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Optimal histone H3 to linker histone H1 chromatin ratio is vital for mesodermal competence in *Xenopus*

Chin Yan Lim[1,*], Bruno Reversade[2], Barbara B. Knowles[1,3] and Davor Solter[1,4]

SUMMARY

Cellular differentiation during embryogenesis involves complex gene regulation to enable the activation and repression of genes. Here, we show that mesodermal competence is inhibited in *Xenopus* embryos depleted of histones H3 and H3.3, which fail to respond to Nodal/Activin signaling and exhibit concomitant loss of mesodermal gene expression. We find that transcriptional activation in gastrula embryos does not correlate with promoter deposition of H3.3. Instead, gastrulation defects in H3.3/H3-deficient embryos are partially rescued with concurrent depletion of the linker histone H1A. In addition, we show that linker histone H1-induced premature loss of mesodermal competence in animal cap explants can be abrogated with the overexpression of nucleosomal H3.3/H3. Our findings establish a chromatin-mediated regulatory mechanism in which a threshold level of H3 is required to prevent H1-induced gene repression, and thus facilitate mesodermal differentiation in response to inductive signaling.

KEY WORDS: *Xenopus* development, Mesoderm differentiation, Nucleosome spacing

INTRODUCTION

Eukaryotic DNA is packaged into chromatin on which all DNA-related processes such as transcription, replication and repair are carried out. Chromatin is assembled from arrays of nucleosomes, consisting of 146 bp of DNA wrapped around an octameric complex of histones H2A, H2B, H3 and H4 (Kornberg, 1974; Luger et al., 1997). In metazoans, higher-order compaction of chromatin is further mediated by an additional linker histone H1, which interacts with the nucleosomal core and the DNA between nucleosomes (Bednar et al., 1998; Thomas, 1999). Chromatin structure can be dynamically modulated via a large repertoire of post-translational modifications on histones, remodeling of nucleosome distribution and the selective incorporation of variant histones (Wolffe, 1998).

Histone variants, identified based on protein sequence divergence, exhibit differential expression patterns and chromatin incorporation dynamics (Sarma and Reinberg, 2005). Encoded by genes located outside of core histone gene clusters, variant histones, unlike canonical histones, are synthesized throughout the cell cycle, and are incorporated into chromatin in a DNA replication-independent manner (Ahmad and Henikoff, 2002; Tagami et al., 2004). One of the best-studied histone variant is H3.3, which differs from canonical H3.1/2 in only four amino acid residues (Elsaesser et al., 2010; Szenker et al., 2011). This small sequence disparity results in decreased stability of H3.3-containing nucleosomes, leading to the hypothesis that H3.3 marks open chromatin and facilitates active gene transcription (Hake and Allis, 2006; Jin and Felsenfeld, 2007). Indeed, H3.3 localizes to transcribed genomic loci in *Drosophila* and mammalian cells, and is enriched for covalent modifications associated with active chromatin (Chow et al., 2005; Hake et al., 2006; Jin and Felsenfeld, 2006; McKittrick et al., 2004; Mito et al., 2007). However, recent findings, demonstrating the presence of H3.3 in pericentric heterochromatin and telomeres of mammalian cells, raised doubts about a singular role for H3.3 in transcriptional activation (Goldberg et al., 2010; Santenard et al., 2010; Wong et al., 2009).

Genetic studies in *Drosophila* and the mouse have highlighted different aspects of H3.3 function *in vivo*. Mutant flies lacking H3.3 are viable but sterile, revealing an indispensable function for H3.3 in germ cells (Hödl and Basler, 2009; Sakai et al., 2009). In mice, disruption of the *H3f3a* gene resulted in partial neonatal lethality, while surviving mutants exhibited severe growth and fertility defects (Couldrey et al., 1999). In addition, H3.3 incorporation into the pericentric heterochromatin of the paternal pronucleus is necessary for the development of mouse embryos (Santenard et al., 2010). Thus, H3.3 function appears to be vital for mammalian embryogenesis.

In this study, we sought to extend analysis of H3.3 function to its biological role in *Xenopus* embryonic development. We find that partial depletion of H3.3 results in abnormal development, whereas a distinct gastrulation arrest phenotype was observed upon substantial depletion of both H3.3 and canonical H3. Using lineage marker analyses and animal cap experiments, we find that mesodermal differentiation is impaired in the H3.3/H3-depleted embryos owing to the loss of competence to respond to mesoderm-inducing signals. We show that deficient mesoderm competence resulted from perturbations in chromatin organization brought about by the loss of nucleosomal H3 and increased somatic linker histone H1A incorporation, arguing that an optimal histone H3 to linker histone H1 chromatin ratio is vital for mesodermal competence in *Xenopus* embryos.

MATERIALS AND METHODS

Morpholino design

Translation-blocking morpholinos to *Xenopus laevis* H3 MO1 (5′ TGTACGGGCCATTTCCCTTTAATCG 3′), H3 MO2 (5′ GCGGTCTG-CTTGGTACGAGCCAT 3′) and H1A (5′ ATTCGGCGGCTTCAGC-CATTGCAGA 3′) were purchased from Gene Tools. H3.3 MO1 and MO2 were mixed at 1:1 ratio; 65 ng of the mixture (H3 MOs) was injected per embryo. For H1A depletion, 135 ng of H1A MO was injected per embryo.

[1]Mammalian Development Laboratory, Institute of Medical Biology, Singapore. [2]Human Embryology Laboratory, Institute of Medical Biology, Singapore. [3]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore. [4]Cancer and Stem Cell Biology Program, DUKE-NUS Graduate Medical School, Singapore.

*Author for correspondence (chinyan.lim@imb.a-star.edu.sg)

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Embryo manipulations

A total of 16 nl of each MO was injected at the two- (2×4 nl per blastomere), four- or eight- (1×4 nl per blastomere) cell stage. Whole-mount *in situ* hybridization was performed as previously described (Reversade et al., 2005). Animal caps were dissected at early blastula and cultured with or without 5 ng/ml Activin A (R&D Systems) to stage 25. Animal caps at stages 9 and 10 were mechanically reopened to facilitate exposure to Activin A, as described previously (Steinbach et al., 1997).

Synthesis of mRNAs and RNA probes

The coding sequences of mouse *H3f3a*, *H3.1* and *H2Az* and *Xenopus H1A* were amplified by PCR from cDNA with primers that resulted in the introduction of a HA-Flag epitope-tag to the C termini of these proteins. The PCR products were subcloned into the pCS2+ vector. Point mutations at specific lysine residues in *H3f3a* were introduced by site-directed mutagenesis (Stratagene). Capped mRNAs were synthesized by *in vitro* transcription using the SP6 mMESSAGE mMACHINE kit (Ambion). The cDNA sequences of *Xenopus H3f3a* and *H3f3b* were PCR amplified and subcloned into pCS2+ at *Eco*RI/*Xho*I sites. The plasmids were linearized with *Hin*dIII (*H3f3a*) or *Pst*I (*H3f3b*) and transcribed with T7 RNA polymerase for the generation of antisense DIG-labeled RNA probes (Roche). Primer sequences are provided in supplementary material Table S2.

Cellular extraction and western blotting

Cellular extracts were prepared from 15 gastrula stage embryos by homogenization in RIPA buffer with pellet pestles. Acid extraction of histones was performed by 0.2 N sulfuric acid extraction of the nuclear pellet at 4°C overnight. Extracted histones were precipitated with 10 volumes of acetone at −20°C. Antibodies for western blotting: anti-H3.3 (ab97968, Abcam), anti-H3 (ab1791, Abcam), anti-Histone H1 (clone AE-4, Millipore), anti-HA (sc-805, Santa Cruz), anti-actin (MAB1501R, Millipore), anti-pSmad2 (custom), anti-pSmad1 (#9511, Cell Signaling) and anti-Smad1 XP (#6944, Cell Signaling).

Chromatin immunoprecipitation and PCR

Chromatin immunoprecipitation (ChIP) assays to analyze H3.3 genomic localization were performed on nuclei prepared from 50 embryos, injected with 750 pg of mouse *H3.3a*-HA mRNA at the two-cell stage and cultured to appropriate developmental stages, as previously described (Blythe et al., 2009). ChIP was carried out using 5 μg of anti-HA antibody (sc-805, Santa Cruz) or rabbit IgG (NI01, Millipore). RNA polymerase II ChIP assays were performed on 50 stage 10 control or H3 MO-injected embryos, using either 10 μg of anti-RNA polymerase II CTD antibody, clone 8WG16 (05-952, Millipore) or mouse IgG (Jackson ImmunoResearch). PCR primer sequences are provided in supplementary material Table S2.

Micrococcal nuclease digestion assay

Nuclei were prepared from 45 control or injected embryos by homogenization in MNB+ [15 mM Tris-HCl (pH 7.5), 250 mM sucrose, 60 mM KCl, 15 mM NaCl, 3 mM CaCl2, 0.5% Triton-X100, 0.5 mM dithiothreitol] with pellet pestles. After a 30-minute incubation on ice, lysates were centrifuged for 30 seconds at 10,000 ***g***. The nuclear pellets were washed once in MNB– (MNB+ without Triton-X100), resuspended in MNB– and digested with 1 U micrococcal nuclease (Sigma) for 3 minutes at room temperature. The reactions were stopped with EDTA, and treated with RNase A for 2 hours, followed by proteinase K treatment at 55°C overnight. DNA fragments were ethanol precipitated after two rounds of phenol:chloroform extraction, and electrophoresed on 1.5% agarose gels.

RESULTS

Histones H3.3 and H3 depletion leads to gastrulation defects in *Xenopus* embryos

H3.3 is highly conserved in higher eukaryotes, with 100% identity in protein sequence between human, mouse and frog. In *Xenopus* embryos, *H3f3a* and *H3f3b* transcripts are maternally supplied and after the mid-blastula transition, both H3f3 genes are expressed throughout development to tailbud stages (supplementary material Fig. S1). We tested the effects of overexpressing exogenous H3.3 in developing embryos, by injecting different amounts of *H3.3-HA* transcripts. We found that overexpression of H3.3 from 2 ng, but not from 1 ng, of *H3.3-HA* mRNA led to defective cell division in blastula- and gastrula-stage embryos, abnormal gastrulation and death by the late gastrula stage (supplementary material Fig. S2A-C). We also observed that expression of the pan-mesodermal marker *Xbra* in these embryos was restricted to the dorsal and ventral mesodermal regions (supplementary material Fig. S2A). By contrast, injection of 2 ng of *H3.1-HA* transcripts did not perturb gastrulation or *Xbra* expression, though the H3.1-overexpressing embryos exhibited abnormalities at later stages of development (supplementary material Fig. S2A). These results suggest that normal *Xenopus* embryonic development requires an optimal regulated amount of H3.3, which may have essential functions that are distinct from the canonical H3.1 histones.

To further examine the functional requirement of H3.3 during *Xenopus* development, we sought to deplete H3.3 proteins with a morpholino oligonucleotide (H3 MO1) designed to block the translation of *h3f3a* transcripts (supplementary material Fig. S3A). The H3 MO1 morpholino was unable to completely abrogate endogenous H3.3 protein levels, as shown by western blot analysis of embryonic extracts from injected embryos (Fig. 1A). To achieve a complete knockdown of H3.3, a second morpholino, H3 MO2, with optimal complementarity to the *h3f3b* transcript was designed (supplementary material Fig. S3A). Although the H3 MO2 fully depleted endogenous H3.3 protein, it also resulted in a significant decrease in the total H3 levels in the injected embryos (Fig. 1A; supplementary material Fig. S3B). Consequently, when both MO1 and MO2 were injected, H3.3 and canonical H3 were substantially depleted. Owing to high sequence conservation in the 5′ untranslated and coding regions of the H3.3 and H3 genes, we could not design morpholinos that fully depleted H3.3 proteins without affecting the levels of the canonical H3 histones.

Partial depletion of H3.3 following injection of H3 MO1 resulted in delayed blastopore closure. Despite this delay, morphant embryos developed to tailbud stages with a shortened axis (supplementary material Fig. S3C). These observations are consistent with the developmental defects recently reported for the specific depletion of H3.3 and the H3.3 chaperone, HIRA (Szenker et al., 2012). By contrast, knockdown of both H3.3 and canonical H3 led to early- to mid-gastrulation arrest in embryos injected with both MO1 and MO2, whereas control embryos developed normally (Fig. 1B; supplementary material Fig. S3C). Notably, this phenotype differs significantly from the early developmental arrest, prior to the mid-blastula transition, observed in embryos rendered deficient in chromatin assembly through the disruption of the H3 chaperone CAF-1 activity (Quivy et al., 2001). Hence, though the H3 MOs resulted in a significant knockdown of histone H3, the results suggest sufficient amounts of canonical H3 remained to support development of these embryos, and the gastrulation arrest could be a specific phenotype of H3.3 loss within a sub-optimal chromatin context. Thus, we sought to further characterize the effects of maximal H3.3 loss induced by the co-injection of MO1 and MO2 (hereafter referred to as H3 MOs), which is coupled to perturbed chromatin organization as a result of canonical H3 knockdown.

To ascertain whether gastrulation defects in injected embryos could have resulted from off-target effects of the H3 MOs, we also attempted to rescue the depletion by co-injecting H3 MOs with mRNA encoding HA-epitope-tagged mouse H3.3. Though mouse and *Xenopus* H3.3 proteins are identical, the mouse *H3f3a* mRNA is not targeted by the H3 MOs owing to sequence differences in the

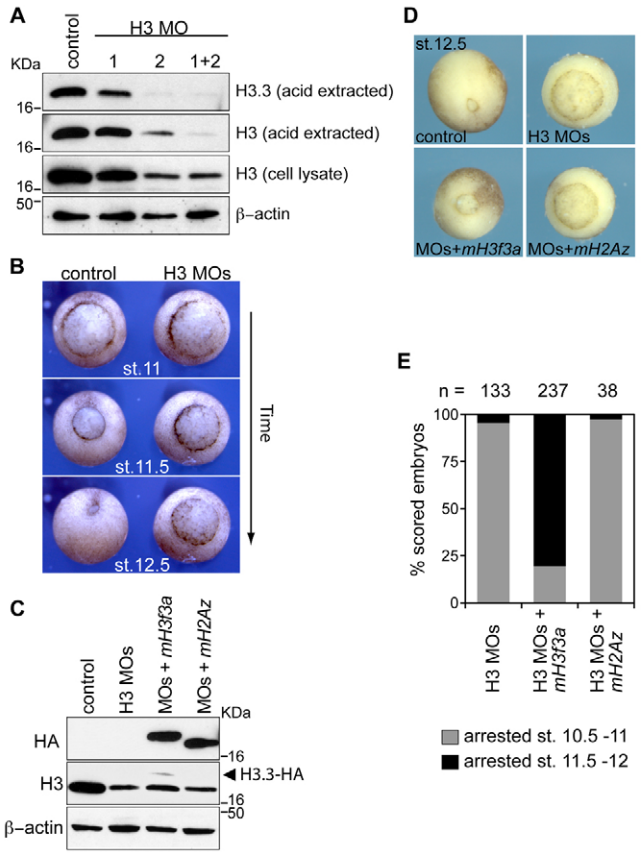

Fig. 1. Loss of H3.3/H3 leads to gastrulation arrest in *Xenopus* embryos. (**A**) Western blot analysis of cell lysates (H3 and β-actin) or acid-extracted nuclear fractions (H3.3 and H3) from stage 11 control or H3 MO-injected embryos. (**B**) Time-lapse images tracking the development of a representative pair of control and H3 MOs-injected embryos from early to late gastrulation. (**C**) Protein synthesis of injected mRNAs shown by western blot analysis of cellular extracts from embryos injected with H3 MOs and 750 pg of mRNA encoding either HA-epitope-tagged mouse H3.3 or H2Az. (**D**) Morphology of injected embryos and control siblings at stage 12.5. (**E**) Percentage of injected embryos arrested at early (stage 10.5-11) or late (stage 11.5-12) gastrula stages.

5′ untranslated region. The injected mouse *H3f3a-HA* transcripts were translated (Fig. 1C) and led to partial rescue of the H3 MO phenotype; 80% of embryos co-injected with the H3 MOs and mouse *H3f3a-HA* mRNA progressed through stage 11 and arrested at late gastrula (Fig. 1D,E). By contrast, embryos co-injected with mRNA encoding histone H2Az were not rescued, and remained arrested at stages 10.5-11, like the H3.3/H3-depleted embryos, demonstrating that the H3 MO-induced phenotype can only be specifically rescued by the expression of H3 histones.

Mesoderm competence, but not induction, is impaired upon H3.3/H3 knockdown

Gastrulation marks the process of cellular differentiation and movement that result in the organization of the three primary germ layers: ectoderm, mesoderm and endoderm. To characterize the gastrulation defects observed, we examined expression of marker genes of the three germ layers by whole-mount *in situ* hybridization. Transcripts of the pan-mesodermal marker *Xbra*, as well as dorsal mesodermal markers *goosecoid* (*gsc*) and *myf5*, were undetectable in the H3 MOs-injected embryos (Fig. 2A-C′). By contrast, H3.3/H3 knockdown resulted in decreased but detectable levels of the pan-endodermal marker *sox17α*, and anterior endoderm markers *hhex* and *cerberus* (Fig. 2D-F′). In addition, we observed modest downregulation of *chordin*, a key molecule in the specification of dorsal neuroectodermal tissues, and *cytokeratin*, a pan-epidermal marker (Fig. 2G-H′). By contrast, the expression of *foxi1* (also known as *Xema*), an ectodermal transcription factor that functions to inhibit mesoderm specification (Suri et al., 2005), was upregulated (Fig. 2I-I′). The upregulation of *foxi1*, and other genes such as *Xnr5-14* and *ina*, was further confirmed by quantitative RT-PCR analysis (Fig. 2J). By contrast, we detected significant decreases in the transcript levels of many mesodermal-expressed genes, such as *dlc*, *lhx1* and *wnt8a*, in the H3.3/H3-depleted embryos (Fig. 2J). We then sought to examine how the loss of H3.3/H3 affected transcription at some genes but not at others, by characterizing the binding of RNA polymerase II at the promoters of *Xbra*, *myf5* and *cebpa* in stage 10 control and H3 MO-injected embryos. At the promoters of the two mesodermal genes *Xbra* and *myf5*, RNA polymerase II binding was significantly decreased by more than fourfold in the H3 MOs-injected embryos (supplementary material Fig. S4A). By contrast, RNA polymerase II remained enriched at the promoter region of the basally expressed *cebpa*, in both control and H3.3/H3-depleted embryos (supplementary material Fig. S4A). Taken together, our analysis on these zygotically expressed lineage marker genes indicates the maximal depletion of H3.3 and partial knockdown of canonical H3 did not lead to global loss of transcription in the developing embryo. Instead, loss of H3.3/H3 perturbed the expression of mesodermal genes, suggesting a specific role of H3.3 and/or proper chromatin organization in the process of mesoderm formation.

Mesoderm specification in *Xenopus* is initiated by ligands of the TGFβ superfamily, produced by the vegetal cells of late blastula embryos (Takahashi et al., 2000; Zhang et al., 1998). Inductive signals from these extracellular ligands, including Nodal and Activin family members, are transduced through receptor-mediated phosphorylation of Smad2/3 proteins. Phosphorylated Smads then translocate into the nucleus to regulate mesodermal gene expression in combination with other transcription factors (Hill, 2001). We hypothesized that the mesoderm formation defects observed in H3 MO-injected embryos could result from either decreased inductive signaling from the vegetal cells or from the failure of animal pole cells to differentiate in response to these signals.

To test which of these two processes is most inhibited by H3.3/H3 depletion, we injected H3 MOs into the four vegetal blastomeres or the four animal blastomeres of eight-cell stage embryos (Fig. 3A). Embryos (n=40) depleted of H3.3/H3 in the animal pole cells arrested at early- to mid-gastrula stage, a similar phenotype to whole embryo knockdown at the four-cell stage (Fig. 3A). By contrast, embryos (39/40) depleted of H3.3/H3 in the vegetal endodermal cells were able to develop to tailbud stages. These findings point to a role of H3.3 and/or proper chromatin organization in mesodermal competence and differentiation of ectodermal cells rather than inductive signaling from the vegetal endoderm. We confirmed our results by assessing the levels of the downstream effectors of Nodal/Activin and BMP signaling, and observed similar levels of phosphorylated Smad2 and Smad1 in both control and H3 MOs-injected embryos, indicating that the mesoderm-inducing and dorsal-ventral signaling pathways remained active in these embryos (Fig. 3B).

To assess directly the effect of H3.3/H3 knockdown on mesodermal competence, we next isolated naïve animal cap (AC)

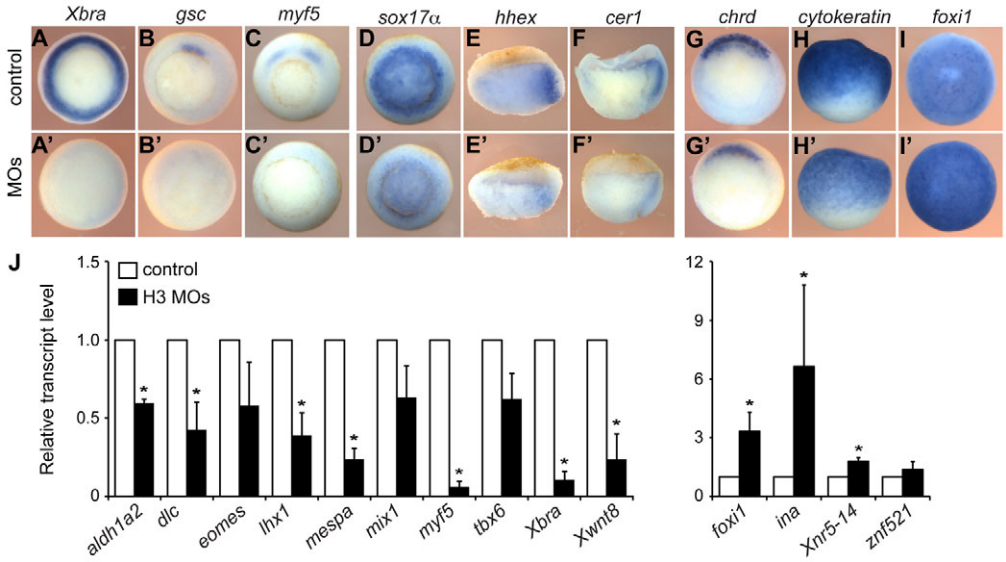

Fig. 2. H3.3/H3-depleted *Xenopus* embryos fail to express mesodermal marker genes. (**A-I'**) Control and injected embryos fixed at early gastrula were subjected to RNA *in situ* hybridization for the analysis of mesodermal (A-C'), endodermal (D-F') and ectodermal (G-I') marker gene expression. Representative embryos from three experiments are shown. (A-D,G) Vegetal views; (E,F) lateral views of bisected embryos, dorsal towards the right; (H) lateral view; (I) animal view. (**J**) Expression of selected genes in control and H3 MO-injected embryos at stage 10.5 was measured by qRT-PCR. All values were normalized to *ornithine decarboxylase* (*ODC*) and plotted relative to the respective transcript levels in control embryos. Error bars indicate s.d. of three independent experiments. **P*<0.05 using a two-tailed Student's *t*-test.

explants from early blastula control or H3 MO-injected embryos and tested their response to Activin A treatment *ex vivo*. *Xbra* expression by *in situ* hybridization and elongation of the AC were scored. Without Activin A, both control and H3 MOs-injected explants remained spherical and did not express *Xbra* (Fig. 3C). When control explants were treated with Activin A, the naïve ectodermal cells differentiated to form elongated mesodermal structures, with concomitant *Xbra* expression. By contrast, animal caps derived from H3 MO-injected embryos did not differentiate and failed to induce *Xbra* expression. Furthermore, we observed rescue of mesodermal differentiation and *Xbra* expression in explants obtained from H3 MOs-treated embryos that were co-injected with the mouse *H3f3a-HA* mRNA (Fig. 3C). These findings indicate that H3.3 function and/or optimal chromatin organization is necessary for ectodermal cells to respond to mesoderm-inducing signals, in a cell-autonomous manner.

Incorporation of H3.3 at promoter regions is not correlated with transcriptional activation

We then sought to determine the contribution of H3.3 function versus chromatin organization to the regulation of mesodermal gene expression and differentiation. As H3.3 incorporation at promoter regions has been proposed to facilitate active gene transcription, we first examined whether H3.3 is present at active gene promoters during mesodermal differentiation. To this end, we analyzed H3.3 localization at the promoters of *Xbra*, *siamois* and *cebpa*, which are expressed at high, intermediate and low levels respectively, in stage 11 embryos. We performed chromatin immunoprecipitation (ChIP) on embryos injected with mouse *H3f3a-HA* mRNA, using an antibody against the HA epitope, and found H3.3 to be present at all three promoters (supplementary material Fig. S4B).

We then examined whether H3.3 deposition at these promoters is correlated with transcriptional activation at different stages of

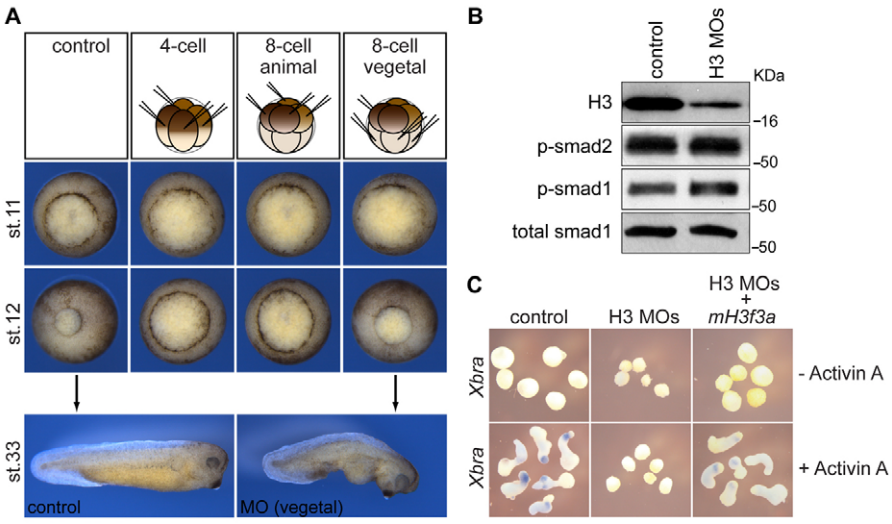

Fig. 3. H3.3/H3 depletion leads to the loss of mesodermal competence in ectodermal cells. (**A**) Time-lapse images show the development of control and embryos injected with H3 MOs in either all blastomeres at the four-cell stage, or four animal or vegetal blastomeres at the eight-cell stage. (**B**) Immunoblots of phospho-Smad2 and phospho-Smad1 in stage 10.5 control and H3 MOs-injected embryos. (**C**) Animal caps excised from control, H3 MOs, and H3 MOs + mouse *H3.3a* mRNA-injected embryos at stage 8 were cultured in the absence or presence of Activin A. At stage 15, the explants were fixed and analyzed for *Xbra* expression by *in situ* hybridization.

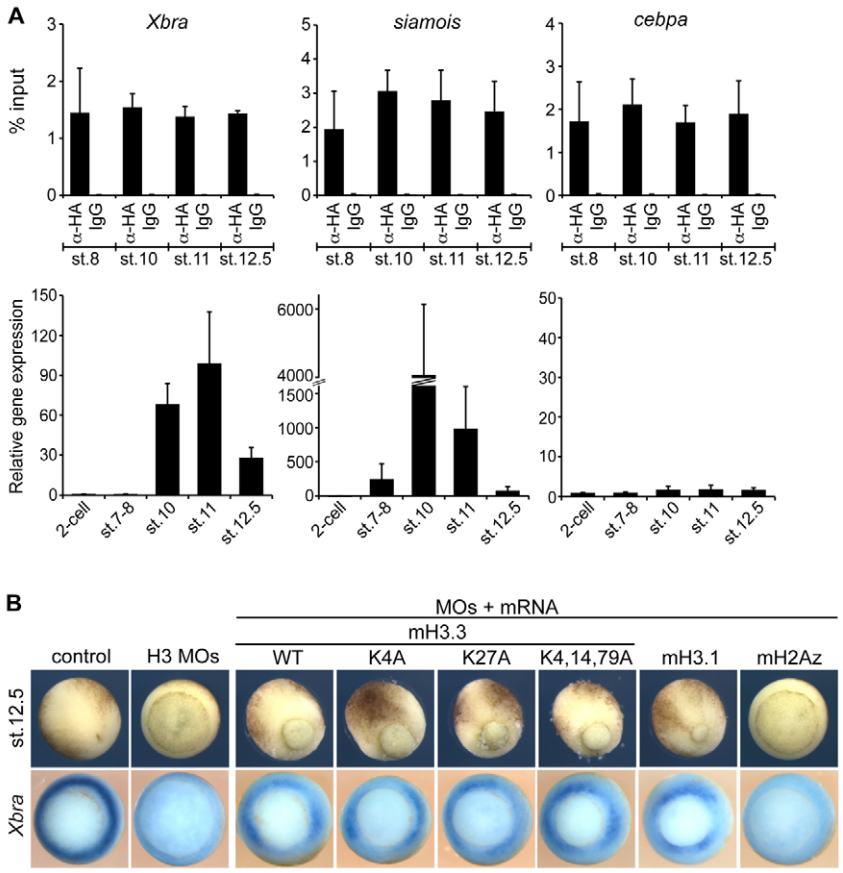

Fig. 4. Mesodermal gene activation is independent of post-translational modifications on H3.3 and incorporation at promoters. (**A**) Enrichment of H3.3-HA at the promoters of *Xbra*, *siamois* and *cebpa* was examined by ChIP-qPCR analysis of embryos injected with 750 pg of mouse *H3.3a*-HA mRNA at blastula (stage 8), early- (stage 10), mid- (stage 11) and late- (stage 12.5) gastrula stages. Level of enrichment is determined as a percentage of input. Expression of these genes at different developmental stages was measured by qRT-PCR. All values were normalized to *ornithine decarboxylase* (*ODC*) and plotted relative to the respective transcript levels in two-cell stage embryos. Error bars indicate s.d. of three independent experiments. (**B**) Rescue experiments were performed by co-injecting H3.3 MO with mRNA encoding HA-epitope tagged wild-type or mutant H3.3, H3.1 or H2Az. Injected embryos were either allowed to develop to late gastrula stage 12.5 (top panels), or fixed at stage 10.5 and subjected to whole-mount *in situ* hybridization analysis of *Xbra* expression (bottom panels).

development. ChIP-qPCR analysis revealed similar levels of H3.3 enrichment at these promoters at blastula, early-, mid- and late-gastrula stages (Fig. 4A). Notably, at the *Xbra* promoter, there were no significant differences in H3.3 enrichment at blastula stage 8, when the gene was not expressed, and at gastrula stages 10 and 11, when *Xbra* became transcriptionally active (Fig. 4A). These results suggest that H3.3 incorporation at specific promoters is not selective, occurs independently of transcription and is not a marker of gene activation in these embryos. Thus, though H3.3 is widely hypothesized to be a general marker of transcription owing to its extensive association with active promoters and genes (Chow et al., 2005; Goldberg et al., 2010; Jin and Felsenfeld, 2006; Mito et al., 2007), our findings indicate that H3.3 incorporation is not directly correlated with transcriptional activation during gastrulation in *Xenopus* embryos.

Specific covalent modifications are not required for function in mesoderm formation

We also assessed the requirement for specific covalent modifications on histone H3.3 that are typically associated with transcriptional regulation, in the control of mesodermal gene expression. Consistent with the developmental rescue observed, we obtained modest recovery of *Xbra* and *gsc* expression in H3.3/H3-depleted embryos when mouse *H3f3a-HA* mRNA were co-injected with the morpholinos (supplementary material Fig. S5A). We then tested the ability of H3.3 mutated singly at K4 and K27 or triply at K4, K14 and K79, to rescue the gastrulation arrest phenotype. We found embryos co-injected with mouse *H3f3K4A-HA*, *H3f3K27A-HA* or *H3f3K4A,K14A,K79A-HA* mRNA developed to late gastrula stages with a concomitant rescue of *Xbra* expression (Fig. 4B). Our findings show that all three H3.3 mutants were able to rescue the MO phenotype, and suggest a function in mesoderm formation that is independent of modifications at these lysine residues. In addition, we were able to rescue the gastrulation-arrest phenotype and *Xbra* expression by co-injecting mRNA encoding the mouse H3.1 protein, but not histone H2Az (Fig. 4B; supplementary material Fig. S5B). We also found that co-expression of both H3.1 and H3.3 did not result in a stronger rescue of the MO phenotype (supplementary material Fig. S5C,D), compared with rescues with equal amounts of individual H3.1 or H3.3. These results indicate that an optimal level of histone H3 is crucial to mesoderm differentiation in *Xenopus* embryos.

Mesodermal competence is regulated by the interplay of nucleosomal H3 and linker histone H1

Taken together, our findings suggest that threshold levels of histone H3 are required to maintain a favorable chromatin organization during the process of mesoderm differentiation in *Xenopus* embryos, and the regulation of mesodermal gene expression is largely influenced by changes in chromatin conformation, rather than H3.3 deposition. Thus, we sought to further assess how loss of H3.3/H3 affects chromatin structure by performing micrococcal nuclease digestion of nuclei prepared from control and H3 MOs-injected stage 10.5 embryos. Limited digestion generated nucleosomal ladders with an estimated nucleosomal repeat length (NRL) of ~176 bp in control embryos (Fig. 5A,B; supplementary material Table S1). In H3.3/H3-depleted nuclei, the nucleosomal ladders were much less defined and exhibited an upward shift corresponding to an ~11 bp increase in NRL. These findings indicate that decreased H3.3/H3 led to more disorganized chromatin with significant perturbations in nucleosomal spacing in gastrula embryos.

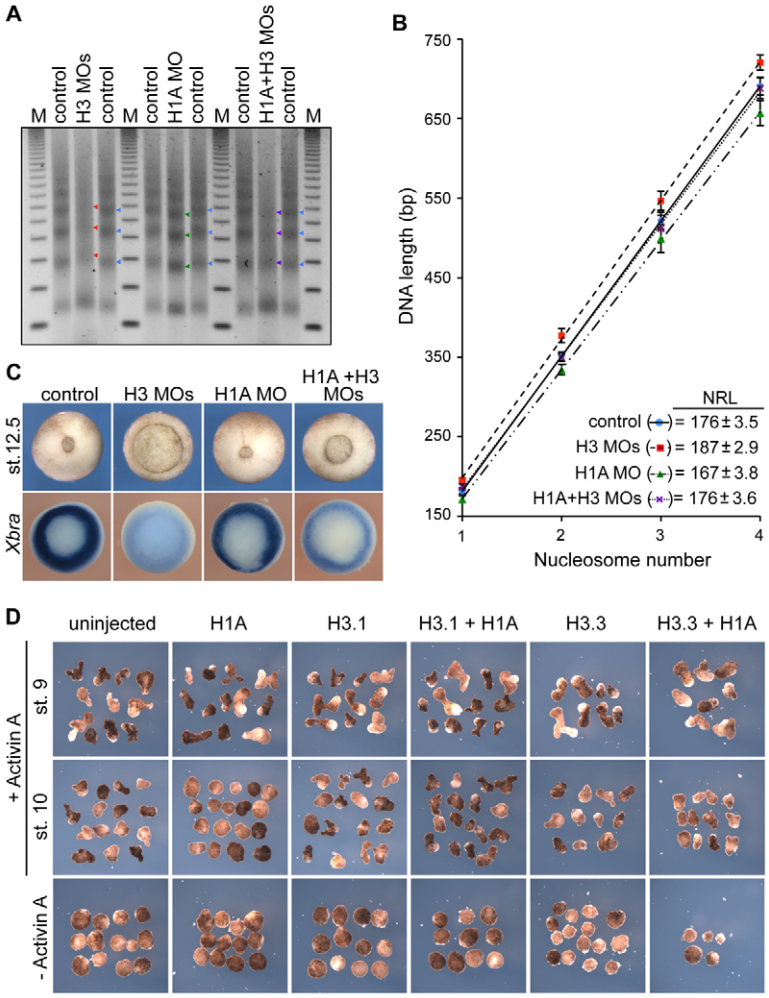

Fig. 5. Mesodermal competence is regulated by interplay of nucleosomal H3 and linker histone H1. (**A**) Nucleosomal ladders resulting from micrococcal nuclease digests of nuclei from control, H3.3–, H1A– and H3.3+H1A–knockdown embryos. Arrows point to di-, tri- and tetra-nucleosomes; M is the 123bp DNA ladder. (**B**) Oligomeric DNA length was calculated and plotted against the nucleosome number. Error bars indicate s.d. of six independent experiments. The mean nucleosomal repeat length (NRL) in each set of embryos is indicated with s.d. (**C**) Embryos injected with H3 MOs alone, H1A MO alone or H3 and H1A MOs were either cultured to late gastrula stage 12.5 (top panels), or fixed at stage 10.5 and subjected to *in situ* hybridization analysis of *Xbra* expression (bottom panels). (**D**) Animal caps excised from stage 8 control or embryos injected with H1A (1.5 ng), H3.1 (1.5 ng), H3.3 (1 ng) or combinations of H1A+H3.1/H3.3 mRNAs, were exposed to Activin A at late blastula and early gastrula stages, and cultured until stage 25.

As linker histone H1 association with the chromatin is a key determinant of nucleosomal spacing (Fan et al., 2005; Woodcock et al., 2006), we sought to examine the relationship between H3.3/H3 and H1 chromatin incorporation in *Xenopus* embryos. To this end, we depleted somatic linker H1 histones in embryos with a translation-blocking morpholino against the H1A isoform (H1A MO). H1A levels are upregulated at the start of gastrulation, and contribute to ~95% of total somatic H1 at this stage (Bouvet et al., 1994; Dworkin-Rastl et al., 1994). The H1A MO effectively reduced total H1 levels to ~30% of control at stage 11, but did not affect the levels of H3 proteins; H1A-depleted embryos did not show overt defects as they developed to tailbud stages (supplementary material Fig. S6A-C). Although H3.3/H3 loss resulted in an increase in average NRL, depletion of H1A led to a distinct shortening of the NRL (Fig. 5A,B). However, when H1A and H3.3/H3 were concurrently depleted, we obtained a more defined nucleosomal ladder that revealed restoration of nucleosomal spacing to that of control. These findings suggest that perturbations to the chromatin organization and nucleosomal spacing upon H3.3/H3 depletion are correlated with increased H1 association to the chromatin.

To address whether the altered chromatin structure, caused by increased H1 incorporation into H3.3/H3-depleted chromatin, directly contributes to the gastrulation defects observed, we tested whether the phenotype could be rescued by concurrent depletion of H1A in H3.3/H3-deficient embryos. Although injection of H3 MOs alone led to early gastrulation arrest, embryos depleted of both H3.3/H3 and H1A developed normally to stage 10.5, when development became delayed, and subsequently arrested at late gastrula stages (Fig. 5C). Notably, expression of *Xbra* was also significantly increased in these H3.3/H3+H1A knockdown embryos (30/37) compared with H3.3/H3 knockdown alone (Fig. 5C; supplementary material Fig. S6D). These results indicate that mesodermal competence can be partially restored by preventing H1-induced changes to the chromatin structure, and lend support to an interplay between nucleosomal H3 and linker H1 histone incorporation in regulating mesodermal differentiation in *Xenopus* embryos.

Precocious accumulation of linker H1 histones achieved by injecting embryos with H1 mRNA has been shown to cause premature loss of mesodermal competence, reflected by the inability of animal caps to respond to Activin A at an earlier developmental stage compared with controls (Steinbach et al., 1997). To further test our hypothesis of an antagonistic functional interplay between nucleosomal H3 and linker H1 histones incorporation, we sought to assess whether co-expression of nucleosomal H3 can abrogate the repressive effects of linker H1 overexpression on the mesodermal differentiation of animal caps. Consistent with other studies (Grainger and Gurdon, 1989; Steinbach et al., 1997), we found that all explants were competent to respond to mesoderm-inducing signals at the late blastula stage (Fig. 5D). By contrast, when treated with Activin A at the early

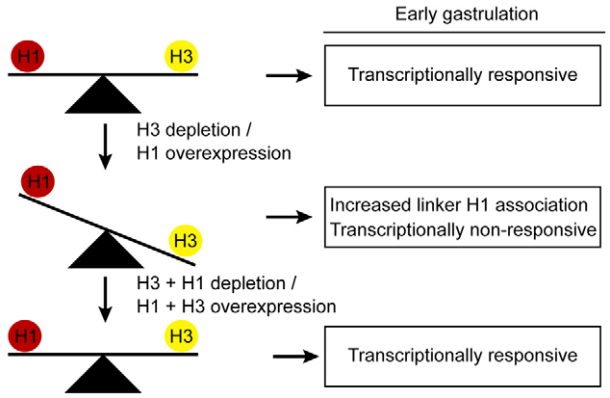

Fig. 6. Model of H3:H1-mediated regulation of mesodermal differentiation. In wild-type embryos, a normal ratio of nucleosomal H3 to linker histone H1 incorporation maintains chromatin in a transcriptionally responsive state to facilitate mesodermal differentiation. In embryos depleted of H3.3/H3 or overexpressing linker histone H1, increased H1 association results in repressed chromatin that is non-responsive to inductive signaling. Simultaneous depletion of histone H1 with H3.3/H3 or overexpression of nucleosomal H3 with linker H1 restores the H3/H1 ratio and alleviates the H1-induced repressive effects on mesodermal competence.

gastrula stage, the animal caps of H1A mRNA-injected embryos failed to respond and remained spherical, whereas control and H3.1- or H3.3-overexpressing explants differentiated to extended structures (Fig. 5D; supplementary material Fig. S6E). More importantly, we observed that explants derived from embryos injected with both H1A and H3.1 or H3.3 mRNAs were also competent to differentiate upon Activin A stimulation at this stage (Fig. 5D; supplementary material Fig. S6E). It is important to point out that though the rescue appeared to be less robust in the H3.3+H1A explants, this weaker effect could be attributed to the injection of less *H3.3* mRNA, which was necessitated by our observations that higher levels of H3.3 overexpression severely affected cell division and development (Fig. 5D; supplementary material Fig. S2A). Taken together, these results indicate that the inhibitory effects of aberrant linker H1 expression on mesodermal competence can be negated by the simultaneous overexpression of nucleosomal H3.1 or H3.3. These findings provide further support for a model whereby equilibrium levels of nucleosomal H3 and linker histone H1 maintain the chromatin in a malleable state to facilitate cellular differentiation in response to signals in developing embryos (Fig. 6).

DISCUSSION

We show here that changes in the levels of nucleosomal H3.3/H3 histones lead to perturbed development of *Xenopus* embryos. The overexpression of H3.3 resulted in severe defects in cellular division early in development, and death of the embryos by late gastrula stage. In addition, though a complete knockdown of H3.3 could not be achieved without affecting the levels of canonical H3, abnormalities observed in embryos partially depleted of H3.3 pointed to a specific function of H3.3 in *Xenopus* development. These findings correlated well with a recent study reporting gastrulation and blastopore closure defects in *Xenopus* embryos depleted of H3.3 and its specific histone chaperone HIRA (Szenker et al., 2012), indicating vital functions for H3.3 and HIRA at late gastrulation. More importantly, we find that maximal H3.3 depletion with concomitant H3 loss strongly impedes mesoderm formation. Vertebrate mesoderm formation is a highly regulated process involving a complex interplay of morphogen-mediated signals and transcriptional responses to direct cellular differentiation in a temporal and spatial manner (Hill, 2001). We find that H3.3/H3-depleted animal cap cells were unable to differentiate upon Activin A stimulation, suggesting that H3.3/H3 depletion directly affects transcriptional responses downstream of the mesoderm-inducing signals. By contrast, levels of phosphorylated Smad2 and Smad1 were unchanged in H3.3/H3-depleted embryos, indicating that the Nodal/BMP pathways remained active in these embryos and were unaffected by the loss of nucleosomal H3.3/H3 histones.

Our study shows that disruption of the global chromatin organization, as a result of H3.3/H3 depletion, can be rescued by the simultaneous depletion of linker histone H1. These findings point to a possible regulatory mechanism whereby a threshold level of nucleosomal H3 incorporation is needed to prevent H1-induced changes to the chromatin structure that result in gene repression, thus facilitating mesodermal differentiation in response to inductive signaling. This hypothesis is further supported by our findings that the premature loss of mesoderm competence induced by precocious linker H1 histones expression can be countered by concurrent overexpression of nucleosomal H3. These results also shed light on the previously published, but unexplained, observations that overexpression of somatic linker H1 causes loss of mesodermal competence in *Xenopus* animal cap explants (Steinbach et al., 1997), a phenotype that corresponds to the mesoderm formation defects we observe in H3.3/H3-deficient embryos. The exclusion of H1 incorporation at specialized genomic regions may be required to maintain these sites in a fluid chromatin state that is accessible and responsive to regulatory factors that drive mesodermal differentiation. Indeed, incorporation of linker histones can lead to decreased nucleosome mobility and transcriptional repression (Pennings et al., 1994; Ura et al., 1995). In addition, it has been shown that transcriptional activation by TFIIA occurs more readily in chromatin depleted of H1 (Bouvet et al., 1994). Thus, we propose that a threshold level of nucleosomal H3 to linker histone H1 is necessary to maintain chromatin organization at specific genomic loci in a state that is accessible and responsive to factors driving mesodermal differentiation (Fig. 6). Genetic manipulations leading to reduced H3:H1 ratios, such as depletion of H3 or overexpression of H1, would thus result in decreased transcription of specific genes, and lead to the loss of mesodermal competence.

A recent study that compared the genomic occupancy of H1 and H3.3 in *Drosophila* embryonic cells, has also revealed a negative correlation between H3.3 and H1 binding, particularly around gene regulatory sites (Braunschweig et al., 2009). Cells partially depleted of H3.3 were also shown to exhibit increased nucleosomal spacing and linker H1 association with the chromatin (Braunschweig et al., 2009). These observations are highly consistent with our findings in *Xenopus* embryos. However, although it was not directly addressed in *Drosophila* cells, we show that these effects may not be H3.3-specific but rather that responsive chromatin organization in these cells is achieved by the modulation of nucleosomal H3 and linker histone H1 levels. More importantly, our study demonstrates, for the first time, a functional relevance to the inverse chromatin association of H3.3/H3 and H1 observed in both *Drosophila* embryonic cells and *Xenopus* embryos. Based on these findings, we speculate that active exclusion of linker histone H1 from chromatin by nucleosomal H3.3/H3 to maintain transcriptional competence in embryonic cells

may be an evolutionarily conserved mechanism, which prevents premature gene silencing and facilitates transcriptional response during cellular differentiation.

In this study, we have illustrated a unique chromatin-mediated mechanism in the regulation of cellular differentiation during embryonic development. Our findings suggest that mesodermal competence can be directly modulated via changes to the nucleosomal H3:linker histone H1 ratio. Challenges for the future will be to understand why, although widespread changes in chromatin structure take place upon perturbations of H3.3/H3 and H1 levels, transcriptional regulation mediated by these molecules is localized and affects only a subset of genes. It is nevertheless possible to speculate that the stage of transition from oocyte-specific to somatic forms of linker H1 is especially sensitive to perturbations of the H3/H1 ratio, and the genes that must be activated at this time will be most affected.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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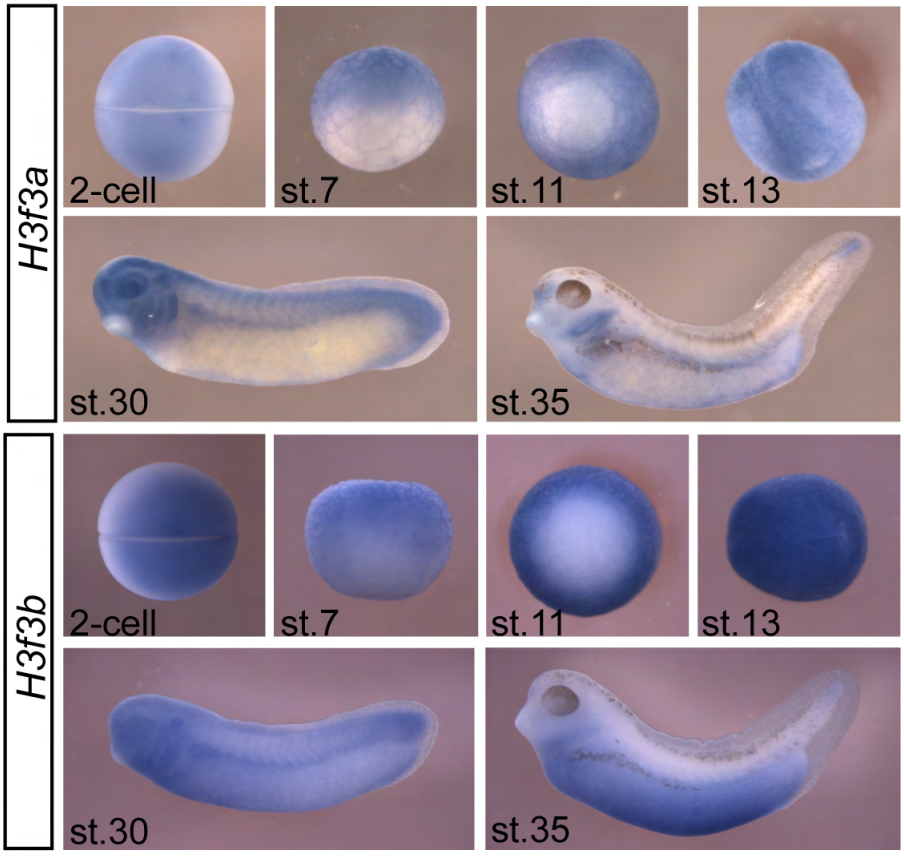

Fig. S1. *H3f3a* and *H3f3b* genes are co-expressed during early embryonic development. Expression patterns of *H3f3a* and *H3f3b* determined by whole-mount *in situ* hybridization.

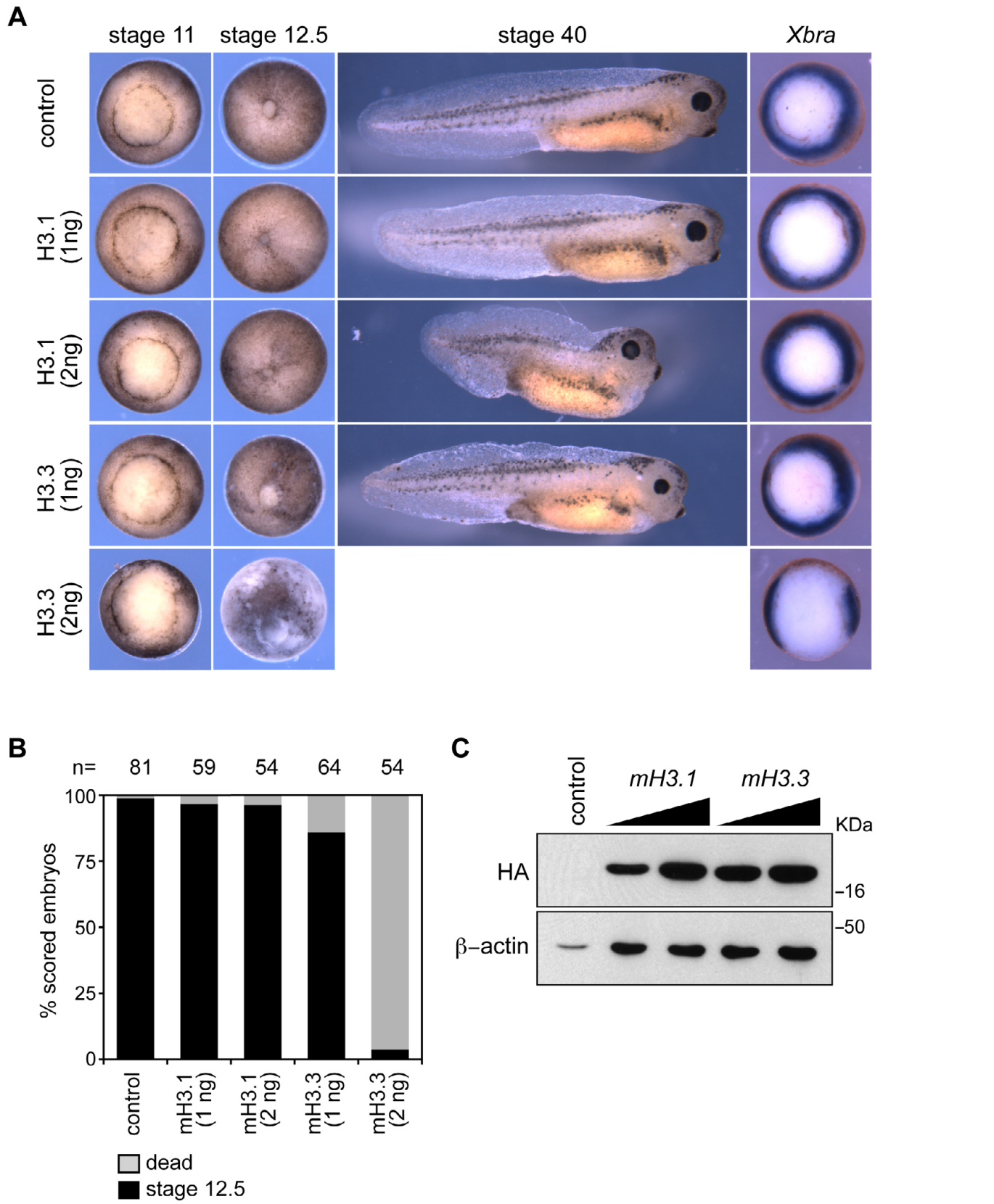

Fig. S2. Overexpression of H3.3 leads to early embryonic lethality. (**A**) Embryos at the two- to four-cell stages were injected with 1 or 2 ng of mRNA encoding HA-tagged H3.1 or H3.3. Injected embryos were either allowed to develop to late tailbud stage, or fixed at stage 10.5 and subjected to whole-mount *in situ* hybridization analysis of *Xbra* expression. (**B**) Percentage of injected embryos that developed normally to late gastrula stage 12.5. (**C**) Synthesis of exogenous H3.1-HA or H3.3-HA proteins in injected embryos detected by immunoblotting for the HA epitope.

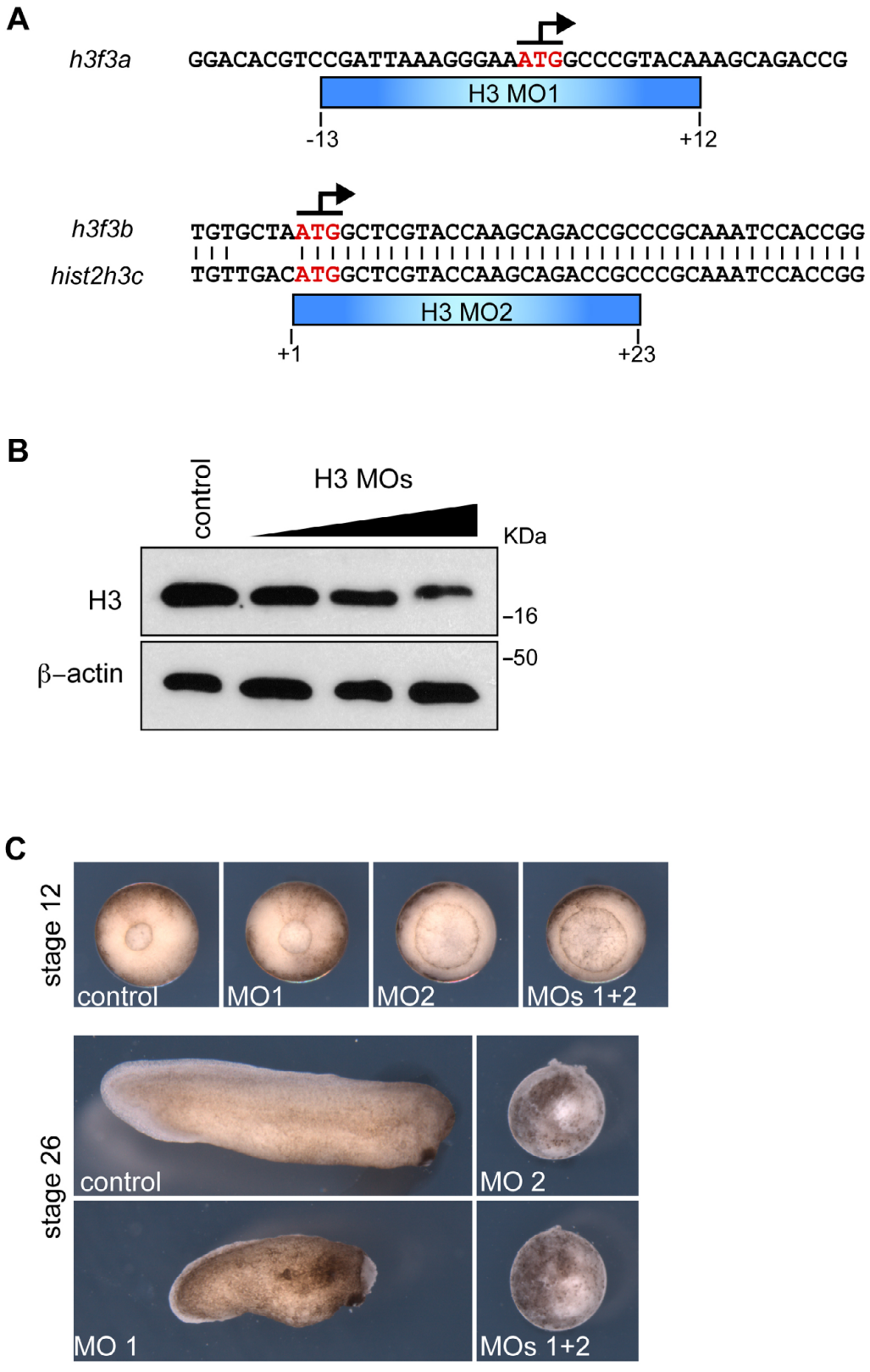

Fig. S3. Developmental defects in embryos partially or completely depleted of H3.3. (**A**) Target regions of H3 MO1 and MO2. (**B**) Western blot analysis showing dose-dependent depletion of endogenous H3 proteins in H3 MO-injected embryos. Doses of H3 MOs tested were 6.5 ng, 26 ng and 65 ng. (C) Development of control or embryos injected with H3 MO1 or MO2 alone or MO1+MO2 at stages 12 and 26.

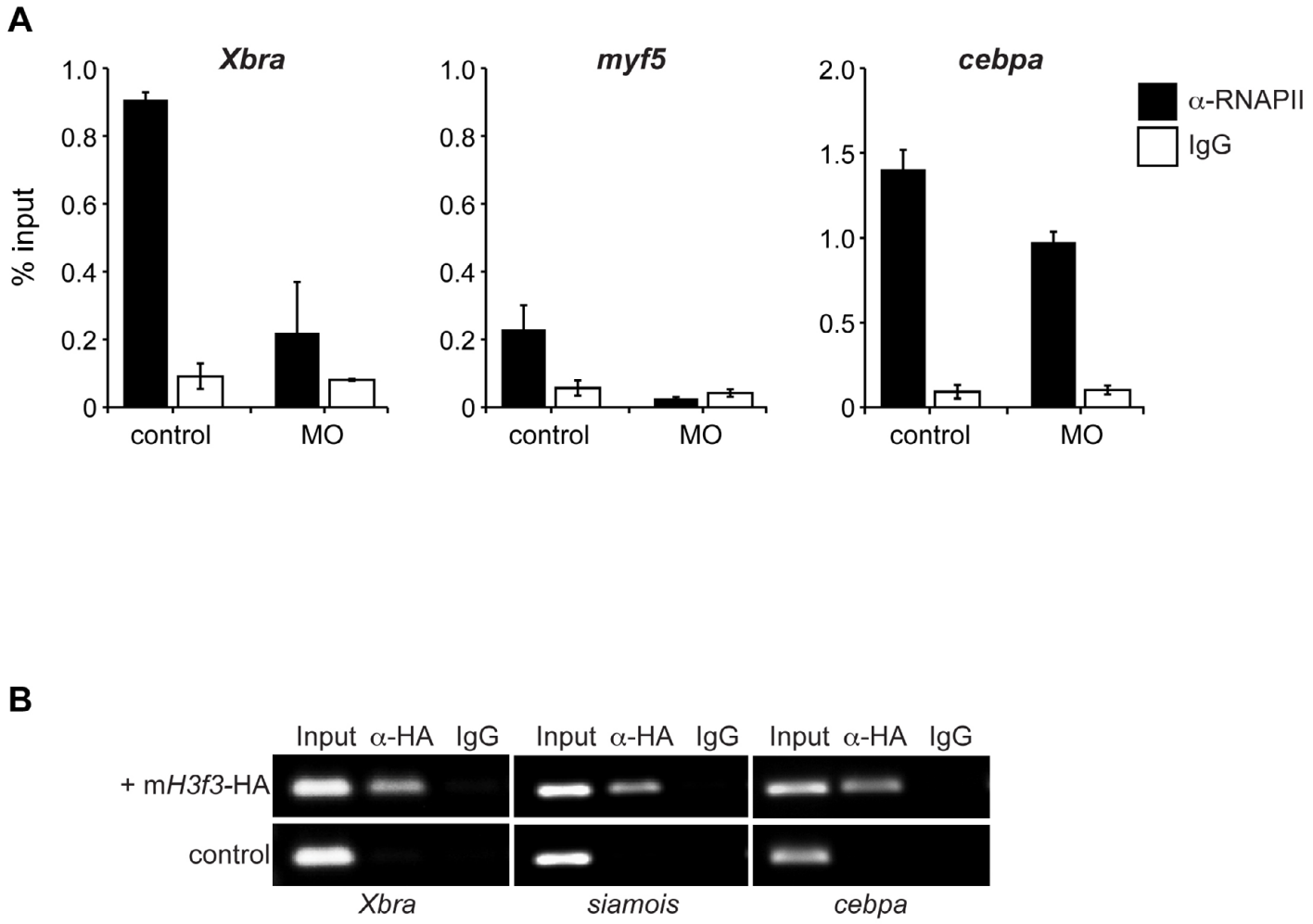

Fig. S4. Presence of RNA polymerase II and H3.3 at the promoters of active genes in gastrula embryos. (**A**) Presence of endogenous RNA polymerase II at the promoters of *Xbra*, *myf5* and *cebpa* were analyzed by ChIP-qPCR analysis of stage 10.5 control and H3 MOs-injected embryos. Level of enrichment is determined as a percentage of input. (**B**) Chromatin immunoprecipitation using anti-HA antibody was performed on stage 10.5 control and embryos injected with 750 pg of *mH3.3a*-HA mRNA. Presence of H3.3-HA at the promoters of *Xbra*, *siamois* and *cebpa* were analyzed by PCR using promoter-specific primers.

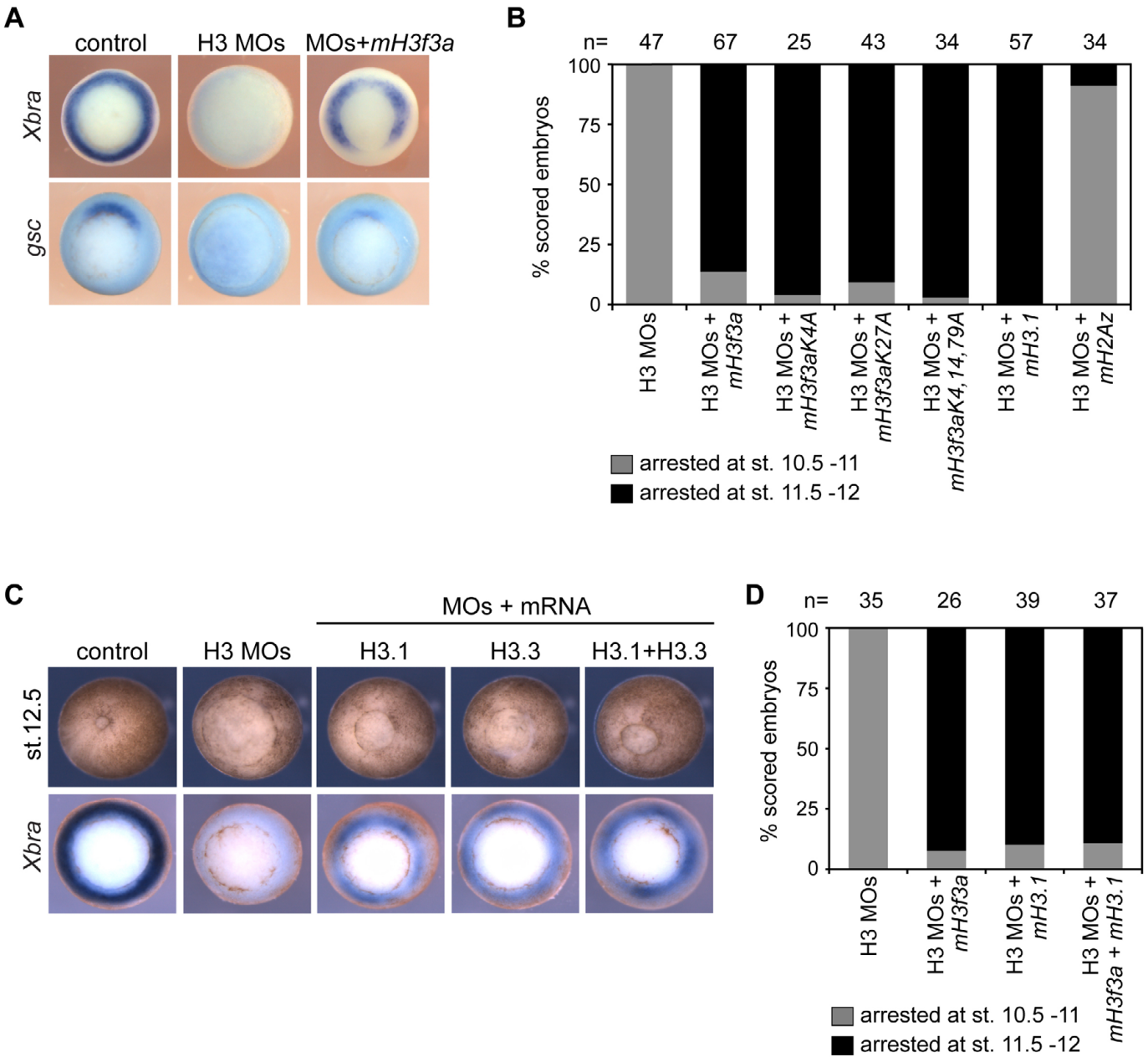

Fig. S5. Mesoderm formation and gene expression in H3 MOs morphants is rescued by co-injection of mouse *H3f3a* mRNA. (**A**) Control and injected embryos fixed at early gastrula were subjected to RNA *in situ* hybridization analysis of *Xbra* and *gsc* transcripts. (**B**) Percentage of embryos co-injected with mRNA encoding HA-tagged wild-type or mutant H3.3, or H3.1 that arrested at early (stage 10.5-11) or late (stage 11.5-12) gastrula stages. (**C**) Rescue experiments were performed by co-injecting H3.3 MO with a total amount of 750 pg of mRNA encoding HA-tagged H3.1 or H3.3, or both. Injected embryos were either allowed to develop to late gastrula stage 12.5 (top panels), or fixed at stage 10.5 and subjected to whole-mount *in situ* hybridization analysis of *Xbra* expression (bottom panels). (**D**) Percentage of injected embryos that arrested at early (stage 10.5-11) or late (stage 11.5-12) gastrula stages.

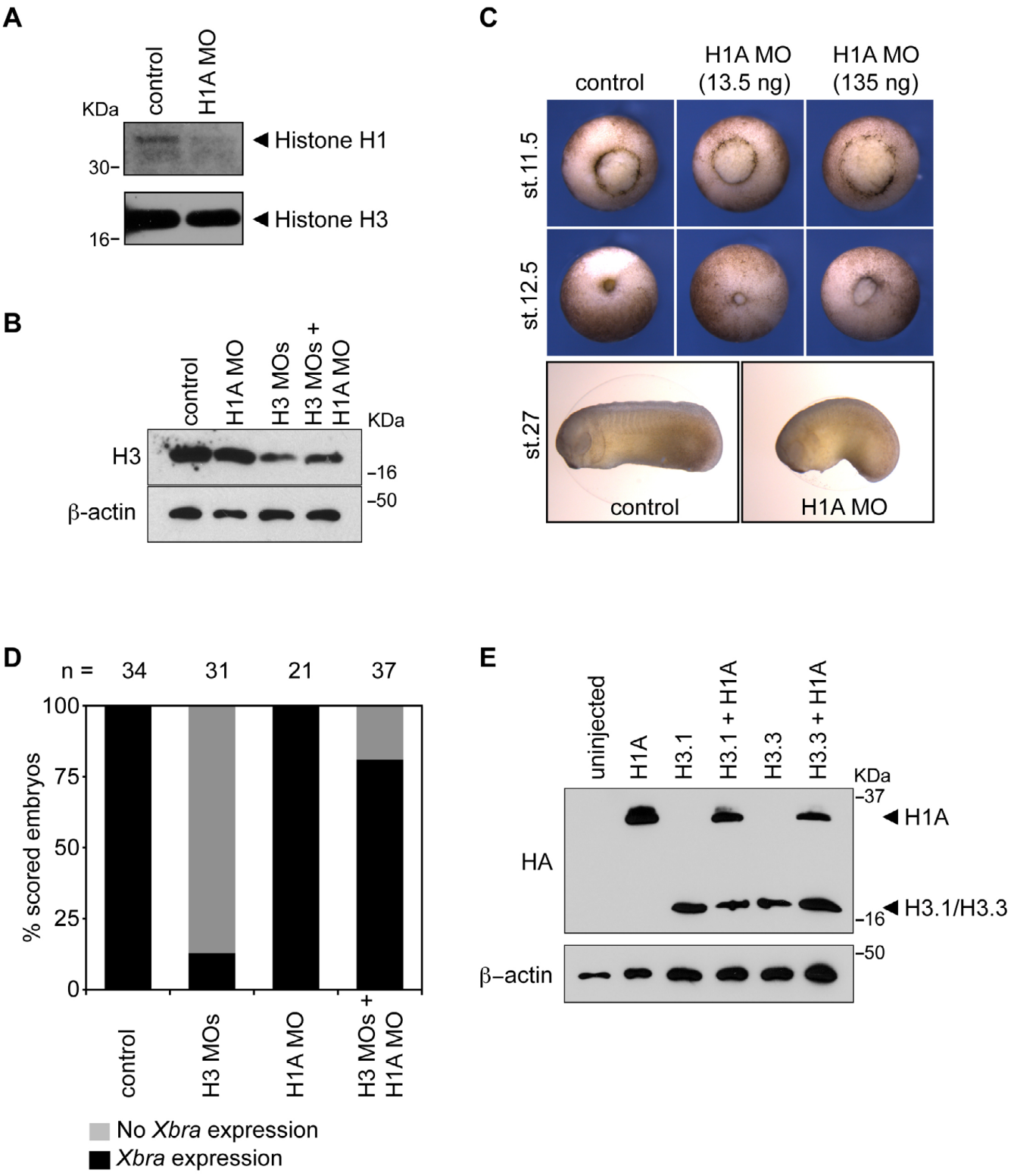

Fig. S6. Mesodermal competence is regulated by interplay between nucleosomal H3 and linker histone H1. (**A**) Synthesis of somatic linker histone H1A is inhibited by H1A antisense MO. Western blot analysis of acid nuclear extracts from gastrula stage control or H1A MO-injected embryos shows ~70% knockdown of histone H1 (arrow). (**B**) Western blot analysis of endogenous H3 protein levels in gastrula stage control and injected embryos. (**C**) Time-lapse images tracking development of control and H1A-depleted embryos. Development of H1A knockdown embryos was slightly delayed but otherwise normal. (**D**) Percentage of control and injected embryos that express *Xbra*. Embryos from two independent experiments were fixed at early gastrula and subjected to RNA *in situ* hybridization analysis of *Xbra* transcripts. (**E**) Protein synthesis of injected mRNAs shown by western blot analysis of cellular extracts from stage 10.5 embryos.

Table S1. The mean sizes of micrococcal nuclease digested DNA fragments from control or injected early gastrula stage 10.5 embryos

Nucleosome number	Control (bp)	H3.3 MO (bp)	H1A MO (bp)	H1A + H3.3 MO (bp)
1	182	196	171	187
2	351	377	334	350
3	521	546	499	512
4	691	720	657	688
*NRL (bp±s.d)	176±3.5	187±2.9‡	167±3.8‡	176±3.6§

*The nucleosomal repeat length (NRL) was calculated from the length of oligomeric DNA divided by the nucleosome number. Oligomeric DNA fragment sizes and standard deviation (s.d) of NRL were calculated from six independent experiments.

‡Differences in NRL of control versus injected embryos are statistically significant ($P<0.0001$, two-tailed paired t-test).

§NRL of control versus injected embryos are not significantly different ($P=0.78$).

Table S2. Primer sequences

Cloning of *Xenopus* H3f3a and H3f3b cDNA		
	Forward	Reverse
H3f3a	TTCGAATTCAGAGGACACGTCCGATTAAAG	AGGCTCGAGACTTACAGGAACAGCACAG
H3f3b	TTCGAATTCTGCAGGAGGCTAGTGAGGCT	AGGCTCGAGACACTCACCAACTATCTG
Cloning of mouse H3f3a-HA-Flag		
	Forward	Reverse
mH3f3a	GAATTCACCATGGCTCGTACAAAGCAGAC	GAATTCTTACTTATCGTCGTCATCCTTGTAATCAGCGTAATCTGGAACATCGTATGGGTAAGCACGTTCTCCGCGTATG

ChIP-qPCR primers		
	Forward	Reverse
Xbra promoter	TGAACAATCTATCCAGGCCACCT	AGAGAGCTCTATGATAATCCTGGGA
siamois promoter	GGGACTTTGAAGTCTTGCCA	TCTGATGACACGTGTTTCCC
cebpa promoter	ACAGGTGCAGAGATACATTCAGGCT	ACCTGCTTCAATGCAGCTACTTGCA
myf5 promoter	TGAGGCTGTGTTGAGATAAAGTATGCTAA	AATCAGGTTTCAGGAAATCTCTTAGGAGT

RT-PCR primers		
	Forward	Reverse
Xbra	TTCTGAAGGTGAGCATGTCG	GTTTGACTTTGCTAAAAGAGACAGG
siamois	TCTCCAGCCACCAGTACCAGATCTA	TGTATCCTGGGCTCAGGAATGCCAGT
cebpa	ACATCATCACCACCATCACCTGCA	CTGTACTCGTTGCTGTTCTTGTCCA
ODC	GATGTGAAACTGAAGTTTGAAGAGAT	CCAGAATCTGCTGGGAAGTATT
aldh1a2	TGTAGCAGATGATATGCGGATTGCC	ACAGTTCCTGCTTGCATTGCTGAAG
dlc	AGATGCCTGTAGTTCCAAACCCTGCA	AGCAGTCCCAGACAAACCACCATGGT
eomes	TCAGGTTTCTCAGAAGATCTCCTAC	TAGTCCTCAGTGCCAATGTCTTCAC
foxi1	AGATGAAGATGATCCAGGCAAGGGCA	AGCGGGCTCCCTTCAGGCTTGTCAGA
ina	ACCATCGGCCAGCTTGACAATGCTT	ATGGGTAGCCAATACCGAACATGCT
lhx1	CACCCATCTAGTGACGCTCAGAGGT	TGGTTGCCATAACCTCCATTGACTG
mespa	TCCGTGCCTTTCTCTGAAGCAAGAT	TGACTGACAGGCGTAGCTACTGTGCT
mix1	GCCCAACAGGAAAGAAGTCA	GACATTTTGGACTGGGCTCT

myf5	ATGGAGATGGTAGATAGCTGCCATT	ACCAATAGGTGCTCTGACATGCTCGT
nkx6	AGAGACACGCAGCAGAGATGGCCAC	CTGCAAGGGCTGAGAAGGTTCAAGT
tbx6	AGGAGTCGCCTCACATCAGCCACAGT	AGTTCATGGAGAAACCTCTGCCCAT
Xwnt8	GCTACCCACAATGGACTTCG	GAGCTAATGGCATGCACAAA
Xnr5-14	TGCTTATCGATGTGAAGGATCCTGC	CCTTCATACATGAGCATTGACAGA
znf521	GACTCGATGTTCTAGCTGCAATGTT	TCAGATTGAGGACTTATCCTGGCCA