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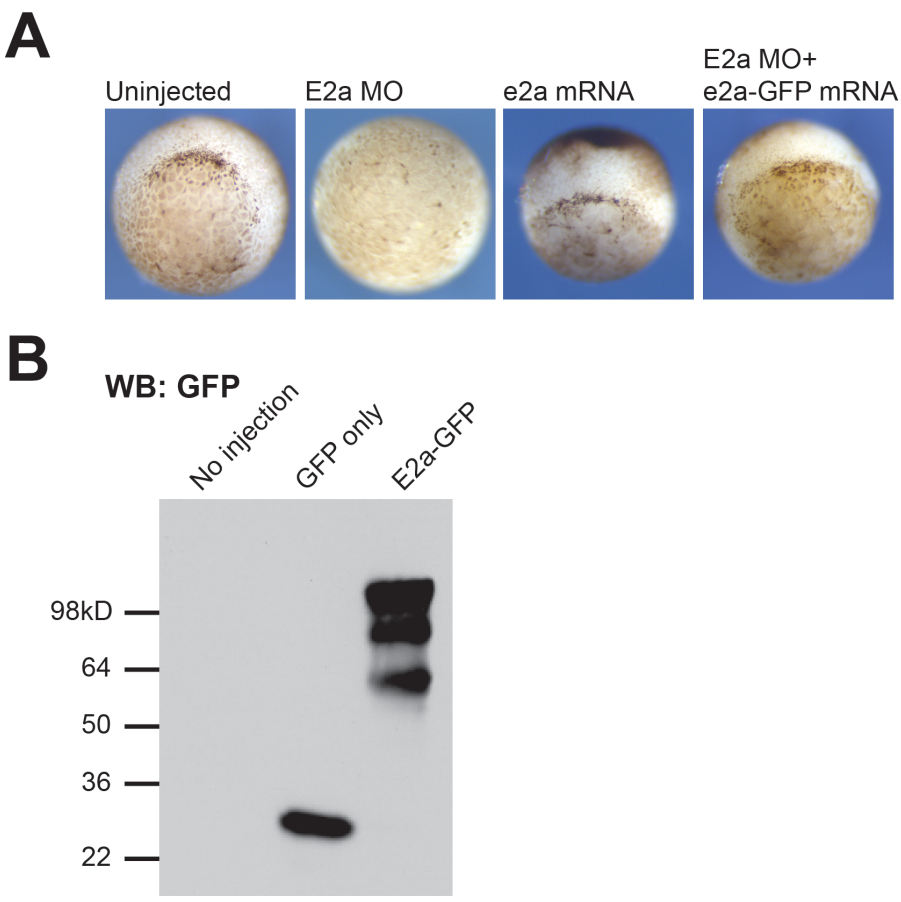
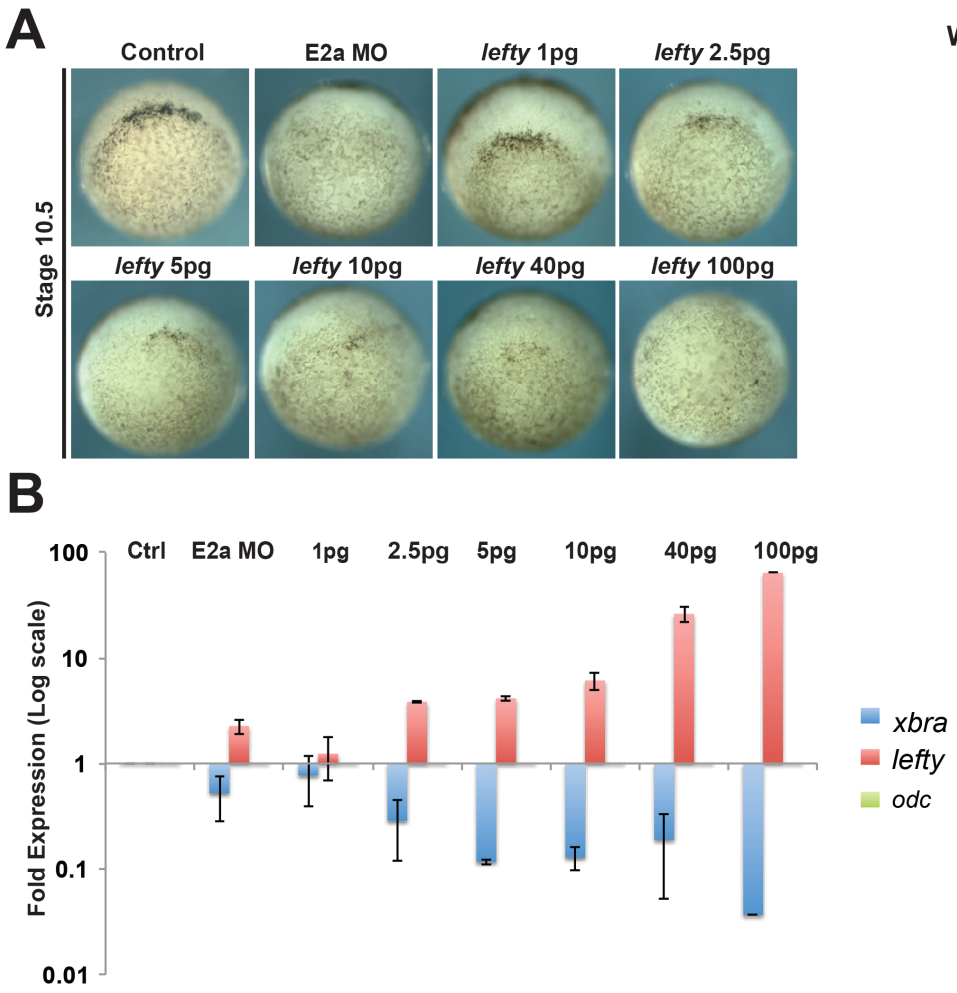


Supplemental Figure S1, related to Figure 2.

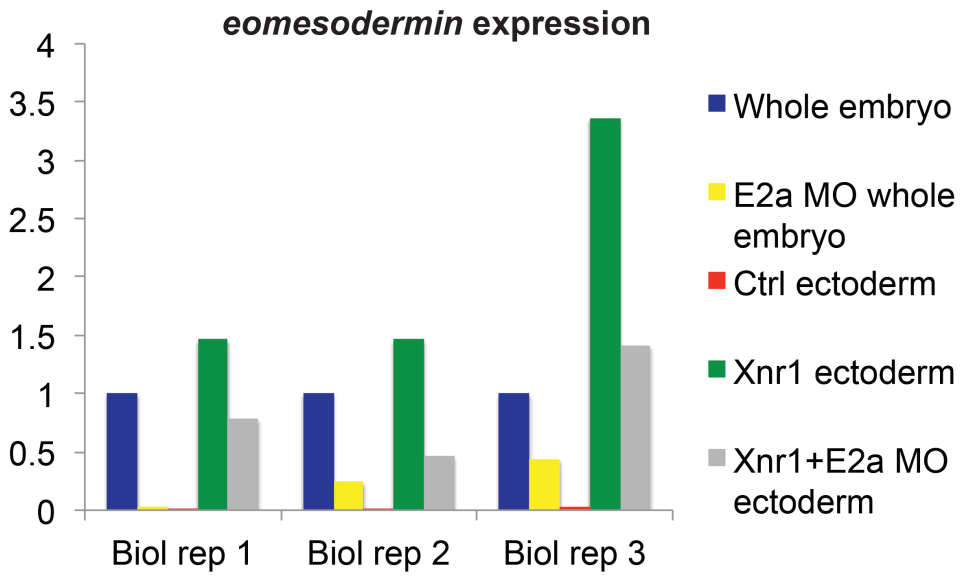
The E2a-GFP fusion protein is functional. A) Embryos were injected at the two cell stage with E2a MO, E2a-GFP mRNA, or E2a MO and E2a-GFP mRNA. Embryos injected with E2a MO fail to form blastopores. E2a-GFP RNA alone has no effect on blastopore formation. Embryos injected with both E2a MO and E2a-GFP have rescued bottle cell formation. B) Western blot for GFP. Embryo lysates were collected from embryos injected with mRNA encoding GFP alone, or E2a-GFP.



Supplemental Figure S2, related to Figure 3.

Expression of *lefty* in E2a depleted embryos is not high enough to cause loss of bottle cells. A) Dose curve of phenotypic effects of *lefty* mRNA injection. Increasing levels of *lefty* mRNA injection (both dorsal blastomeres at the 4-cell stage; total mRNA dose shown) result in progressively more severe inhibition of

bottle cell and blastopore formation. At 1pg total *lefty* mRNA, blastopore formation is not inhibited, and at 2.5pg, it is only modestly inhibited, while blastopore formation is significantly impaired at 10pg or more of *lefty* mRNA. B) qRT-PCR comparing the effects of E2a MO injection with *lefty* mRNA injection at a range of doses. The amount of injected *lefty* mRNA is indicated across the top of the graph. The amount of *lefty* mRNA expressed in E2a morphants is comparable to injection of between 1 and 2.5pg *lefty* mRNA.



Supplemental Figure S3, related to Figure 4.

Expression of *eomesodermin* is reproducibly reduced by E2a MO injection. Related to Figure 4. qRT-PCR of *eomesodermin* in 3 biological replicate clutches of embryos. These data were combined in Figure 4A of the main text. Note that while *eomes* expression in Xnr1 injected ectoderm is variable between clutches, expression is always reduced in embryos that are injected with both Xnr1 and E2a MO.

Supplemental Table S1, related to Figure 1. Supplied as excel spreadsheet. List of genes associated with each category of Smad2/3 binding, as delineated in Figure 1B. Categories include genes associated with a Smad2/3 occupied region in one or both Control embryo ChIP-Seq libraries but neither E2a-depleted embryo library ("lost"); genes associated with a Smad2/3 occupied region in one or both E2a-depleted ChIP-Seq libraries but neither control library ("gained); and genes that are associated with a Smad2/3 occupied region in both E2a-depleted embryos and control embryos. This latter category is further divided into genes where the Smad2/3 occupied region in control embryos has overlapping coordinates with the Smad2/3-occupied region in E2a depleted embryos ("retained"), and into genes where a Smad2/3 occupied region exists for both control embryos and E2a-depleted embryos, but the regions do not have overlapping coordinates ("shifted"). Smad2/3 occupied regions are defined as called peaks using MACS2 with an FDR cutoff of 0.001. Overlapping regions were defined using the BedIntersect tool of BedTools.

Supplemental Table S2, related to Figure 1. Supplied as excel spreadsheet. RNA-Seq expression data, normalized by FPKM. Expression values and p-values are based on combined expression levels from two biological replicates for Control embryos and three biological replicates for E2a-depleted (E2a MO) embryos. Sheet 1 contains all genes that are significantly upregulated in E2a-depleted embryos. Sheet 2 contains all genes that are significantly downregulated in E2a-depleted embryos.

Supplemental experimental procedures:

Xenopus tropicalis embryo culture:

Adult female *Xenopus tropicalis* were injected 18-30 hours before embryo collection with 10u human chorionic gonadotropin (HCG)(Sigma). Four hours before embryo collection, male and female frogs were injected with 100 units and 400 units of HCG, respectively, and allowed to begin amplexus. 30-45 minutes after the onset of egg laying, embryos were collected and dejellied in 1/9 MR+ 3% cysteine. Embryos were cultured in 1/9 MR at room temperature or at 28°C (generally, embryos analyzed at stage 11 or younger were reared at 28°C to facilitate earlier collection, while embryos analyzed at later stages were reared at room temperature).

Xenopus tropicalis embryo microsurgery

Xenopus tropicalis animal caps were isolated from stage 8 or stage 9 embryos using Dupont #5 forceps, and were cultured at room temperature in 3/4X NAM. Animal caps were cut conservatively to minimize contamination from the marginal mesoderm layer. For Nieuwkoop recombinants, embryos were first transferred to 3/4X NAM in dishes thickly coated with 2% agarose, then the animal cap and vegetal pole (approximately 100 cells) were isolated separately and recombined immediately, before healing of the animal cap could begin. Recombinants were cultured to the desired stage at room temperature in Steinberg's solution or 3/4X NAM, in agarose-coated dishes.

LacZ lineage tracing

100-200pg mRNA encoding nuclear-localized β-galactosidase (nβgal-CS2+) was injected alone or in conjunction with X. tropicalis E2a mRNA. At the desired stage, embryos were fixed for 30 minutes in MEMFA, washed 3X in PBS+0.1% tween 20, then incubated with Red-Gal substrate (Research Organics) at 37° until well-developed (Sive, 2000). Embryos were then washed with PBS+0.1% tween 20, fixed 2 hours in MEMFA, and dehydrated in methanol. Dehydrated stained embryos were subsequently used for *in situ* hybridization.

Plasmids used for in situ hybridization:

Gene:	Source:	Vector:
Xbra	Genbank AL642113	pCS107
Gsc	IMAGE:7536652	pCS108
Lefty	IMAGE:7520937	pCS108
MyoD	Genbank: AL645144	pCS107
Myf5	Genbank: AL654739	pCS107
Shh	Genbank: AL804833	pCS107
Nkx2.5	Genbank: DN008946	pCS107
FoxA1	Genbank: AL681017	pCS107

RNA-Seq library preparation:

Embryos were collected into lysis buffer (0.5% SDS, 5mM EDTA, 50mM Tris pH7.5, 50mM NaCl, 250ug/ml Proteinase K). 20ug of starting total RNA was used for each library. mRNA was prepared from total RNA by two rounds of

purification using oligo-DT Dynabeads (Invitrogen, cat #610.06). 400ng of of poly-A selected RNA was used for library preparation. Libraries for RNA-Seq were prepared according to published methods (Beck et al., 2010), using HPLC or PAGE purified primers, 3% NuSieve GTG agarose for gel purification. Sequencing was performed on the Illumina hiSeq platform, and aligned to the xenTro2 genome using Bowtie. FPKM normalization and expression profile analysis was carried out as described (Tan et al., 2013).

E2a Fusion protein constructs:

Mouse E2a-GFP fusion was made by fusing the carboxy terminus of mouse E2a to the amino terminus of eGFP on a CS2 backbone. Mouse E2a (IMAGE clone 2631291) was amplified using forward primer 5' CTC GAG ACG CAC CCC AGT TCC 3' and reverse primer 5' TCT AGA CCA GGT GCC CGG CTG 3' using Pfx platinum high-fidelity polymerase. The resulting PCR product was ligated into PCR Blunt 2.1 with T4 ligase, then sequence confirmed. This intermediate product was digested with XbaI and XhoI, and subcloned into CS2 eGFP X/P digested with XbaI and XhoI. The finished subcloned product was fully sequence confirmed. mRNA for injection was made by linearizing with NotI and transcribed with Sp6 (mmessage mmachine, Ambion).

X. tropicalis E2a-EnR fusion was made by fusing the Engrailed repressor domain to the carboxy terminus of *X. tropicalis* E2a, on a pCMV Sport6 backbone. *X. tropicalis* E2a (IMAGE 7660124) was digested with SalI/PciI (for coding region) and SalI/XbaI (for backbone). The engrailed repressor domain

was modified to contain PciI and XbaI sites by amplifying with forward primer 5' ACA TGT CAA TGG CCC TGG AGG 3' and reverse primer 5' TCT AGA GCT TAG GGG ATC TGC 3' using Pfx high fidelity polymerase, then ligated into PCR Blunt vector. This intermediate product was digested with XbaI and PciI, and ligated to the SalI/PciI product (E2a coding region). The resulting SalI-E2a-PciI-EnR-XbaI fusion product was then ligated into the SalI/XbaI digested backbone. The finished subcloned product was fully sequence confirmed.

Quantitative RT-PCR

1ul of cDNA was used in each 25 uL PCR reaction, together with 10uM primer mix and SyBR green iQ reaction master mix (BioRad) diluted to a final concentration of 1x. The PCR reaction was as follows: 95° for 5 minutes, 40 repeats with each repeat consisting of 95° for 30s, 55,57, or 59° for 30s (annealing temperatures were determined empirically for each primer set based on quality of melt curves), 72° for 30s. This was followed by 72° for 7 minutes and a melt curve of 10s cycles increasing by 0.5 degrees from 55° to 95°. After data collection, melt curves were analyzed visually to ensure a single peak was present for each primer. Threshold amplification values (ct) were assigned by the iCycler analysis software, and these were converted to expression values following (Zhu et al., 2007). Values were normalized using control stage-matched whole embryo cDNA and odc expression values. Error bars represent standard deviations.

qRT-PCR Primers:

ODC:

F: TTTGGTGCCACCCTTAAAAC

R: CCCATGTCAAAGACACATCG

Xbra:

F: AGGTTCAAGGAGCTCACCAA

R: GAACCCATTCTCCATTCACG

Gsc:

F: CAAAGTGGAGGAGGCAGAAA

R: TGTTCGTCCCCTTTCTCTGT

Lefty:

F: GTCTCTGCCTGATCCTCAGC

R: TCCTCTTCCTCTCCCTGTGA

Eomes:

F: TCGGGAAACTCAAACTCACC

R: TCAGATCCTCCACTCCATCC

EphA4:

F: GACCCAAGTTTGGGCAGATA

R: ATCAAGCACAGAAGCCACCT

Xnr1:

F: TGTCCAATTCCACTCAACGA

R: CATCTCTTGGTGGTGCCTTA

ChIP-qPCR:

Embryos were injected with mRNA encoding E2a-GFP or GFP alone. Embryos were reared to stage 10.5 and collected as described for ChIP-Seq. ChIP was performed using anti-GFP antibody in both cases (life technologies #A11122, lot 1296649). Input samples were also collected for both conditions. ChIP and input DNA was collected as for ChIP-Seq, and quantified using Qbit. qPCR was performed using constant amounts of DNA, as for RT-qPCR. qPCR values were normalized to input and to “off target” negative control DNA regions.

ChIP-qPCR primers:

Lefty region 1 (Scaffold 719: 392500-393900)

F: GATGGGCCCTTAGGAAAATC

R: TCTCGCTGTCTGATACCCCT

Lefty region 2 (Scafold 719: 397000-398000)

F: TCTGGGGCATTAGTGGATTG

R: CAGCGAACAGGTGACTGTGT

Lefty negative region (scaffold 719: 394,476-394,605)

F: AGGGCTACTGTTGCACTTCA

R: AGGCATTCCCCATACTTTCC

Eomes: preserved Smad2/3 peak at scaffold_26: 614342-614718

F: CTAGGGAAGCAGTGGGAGTG

R: GGCAATGCCATTCAGACATA

Epha4: preserved peak at scaffold 224_705811-706124

F: GAGGAGCCTGCAGTGATGA

R: GGACTCAACAGGGGCAACTA

Xbra: preserved peak at scaffold 432: 271300-271600

F: TTGTCCAACGTTTGTATTCTTCA

R: TGCACAAAATAATCAATAACAGACAA

negative control region (scaffold 185: 1829000-1830000)

F: GCTCTGTGTGTCTTCACCCTT

R: CTCGCTGACATGCCATACAC