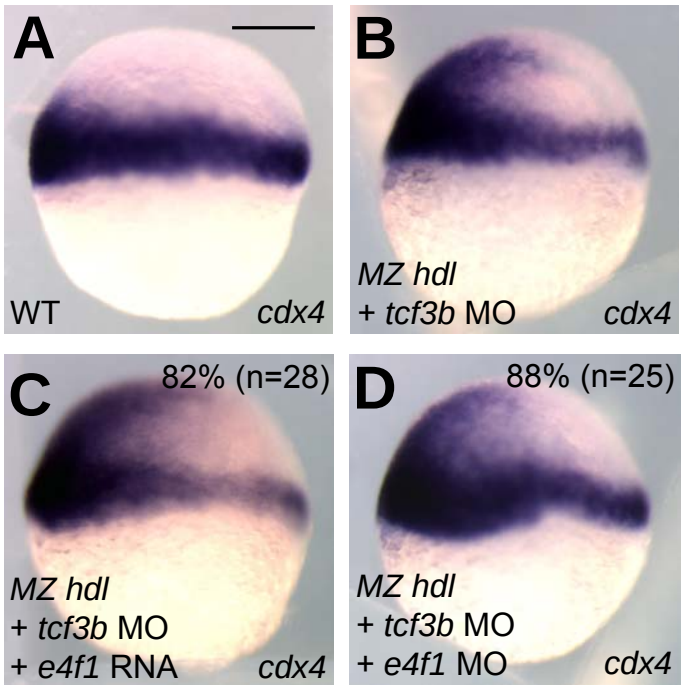

Supplementary Information Fig. S11