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

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The caudal homeobox (*cdx*) gene family is critical for specification of caudal body formation and erythropoiesis. In zebrafish, *cdx4* expression is controlled by the Wnt pathway, but the molecular mechanism of this regulation is not fully understood. Here, we provide evidence that Tcf3 suppresses *cdx4* expression through direct binding to multiple sites in the *cdx4* gene regulatory region. Tcf3 requires corepressor molecules such as Groucho (Gro)/TLE and HDAC1 for activity. Using zebrafish embryos and cultured mammalian cells, we show that the transcription factor E4f1 derepresses *cdx4* by dissociating corepressor proteins from Tcf3 without inhibiting its binding to *cis*-regulatory sites in the DNA. Further, the E3 ubiquitin ligase Lnx2b, acting as a scaffold protein irrespective of its enzymatic activity, counteracts the effects of E4f1. We propose that the modulation of Tcf3 repressor function by E4f1 assures precise and robust regulation of *cdx4* expression in the caudal domain of the embryo.

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Introduction

The process of anteroposterior (AP) axis specification is comparatively well understood in amphibians and fish where a caudal-to-rostral gradient of Wnt activity is instrumental in AP patterning (MacDonald *et al*, 2009). Canonical Wnt signalling stabilizes cytosolic β-catenin, which translocates into the nucleus and binds to LEF/TCF transcription factors to mediate target gene expression. Four evolutionary well-conserved factors (Lef1/LEF1, Tcf3/TCF7L1, Tcf4/TCF7L2 and Tcf7/TCF1) are involved in Wnt/β-catenin signalling. Although LEF/TCF family members exhibit some functional redundancy (Galceran *et al*, 1999; Nagayoshi *et al*, 2008), individual LEF/TCF factors often behave differently during embryonic development. In *Xenopus*, XTcf3 establishes early dorsal polarity while XLef1 has important roles in mesoderm patterning (Brannon *et al*, 1997; Roël *et al*, 2002). In zebrafish, Lef1 regulates brain neurogenesis (Lee *et al*, 2006; Bonkowsky *et al*, 2008) and pectoral fin outgrowth in cooperation with Tcf7 (Nagayoshi *et al*, 2008), and Tcf3 specifies anterior neural tissues (Kim *et al*, 2000; Dorsky *et al*, 2003).

The distinctive functional differences between LEF/TCF family members seem to be due to their intrinsic properties as transcriptional activators or repressors. LEF/TCF factors act as repressors when bound to corepressor proteins such as Gro/TLE and HDAC, and this repression is relieved by β-catenin recruitment (Cavallo *et al*, 1998; Brantjes *et al*, 2001; Hovanes *et al*, 2001; Hurlstone and Clevers, 2002; MacDonald *et al*, 2009). However, Lef1 cannot substitute for Tcf3 repressor function in AP patterning (Kim *et al*, 2000; Dorsky *et al*, 2003), suggesting that the primary embryonic function of Lef1 is transcriptional activation in the presence of β-catenin (Gat *et al*, 1998; Hovanes *et al*, 2001). In contrast, Tcf3 function involves mostly or entirely cooperation with corepressors to act as a transcriptional repressor in embryonic domains in which Wnt signals are limiting (Kim *et al*, 2000; Dorsky *et al*, 2003; Gribble *et al*, 2009). In the developing zebrafish and mouse embryo, AP body patterning largely depends on the transcriptional repressor function of Tcf3 (Kim *et al*, 2000; Dorsky *et al*, 2003; Merrill *et al*, 2004). This is most clearly indicated by the fact that the *tcf3a* mutant *headless (hdl)* shows anterior truncations due to overall posteriorization, similar to embryos with hyperactivated Wnt signalling (Kim *et al*, 2000). In contrast, when Wnt signalling is inhibited, zebrafish embryos show truncated tails reminiscent of the loss-of-function phenotype of *cdx1a* and *cdx4* (Shimizu *et al*, 2005).

The evolutionary conserved Cdx/caudal homeobox transcription factors, known Wnt target genes (Shimizu *et al*, 2005; Pilon *et al*, 2006, 2007), have critical roles in patterning of caudal structures, early endoderm specification, gut AP patterning, establishment of the intestinal epithelium and haematopoiesis, at least in part by regulating expression of certain *Hox* genes (Isaacs *et al*, 1998; van den Akker *et al*, 2002; Davidson *et al*, 2003; Bansal *et al*, 2006; Cheng *et al*, 2008; Flores *et al*, 2008; Chen *et al*, 2009; Faas and Isaacs, 2009; Young and Deschamps, 2009; Young *et al*, 2009; Gao and Kaestner, 2010). Aberrant expression of human CDX2 is frequently detected in AML and paediatric ALL patients, and seems to be causative of leukaemia development via altering *HOX* gene expression (Scholl *et al*, 2007; Riedt *et al*, 2009; Thoene *et al*, 2009). Additionally, Cdx genes function as key factors during haematopoiesis, and ectopic expression of *Cdx4* can trigger leukaemogenesis in mice (Bansal *et al*, 2006; Wang *et al*, 2008). Thus, the expression of *Cdx* genes

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must be tightly regulated during development, but the mechanism leading to the induction of *Cdx* genes in a precise pattern in the embryo is not fully understood.

In zebrafish, Cdx4 is the major factor that governs caudal tissue specification and primitive erythropoiesis (Davidson *et al*, 2003). Here, we provide evidence that Tcf3 in cooperation with Gro/TLE and HDAC1 suppresses *cdx4* expression through direct binding to the *cdx4* gene regulatory region. We show that E4f1, previously characterized as a transcriptional repressor of *cyclin A2* (Fajas *et al*, 2001), derepresses *cdx4* by disrupting a complex between corepressor proteins and Tcf3 while leaving Tcf3 bound to its cognate sites in the *cdx4* regulatory region. As a further mechanism in precise modulation of *cdx4* expression, we find that the multi-PDZ domain-containing E3 ubiquitin ligase Lnx2b (one of the zebrafish homologues of Ligand of Numb protein X-2; Nie *et al*, 2002; Ro and Dawid, 2009; Ro and Dawid, 2010) counteracts E4f1 function by stabilizing the Tcf3–Gro/TLE–HDAC1 repressor complex. These observations introduce a novel mechanism that modulates Tcf3 repressor activity and through this the output of the Wnt signalling pathway in AP patterning. We propose that this mechanism contributes to the establishment of the precise *cdx4* expression domain in the zebrafish embryo, assuring normal development of the caudal body region and erythropoiesis.

Results

E4f1 is a positive factor in tail development

Our interest in the function of E4f1 started from the observations that it is a binding partner of Lnx2b (previously designated Lnx-like), a factor we studied previously (Supplementary Figures S1 and S2) (Ro and Dawid, 2009, 2010). Besides having transcriptional repressor activity (Fajas *et al*, 2001), E4f1 has been reported to act as a BMI1 modulator (Chagraoui *et al*, 2006) and as an atypical ubiquitin ligase (Le Cam *et al*, 2006), suggesting multiple functions for E4f1 during development. While Lnx2b can act as an E3 ubiquitin ligase (Ro and Dawid, 2009), it did not modulate the stability or transcriptional repressor activity of E4f1 (Supplementary Figure S1A and B; unpublished data). Since early embryonic lethality of *E4f1* mutant mice impeded further analysis of its developmental role (Le Cam *et al*, 2004), we exploited zebrafish for studying the function of E4f1. Zebrafish *e4f1* is expressed maternally and zygotically without exhibiting significant spatio-temporal differences (Supplementary Figure S3). Injection of *e4f1* translation-blocking morpholino (MO) caused developmental defects beginning by 11 h post-fertilization (h.p.f.), leading to delayed tail elongation and, starting from early somitogenesis, shortened and curled-up tails (Figure 1A and B). Because the *e4f1* morphant phenotype was largely rescued by the injection of human *E4F1* mRNA that does not contain the MO target site (Figure 1C), we conclude that the MO effects are specific to the function of E4f1 rather than reflecting non-specific effects. Further, these observations suggest that the function of E4f1 is conserved from teleosts to tetrapods. Overexpression of E4f1 by injection of zebrafish *e4f1* mRNA at the same level that achieved rescue of morphants did not result in a visible phenotype by 24 h.p.f. (Figure 1D), possibly due to the fact that the embryo already contains substantial levels of E4f1 (Supplementary Figure S3).

The development of the caudal domain of the embryo is controlled by several signalling pathways among which the Wnt pathway has an important position (Shimizu *et al*, 2005; Nordström *et al*, 2006; Iimura *et al*, 2009; Mallo *et al*, 2009). The formation of short tails by depleting E4f1 suggests that this factor has a positive influence on tail development, as does the Wnt pathway. An embryo with attenuated Wnt signalling might then represent a sensitized test object in which the effect of E4f1 can be analysed. This expectation is borne out by the data in Figure 1E. Classification of tail defects into five categories allowed us to quantify the results. As expected, injection of a moderate level of RNA encoding the Wnt inhibitor Dkk1 led to intermediate levels of tail shortening. These effects were substantially ameliorated by co-injection of *e4f1* mRNA with over 50% of the embryos rescued to normal development; in contrast injection of the *e4f1* MO strongly enhanced the tail phenotype, with over 50% of embryos showing complete loss of the tail (Figure 1E). These results strongly support our conclusion that E4f1 is a positive factor in tail development, and further suggest that it carries out this function in conjunction with the Wnt signalling pathway.

E4f1 together with Wnt regulates cdx4 expression

Cdx transcription factors have a major role in caudal body development in all vertebrates (van den Akker *et al*, 2002; Shimizu *et al*, 2005; Nordström *et al*, 2006; Faas and Isaacs, 2009; Young and Deschamps, 2009; Young *et al*, 2009). Therefore, we asked whether the tail defects in *e4f1* morphants correlate with effects on the expression of *cdx4*, the major caudal determinant in zebrafish. *e4f1* morphants exhibited a modest reduction of the *cdx4* expression domain at the bud stage (49%, $n=37$), similar to embryos injected with a *wnt3a/wnt8.1* MO mixture (94%, $n=32$) (Figure 2A–C). The expression domain of *cdx4* became further compromised when *wnt3a/wnt8.1/e4f1* expression was blocked simultaneously (Figure 2D; 100%, $n=33$). Similar to a previous report, extensive blocking of Wnt signalling through depletion of *wnt3a/wnt8.1/wnt8.2* (100%, $n=24$; Figure 2E) or by overexpression of a high level of Dkk1 (100%, $n=30$; Figure 2G) caused strong reduction of *cdx4* expression (Shimizu *et al*, 2005). Expression of *cdx4* was reduced further when *e4f1* and Wnt function was blocked simultaneously (Figure 2F; 70%, $n=30$ and Figure 2H; 60%, $n=30$). The reduction of *cdx4* expression in the *e4f1* morphants was visible as early as the onset of gastrulation (Supplementary Figure S4A, C and D). In contrast to the effects of the MO, overexpression of E4f1 did not significantly alter the expression level of *cdx4* (Supplementary Figure S4B and D), consistent with the lack of a visible phenotype in embryos injected with the same levels of *e4f1* mRNA (Figure 1D). The expression levels of other caudal genes that are regulated by posterior Wnt signal were also compromised in the *e4f1* morphant embryos (Supplementary Figure S4D) (Dorsky *et al*, 2003; Li *et al*, 2011). These results indicate that E4f1 cooperates with Wnt signalling in the regulation of *cdx4* expression in the caudal domain of the embryo.

Cdx4 regulates caudal development and erythropoiesis through target genes among which several Hox genes hold an important position. Therefore, we examined the expression of *hoxa9a*, a direct target gene of Cdx4 (Davidson *et al*, 2003; Shimizu *et al*, 2005) after manipulating Wnt and E4f1

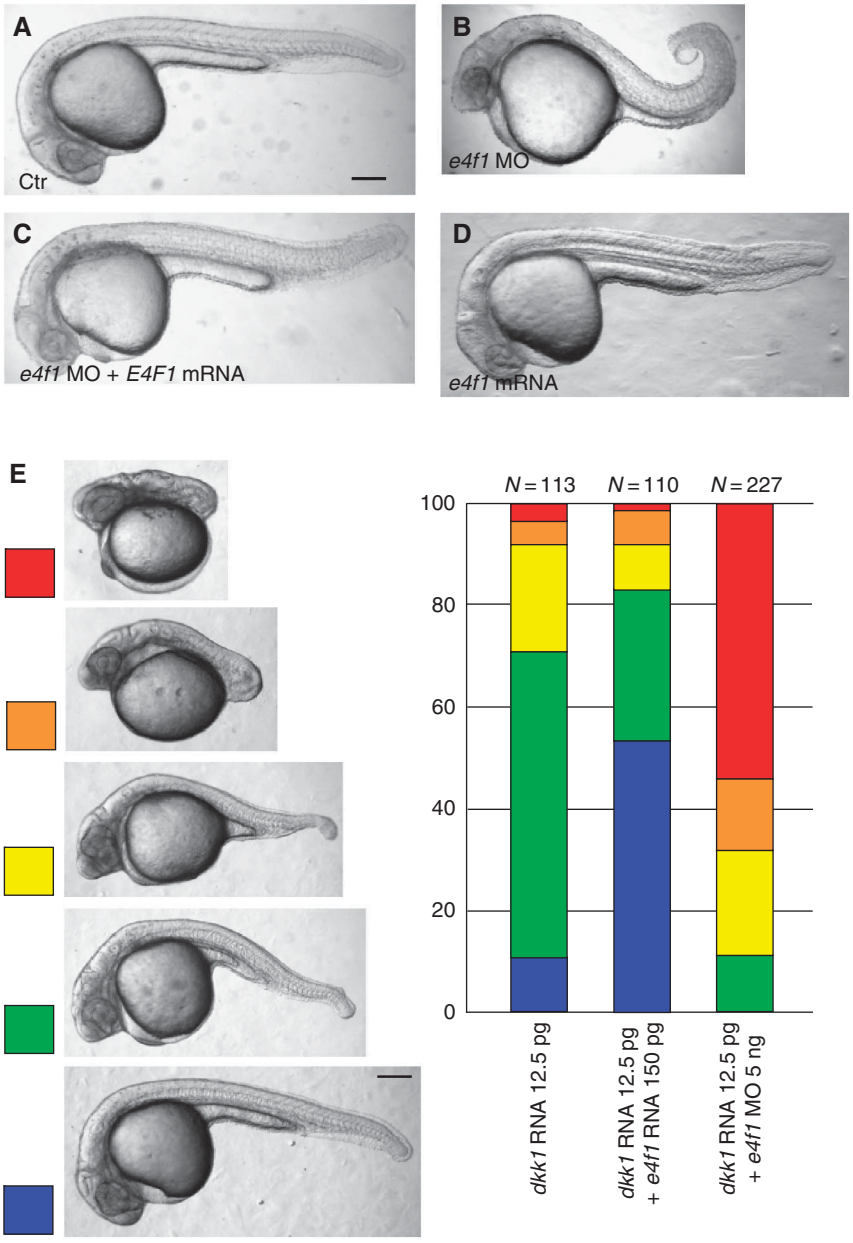

Figure 1 Depletion of E4f1 causes caudal defects. (**A**–**C**) The tail defects of *e4f1* morphants (*e4f1* MO; 5 ng) were largely rescued by injection of 100 pg of human *E4F1* mRNA. Injected reagents are shown at bottom left. (**B**) Tail shortened and kinked in 74% of embryos ($n = 159$). (**C**) Most embryos showed rescued tail (69%, $n = 160$). (**D**) Overexpression of E4f1 by injection of a similar level of zebrafish *e4f1* RNA as used in the rescue experiment (**C**; 100 pg) did not cause a visible phenotype. (**E**) A moderate amount of *dkk1* mRNA (12.5 pg) was injected alone or together with *e4f1* mRNA (150 pg) or *e4f1* MO (5 ng). Tail defects were classified into five levels at 26 h.p.f. Enforced expression of E4f1 mitigated tail defects due to Wnt inhibition, whereas *e4f1* MO injection aggravated the effect. Numbers of embryos (*N* at top) are based on three independent experiments. Lateral views of embryos at 26 h.p.f. Scale bar, 200 μm.

function. *hoxa9a* expression was reduced in *wnt3a/wnt8.1/wnt8.2* morphants (86%, $n = 28$) or Dkk1 overexpressing embryos (100%, $n = 33$), and reduced further by co-injection of *e4f1* MO (*wnt3a/wnt8.1/wnt8.2/e4f1* MO, 45%, $n = 31$; *dkk1* RNA + *e4f1* MO, 64%, $n = 25$) (Figure 2I–R). These observations confirm that the changes in *cdx4* expression noted above have functional consequences for downstream genes. Since Cdx4 is critical for primitive erythropoiesis in zebrafish through the activation of the posterior *hox* gene cluster (Davidson *et al*, 2003), we analysed expression of *gata1*, a marker for primitive erythropoietic progenitors (Davidson and Zon, 2004). *gata1* expression in the posterior lateral plate mesoderm was severely reduced in *cdx4* morphants, similar to the *kgg* mutant (Davidson *et al*, 2003) (Supplementary Figure S5A and B). To visualize synergistic effects, we injected moderate amounts of *dkk1* mRNA to dampen rather than eliminate Wnt signalling; in these embryos, *gata1* expression was slightly compromised, similar to the reduction seen in *e4f1* morphants (Supplementary Figure S5C and D). Notably, *gata1* expression was strongly reduced in embryos co-injected with low-level *dkk1* mRNA plus *e4f1* MO, but could be rescued by co-injection of *cdx4* mRNA (Supplementary Figure S5E and F). By contrast, the *pax2.1* stripe in the lateral cells of the pronephric primordium was

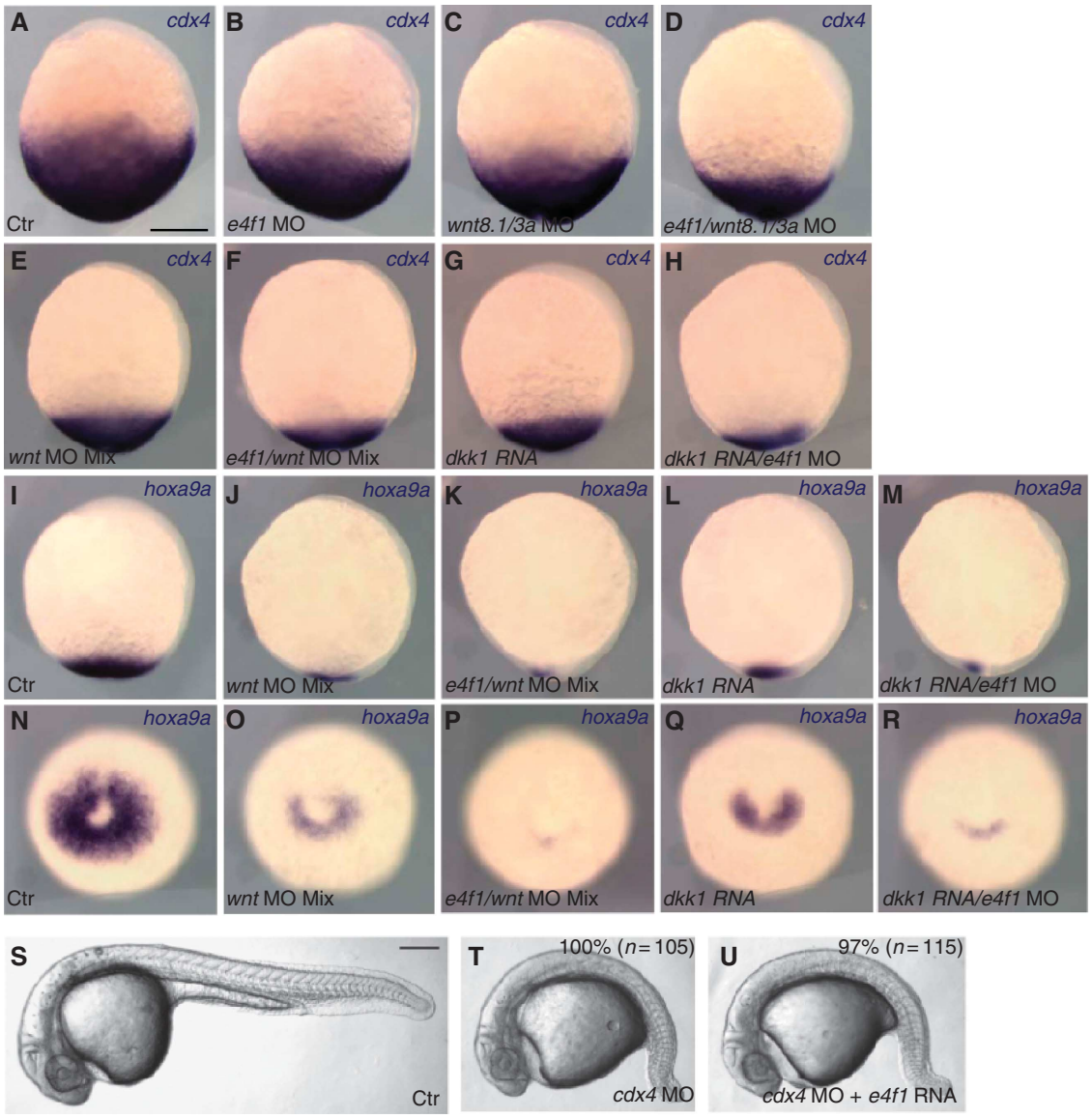

Figure 2 E4f1 modulates Wnt-dependent regulation of *cdx4* and *hoxa9a* expression. (**A**–**H**) *cdx4* expression in the caudal region at the end of gastrulation is reduced by inhibiting Wnt signalling and further attenuated by *e4f1* depletion. (**I**–**R**) Expression of *hoxa9a* at the end of gastrulation is likewise regulated by Wnt signalling and E4f1. Injected reagents are listed at the bottom, probe for *in situ* hybridization at top right. *wnt* MO mix indicates a mixture of *wnt3a* MO (2 ng), *wnt8.1* MO (2 ng) and *wnt 8.2* MO (2 ng); 5 ng of *e4f1* MO and 25 pg of *dkk1* mRNA were injected. (**A**–**M**) Lateral view, anterior is up and dorsal to the right. (**N**–**R**) Posterior view, dorsal is up. (**S**–**U**) E4f1 acts downstream of Cdx4. E4f1 mRNA (150 pg) does not rescue tail defects caused by *cdx4* MO (2 ng). Scale bars, 200 μm.

less sensitive to levels of *e4f1* and Wnt signalling (Supplementary Figure S5G–L). These data indicate that Wnt and E4f1 coordinately modulate caudal tissue formation and primitive erythropoiesis through the regulation of *cdx4* expression. As Wnt signalling regulates caudal development through Cdx4 and we show that E4f1 cooperates with Wnt in regulating *cdx4* expression, we predict that E4f1 should act upstream of Cdx4. This is the case as E4f1 overexpression failed to rescue tail development in *cdx4* morphants (Figure 2S–U).

E4f1 regulates expression of a Cdx4 reporter and is antagonized by Lnx2b

To study *cdx4* regulation further, we constructed a *cdx4* reporter by inserting the *egfp* or *luciferase (luc)* coding region at the translation start site in exon 1 of *cdx4*, retaining ~1.6 kb of upstream region and of intron 1 (Figure 3A). The *cdx4* genomic structure, composed of three exons separated by a relatively long first intron and short second intron, is well conserved from fish to humans (Figure 3A). The *cdx4-egfp* reporter was expressed in a similar domain as the resident *cdx4* gene in stable transgenic fish (Figure 3B and C), validating the use of this reporter to study *cdx4* regulation. A positive role of E4f1 in *cdx4* induction suggested above (Figure 2) was supported by transient transfection assays in 293T cells in which E4f1 increased the activity of *cdx4-luc* (Figure 3D). As we had identified E4f1 as a binding partner of Lnx2b, we tested whether Lnx2b affects E4f1-dependent regulation of the *cdx4-luc* reporter. We found that Lnx2b counteracted E4f1-dependent *cdx4-luc* activation. Because E3 ubiquitin ligase-dead mutant forms of Lnx2b (Lnx2b Mu, Lnx2b ΔN) (Ro and Dawid, 2009) also

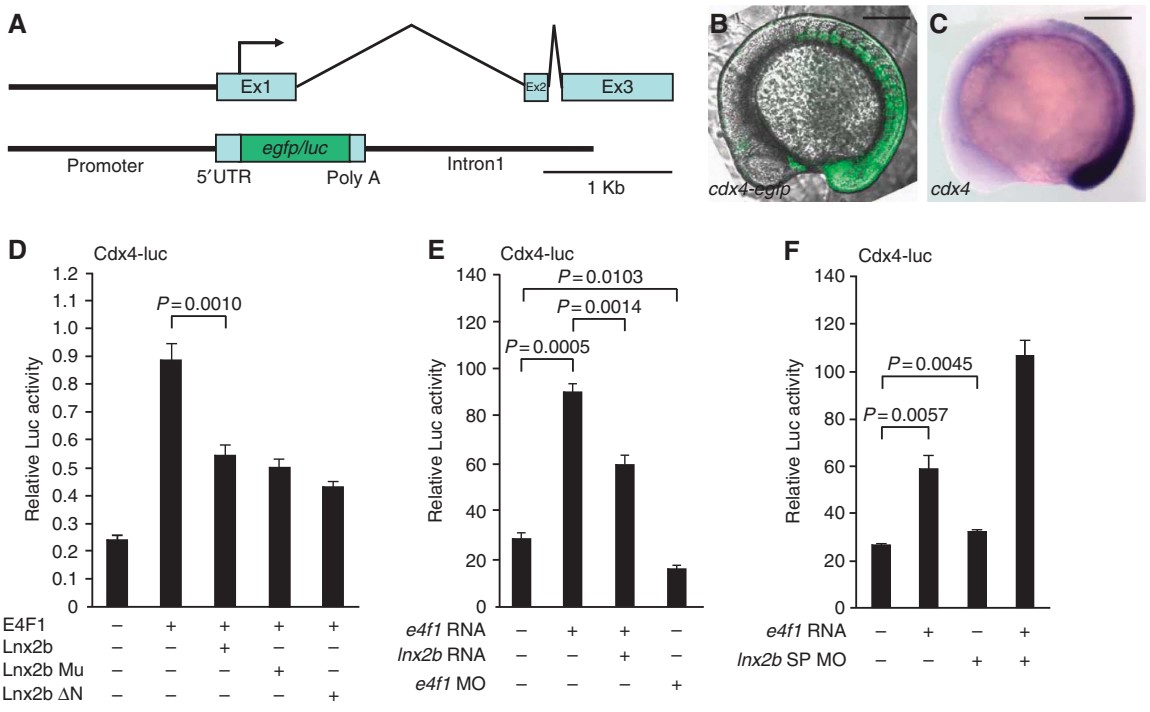

Figure 3 E4f1 and Lnx2b modulate *cdx4* expression. (**A**) The zebrafish *cdx4* genomic region and reporter constructs. Three exons (Ex1 ~ Ex3) and two introns are depicted; the translation initiation site is indicated with an arrow. The *egfp* or firefly luciferase (*luc*) coding region was inserted between the 5′ flanking (−1494 to +181) and the coding region of the first exon plus first intron (1637 bp) of *cdx4*. (**B**) Stable transgenic embryo carrying the *cdx4-egfp* transgene, at the 12-somite stage. (**C**) *cdx4* expression at the equivalent stage as (**B**). (**D**–**F**) Luciferase activity under the control of the *cdx4* regulatory region. (**D**) Luciferase activity of a transiently transfected *cdx4-luc* reporter in 293T cells. 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). (**E**, **F**) *cdx4-luc* reporter plasmid (30 pg) was co-injected with the indicated mRNAs or MOs into zebrafish embryos. *e4f1* mRNA (100 pg), *lnx2b* mRNA (100 pg), *e4f1* MO (5 ng) and *lnx2b* SP-MO (10 ng) were injected at the 1–4 cell stage. Luciferase activity was measured in triplicate or more at the 3–5-somite stage. Statistical significance of differences is shown in the figure. (**B**, **C**) Lateral views, anterior is left and dorsal is up. Scale bars, 200 μm.

antagonized E4f1-dependent reporter activation, we conclude that the E3 ubiquitin ligase activity is dispensable and that Lnx2b modulates E4f1 activity as a scaffold protein (Figure 3D). In zebrafish embryos as in cultured cells, overexpression of E4f1 enhanced *cdx4-luc* activity, depletion of E4f1 attenuated the activity, and Lnx2b counteracted reporter activation (Figure 3E). To assess the physiological relevance of the low-level expression of *lnx2b* in the caudal tissue, we depleted zygotically expressed Lnx2b using splice-blocking MO (SP-MO) in order to avoid defects in dorsoventral axis specification that arise when maternal and zygotic Lnx2b were simultaneously depleted using translation-blocking MOs (Ro and Dawid, 2009; Supplementary Figure S6A). Depletion of zygotically expressed Lnx2b alone was not enough to stimulate *cdx4-luc* activity, presumably due to the low level of Lnx2b expression, but *cdx4-luc* reporter activity was synergistically increased by the co-injection of *lnx2b* SP-MO and *e4f1* mRNA (Figure 3F). These data indicate that E4f1 activates *cdx4* transcription, modulated by the opposing action of Lnx2b.

We also asked whether E4f1 regulates *cdx4* induction through direct binding to the promoter. The *cdx4* 5′ upstream region contains a putative E4f1-binding element TGACGT CAG, although its sequence does not conform exactly to the consensus TGACGTAAC (core sequence is underlined) (Fernandes and Rooney, 1997; Hofmayer *et al*, 2009). Nevertheless, we failed to detect binding of E4f1 to the *cdx4* promoter in electrophoretic mobility shift assays (data not shown). In addition, a *cdx4-luc* reporter in which the putative E4f1-binding element was mutated to TAACGTCCG (mutated nucleotides underlined) could still be activated by E4f1 overexpression as effectively as the wild type (WT) (data not shown). Therefore, we conclude that E4f1-dependent *cdx4* upregulation is likely to be mediated by an indirect mechanism.

As *lnx2b* is expressed in the caudal domain (Ro and Dawid, 2009) where it modulates the regulation of *cdx4* by E4f1, we asked whether *lnx2b* is regulated by Wnt signalling. Wnt depletion abolished *lnx2b* expression in tailbud embryos except in Kupffer's vesicle, and Wnt activation by LiCl enhanced both *lnx2b* and *cdx4* expression (Supplementary Figure S7A–K). In addition, putative LEF/TCF-binding elements are clustered in the upstream region of the *lnx2b* gene, and at least one of these sites binds Tcf3 *in vivo* (Supplementary Figure S6B). To our surprise we found that *lnx2b* expression was strongly enhanced after MO-dependent depletion of *cdx1a/cdx4*, and was further elevated by treatment with LiCl (Supplementary Figure S7L and M). These data indicate that Wnt signalling activates both *cdx4* and *lnx2b* expression, but Cdx proteins suppress *lnx2b*. These relationships suggest a feedback loop that may contribute to robustness of *cdx4* regulation, as further discussed below.

Tcf3 regulates cdx4 by repression and Wnt/E4f1-mediated derepression

Tcf3 is a major mediator of the Wnt pathway in the early embryo, acting as a repressor in this context, as removal of Tcf3 yields the same phenotype as zygotic Wnt activation (Fredieu *et al*, 1997; Kim *et al*, 2000; Dorsky *et al*, 2003; Merrill *et al*, 2004; Gribble *et al*, 2009). We surmised that E4f1 acts by modulating Tcf3 function in *cdx4* regulation. Therefore, we analysed the *cdx4* 5′ flanking region as well as the first intron, and identified several LEF/TCF consensus elements (Figure 4A and B). We then carried out chromatin immunoprecipitation (ChIP) analysis for several regions containing predicted Tcf3-binding elements and found that they are occupied by endogenously expressed Tcf3a in the *cdx4-egfp* transgenic embryo (Figure 4C; Supplementary Figure S8). The specific binding of Tcf3a to the *cdx4* promoter and first intron regions was confirmed by comparing ChIP analysis obtained using WT with Tcf3a mutant zebrafish (*tcf3a/hdl*; maternal/zygotic *MZ hdl* animals were used) (Kim *et al*, 2000) (Figure 4D). The ChIP signal generated by WT fish was robust, but was reduced substantially when mutant fish were used.

Since Tcf3 primarily acts as transcriptional repressor, we reasoned that *cdx4* induction should be suppressed by overexpression of Tcf3. Using the *cdx-luc* reporter we found that Tcf3a suppressed basal activity, whereas E4f1 stimulated it, as

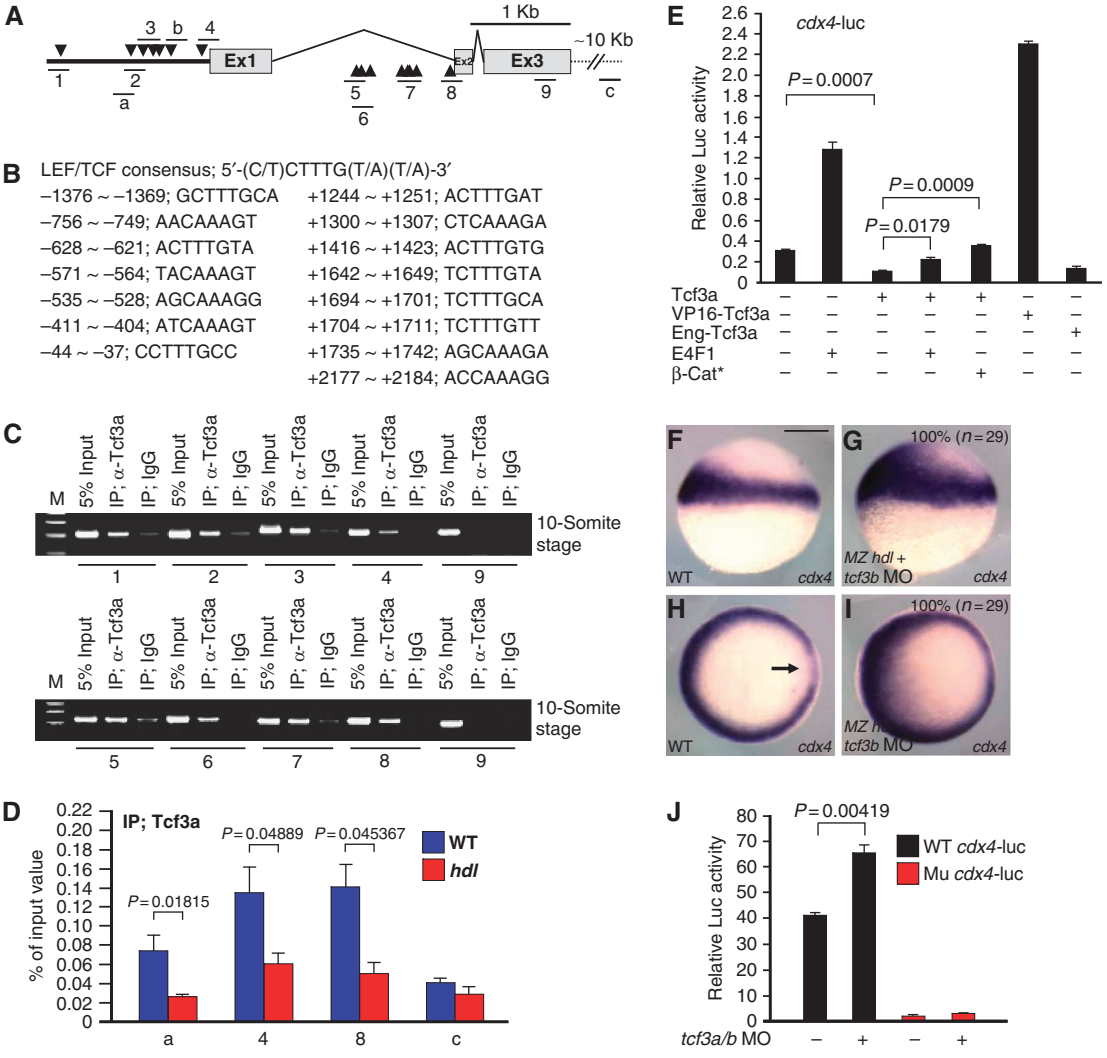

Figure 4 Tcf3 acts as a repressor in regulating *cdx4*. (**A**) Schematic diagram of *cdx4* genomic region. Arrowheads indicate putative LEF/TCF-binding elements. Numbers 1 to 9 and a to c indicate amplicons tested in ChIP experiments. (**B**) LEF/TCF consensus sequence and sequences of the putative binding elements in the *cdx4* gene. (**C**) ChIP of embryo chromatin with anti-Tcf3a antibody. Amplicons 1–8 encompassing LEF/TCF-binding elements were precipitated, but exonic amplicon 9 and amplicon c located ~10 Kb downstream of amplicon 8 were not. IgG was used as negative control. (**D**) Comparison of the q-PCR yield, based on triplicate analysis, of indicated amplicons after ChIP with anti-Tcf3a antibody between WT and *MZ hdl/tcf3a*$^{-/-}$ mutant embryos. Statistical significance is indicated in the figure. (**E**) *cdx4-luc* reporter activity in 293T cells. Combined DNA mixtures were used for transfection as indicated. Tcf3a and En-Tcf3a suppressed *cdx4-luc* transgene activity, while E4f1 and stabilized β-catenin relieved repression. VP16–Tcf3a acts as a strong transcriptional activator. (**F–I**) At the shield stage, *cdx4* is expressed in the margin in a ventral-to-dorsal gradient in uninjected embryos, with a gap in the shield region (arrow in **H**). Depletion of Tcf3 by injection of *tcf3b MO* (2 ng) into *MZ hdl/tcf3a*$^{-/-}$ mutant embryos, expanded *cdx4* expression into the organizer and ventral-animal domain. (**J**) Zebrafish embryos were co-injected with *tcf3a/b* MO and WT *cdx4-luc* or LEF/TCF-binding site null mutant construct (Mu *cdx4-luc*; see Supplementary Figure S9). The embryos were harvested at 6.5 h.p.f. for measurement of reporter activity. Luciferase activity was assayed at least in triplicate. (**F**) Scale bar, 200 μm.

shown above (Figures 3D–F and 4E). Notably, E4f1 and the classical Wnt pathway activator β-catenin (β-cat* is a stabilized form) similarly increased reporter activity to about basal level, suggesting that derepression rather than activation takes place under these conditions. This view is supported by the fact that an artificial activator, a VP16–Tcf3a fusion protein, increased reporter activity strongly whereas the artificial repressor, Eng-Tcf3a, repressed at a similar level as WT Tcf3a (Figure 4E).

If Tcf3 does suppress *cdx4* induction *in vivo*, *cdx4* levels should be increased after depletion of Tcf3. In contrast, if Tcf3 conversion to an activator by Wnt stimuli is a prerequisite for *cdx4* induction, Tcf3 depletion would preclude *cdx4* induction. In gastrula embryos, *cdx4* is expressed around the margin in a ventral-to-dorsal gradient that results in a gap in the organizer region in which relatively low levels of Wnt signalling are maintained (Erter *et al*, 2001; Lekven *et al*, 2001) (Figure 4F and H). To suppress both Tcf3 isoforms, we introduced *tcf3b* MO (Dorsky *et al*, 2003) into *MZ hdl* mutant embryos. These embryos showed increased *cdx4* expression without any gap in the organizer (Figure 4G and I), indicating that Tcf3 functions as a transcriptional repressor to restrict *cdx4* expression to tissues with high levels of Wnt activity. Derepression of *cdx4* by the depletion of Tcf3a/b was further validated by measuring *cdx4-luc* activity. WT *cdx-luc* reporter was stimulated by injecting a *tcf3a/b* MO mixture into the embryos, while a mutant reporter construct lacking LEF/TCF-binding sites (Supplementary Figure S9A) lost basal activity and failed to respond to the MO injection (Figure 4J). In contrast, overexpression of Tcf3a significantly reduced WT *cdx4-luc* reporter activity (Supplementary Figure S9B). Furthermore, whereas both LEF/TCF consensus sites in the upstream region and the first intron contribute to the basal expression of *cdx4*, only the consensus sites in the 5′ flanking region mediate the suppressive action of Tcf3a (Supplementary Figure S9B). Taken together, these data agree with the conclusions of Tcf3 loss-of-function studies (Kim *et al*, 2000; Dorsky *et al*, 2003) and support our view that *cdx4* activation is the result of derepression, in agreement with a recent report (Hikasa *et al*, 2010).

If E4f1 mediates relief of *cdx4* from repression by Tcf3, embryos without functional Tcf3 should be refractory to *e4f1* MO. We tested this prediction of an epistatic relationship between Tcf3 and E4f1 in the sensitive assay of tail formation. As shown above (Figure 1B), E4f1 depletion leads to reduced tail length (Figure 5B and F). Removal of Tcf3 affects head development but has almost no effect on the tail, as previously reported (Kim *et al*, 2000; Dorsky *et al*, 2003) (Figure 5C, D and F). Importantly, embryos lacking Tcf3 (*MZ hdl* embryos injected with *tcf3b* MO) did not show tail defects after depletion of E4f1, whereas WT embryos exhibited shortened tails. In other words, the reduced tail length of *e4f1* morphants was largely rescued by concurrent depletion

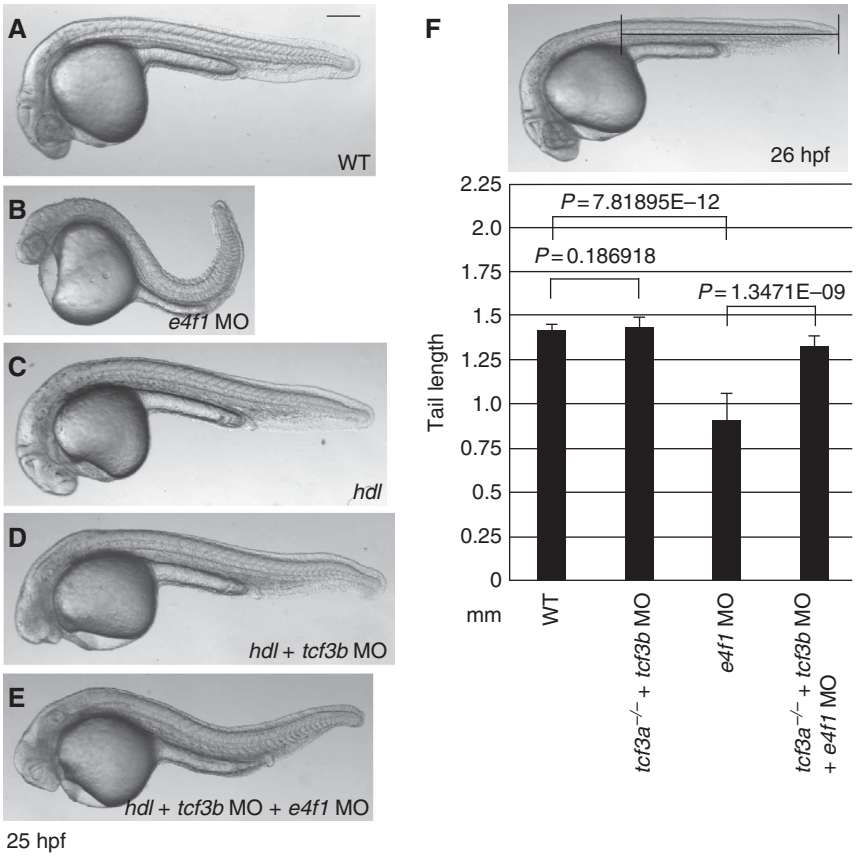

Figure 5 Tcf3 depleted embryos resist *e4f1* MO injection. (**A**–**E**) Morphology of embryos injected as indicated in lower right. (**A**) Control embryo; (**B**) *e4f1* MO (5 ng)-injected embryo; (**C**) *MZ hdl/tcf3a*$^{-/-}$ mutant embryo; (**D**) *tcf3b* MO (2 ng)-injected *MZ hdl/tcf3a*$^{-/-}$ embryo; (**E**) *e4f1* MO (5 ng) was co-injected with *tcf3* MO (2 ng) into *MZ hdl/tcf3a*$^{-/-}$ embryos. Note that the tail defect in (**B**) is largely rescued in (**E**), but the head defect in (**D**) is not. (**F**) The tail length of 20 randomly selected embryos from each group was measured using ImageJ, as indicated in the photograph. Statistical significance is shown in the histogram. Scale bar, 200 μm.

of Tcf3a and b (Figure 5E and F). At the same time the head phenotype resulting from Tcf3a/b depletion was unaffected by the *e4f1* MO (Figure 5D and E). These results strongly support a physiological function of E4f1 in caudal body formation: if *e4f1* MO effects on tail length were due to off-target effects, it should not be possible to rescue tail length by co-depletion of Tcf3. Consistent with the lack of an effect on tail length, alteration of E4f1 levels did not substantially affect the elevated *cdx4* expression in embryos depleted of Tcf3 (Supplementary Figure S10).

E4F1 dissociates HDAC1–Gro/TLE corepressor proteins from Tcf3

The observations described above, in agreement with earlier data, indicate that Wnt signalling governs *cdx4* expression in zebrafish as it does in the mouse (Shimizu *et al*, 2005; Pilon *et al*, 2006). As E4f1 has been shown to be a transcriptional repressor (Fajas *et al*, 2001) and we found that E4f1 does not bind to the *cdx4* regulatory region (see above), we tested whether the stimulation of *cdx4* expression by E4f1 and its attenuation by Lnx2b might be achieved by protein–protein interactions. There is ample evidence that LEF/TCF transcriptional repressor activity depends upon association with Gro/TLE and HDAC1 (Cavallo *et al*, 1998; Billin *et al*, 2000; Brantjes *et al*, 2001; Yamaguchi *et al*, 2005; Ye *et al*, 2009). Initially, we tested whether E4f1 and Lnx2b can associate with Tcf3 and corepressor proteins. E4f1 co-precipitated with HDAC1 as previously reported (Colombo *et al*, 2003) and also with Tcf3, but not with TLE3 (Figure 6A). Lnx2b could be co-precipitated with all proteins tested, albeit with different yields (Figure 6A). To investigate whether Lnx2b, HDAC1 and Gro/TLE form a complex, we performed sequential co-IP (Figure 6B). Cells transfected with HDAC1, Flag-Lnx2b and HA–TLE3 were immunoprecipitated with anti-Flag antibody, eluted using Flag peptide, and subjected to a second IP with anti-HDAC1 antibody; the immune complex contained HA–TLE3 (Figure 6B). Thus, Lnx2b, HDAC1 and Gro/TLE can form a triple complex, independently of the E3 ubiquitin ligase activity of Lnx2b. Next, we tested whether the yield of repressor complex is modified by E4f1, and found that E4f1 destabilized Tcf3/corepressor association, even in the presence of Lnx2b (Figure 6C; key results are outlined). Thus, we conclude that E4f1 acts to dissociate corepressors from Tcf3, which is expected to abolish its repressor function. To check the role of E4f1 at endogenous levels of expression, we tested these protein–protein interactions after depletion of E4F1 with specific siRNAs in 293T cells; because the levels of TCF7L1 (the human Tcf3 orthologue) are low, we co-transfected Myc-tagged Tcf3a (Figure 6D and E). We observed that treatment of the cells with *E4F1* siRNAs resulted in increased association of the endogenously expressed Gro/TLE and HDAC1 with Myc–Tcf3a (Figure 6E), indicating that E4F1 negatively influences Tcf3 repressor complex formation or stability at physiologically relevant levels.

A possible mechanism for E4f1 action in *cdx4* derepression might be interference with Tcf3 binding to *cis*-regulatory DNA elements, similar to the effects of Kaiso, I-mfa and HIPK2 (Snider *et al*, 2001; Ruzov *et al*, 2009; Hikasa *et al*, 2010). To map the critical domains of Tcf3 required for association with E4f1, we constructed several deletion mutants of Tcf3. Even though both the Gro/TLE interaction domain and the HMG DNA-binding domain of Tcf3 are required for its binding to E4f1 (Supplementary Figure S11), ChIP assays revealed that E4f1 does not interfere with the binding of Tcf3 to the regions containing LEF/TCF-binding elements in the *cdx4* gene (Figure 7A). In this respect, E4f1 behaves similarly to β-catenin, extensively studied as modulator of LEF/TCF activity (Hurlstone and Clevers, 2002).

A different mechanism that could explain E4f1-mediated derepression of the *cdx4* gene is based on the protein–protein interaction studies shown in Figure 6. These experiments suggest that derepression could be achieved by dissociation of corepressor proteins from Tcf3, even though Tcf3 remains attached to the promoter. This hypothesis is supported by ChIP experiments that showed removal of the Groucho (Gro) family corepressor TLE3 from the Tcf3-binding sites in the *cdx4* promoter/first intron after the addition of E4f1; this was true for six genomic regions containing Tcf3-binding sites (Figure 7B). Consistently, ChIP yields of LEF/TCF-binding sites in the *cdx4* gene after co-precipitation with endogenously expressed HDAC1 and TLE increased substantially after depletion of E4F1 (Figure 7C). These data support the view that E4f1-dependent *cdx4* derepression is due to the dissociation of corepressor proteins from Tcf3 (Figure 7D). We suggest that E4f1 can act on the *cdx4* promoter in a similar way and with comparable efficiency (Figure 4E) as β-catenin to relieve Tcf3-mediated repression of the *cdx4* gene.

Discussion

E4f1 modulates regulation of cdx4 by Tcf3

Wnt signalling has a major role controlling cell proliferation, differentiation and body patterning and is an important factor in the causation of various tumours (MacDonald *et al*, 2009). Wnt-dependent *Cdx-Hox* gene expression is critical for caudal tissue specification including haematopoiesis (Isaacs *et al*, 1998; Davidson *et al*, 2003; Shimizu *et al*, 2005; Pilon *et al*, 2006, 2007; Lengerke *et al*, 2008; Young and Deschamps, 2009). Here, we describe novel components and interactions that modulate Wnt signalling during caudal body formation in the zebrafish embryo. We identified a mechanism for caudal region development based on the relief of repression of the *cdx4* gene by Tcf3. In agreement with the expected role of a transcriptional repressor (Brannon *et al*, 1997; Kim *et al*, 2000; Dorsky *et al*, 2003; Gribble *et al*, 2009), Tcf3 suppresses *cdx4* induction through direct binding to multiple *cis*-regulatory elements. Removal of Tcf3 by mutation of the *tcf3a* gene and using an MO against *tcf3b*, led to an increase in *cdx4* expression (Figure 4F–I), consistent with a repressor function of Tcf3 in *cdx4* expression. In this respect, Tcf3 acts similarly in caudal body formation as previously reported for head formation where depletion of Tcf3 gives equivalent phenotypes to activation of the Wnt pathway (Fredieu *et al*, 1997; Kim *et al*, 2000; Dorsky *et al*, 2003). Corepressor proteins such as Gro/TLE and HDAC1 confer repressor activity upon Tcf3 (Brannon *et al*, 1997; Kim *et al*, 2000; Dorsky *et al*, 2003; Gribble *et al*, 2009), and we show that Tcf3 associates with corepressors in a complex that can be stabilized by Lnx2b (Figure 6B and C). This Tcf3–HDAC1–Gro/TLE repressor complex, even if stabilized by Lnx2b, can be dissociated by E4f1 to mediate derepression of *cdx4* (Figure 6C). Results of ChIP experiments indicate that the corepressor complex is bound to LEF/TCF-binding sites in the *cdx4* promoter (Figure 7B), and that Tcf3 remains associated with the DNA

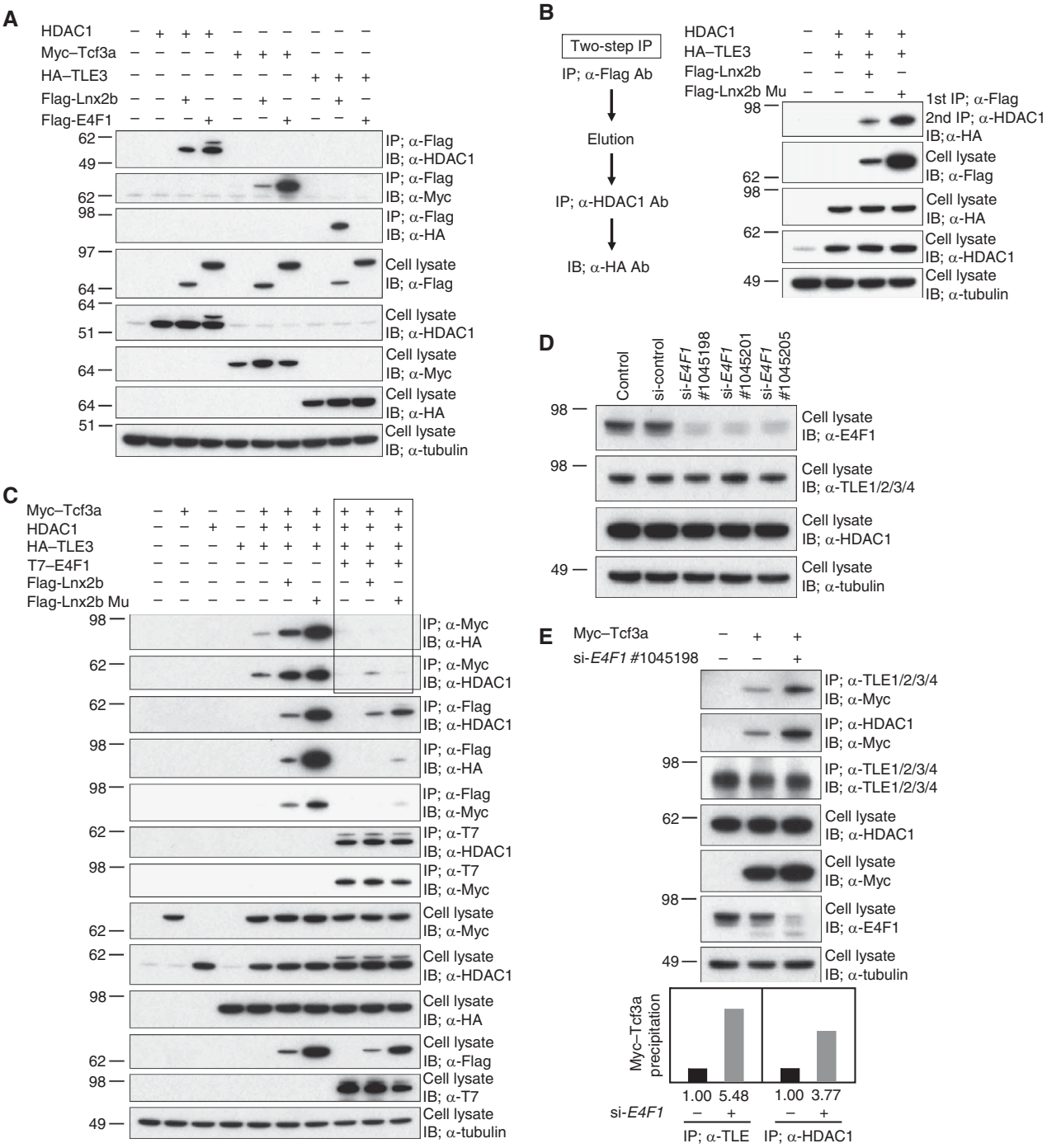

Figure 6 E4f1 and Lnx2b modulate binding of Tcf3 to corepressor molecules. (**A**–**E**) 293T cells were transfected with the indicated plasmids. (**A**) Co-IP at 48 h post-transfection and blotting was carried out with the antibodies indicated. Lnx2b co-precipitated with Tcf3, TLE3 and HDAC1, while E4f1 co-precipitated with HDAC1 and Tcf3 but not with TLE3. Note that Lnx2b mutant accumulated to a higher level than WT due to self-ubiquitylation and destabilization of the latter (Ro and Dawid, 2009). (**B**) Two-Step IP. Lysates of cells transfected with plasmids encoding HDAC1 and HA-tagged TLE3, plus Flag-Lnx2b or Flag-Lnx2b Mu, were first precipitated with anti-Flag antibody. Proteins eluted by 3xFlag peptide were subjected to a second IP with anti-HDAC1 antibody; co-precipitated TLE3 was detected by anti-HA blotting. (**C**) Lnx2b, WT or mutant, enhanced co-precipitation yields of Tcf3a with HDAC1 or TLE3. By contrast, E4f1 overexpression dissociated the corepressors and WT or Mu Lnx2b from Tcf3a; the lanes showing this critical observation are outlined in the figure. Association between E4f1 and Tcf3a and between E4f1 and HDAC1 was not altered by the presence of Lnx2b, and the association between Lnx2b/Lnx2b Mu and HDAC1 was not greatly altered by E4f1. (**D**) All three *E4F1* siRNAs tested knocked downed E4F1 levels. (**E**) Depletion of E4F1 facilitated binding of endogenous HDAC1 and TLE family proteins to Tcf3a. The histogram at the bottom shows quantification of this experiment using ImageJ.

when E4f1 replaces the corepressors in the complex (Figure 7A–C). Thus, E4f1 exerts its effect on *cdx4* expression by protein–protein interaction rather than by binding to the promoter. It should be emphasized that overexpression of stabilized β-catenin did not activate the *cdx4-luc* transgene when Tcf3 was co-expressed, but instead restored the basal level of expression (Figure 4E). These data are consistent with the view that β-catenin and E4f1 act in a similar manner

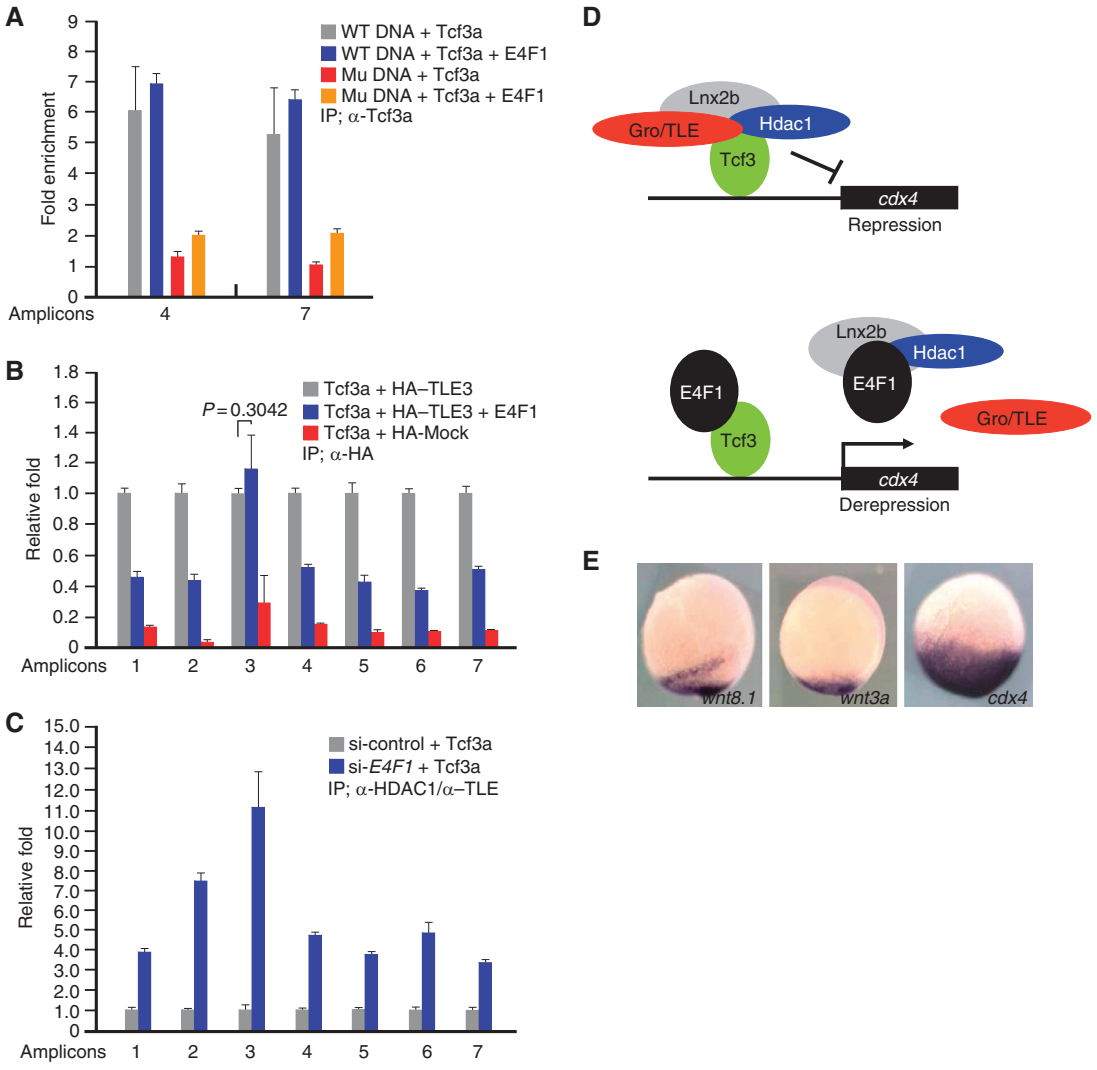

Figure 7 Tcf3 remains bound to the *cdx4* promoter in the presence of E4f1 while the corepressors are removed. (**A**–**C**) ChIP analyses in 293T cells transfected with the indicated DNAs. Amplicons are identified in Figure 4A. (**A**) PCR amplified DNA fragments including WT or mutant *cdx4* regulatory elements (Supplementary Figure S9Aa and d, respectively) were co-transfected with Tcf3a with or without E4F1, as indicated. Samples were immunoprecipitated with anti-Tcf3a antibody, or IgG as control. Amplicons 4 and 7 were used for q-PCR. Fold enrichment is calculated setting the IgG control as one. Note that E4F1 did not interfere with Tcf3a binding to the DNA, while mutant DNA was recovered at low yield. (**B**) ChIP q-PCR assay showing that E4F1 dissociates TLE3 from the Tcf3-binding sites in the *cdx4* promoter; the aberrant behaviour of amplicon 3 is not understood. (**C**) ChIP q-PCR analysis of *cdx4* regulatory region after IP with anti-HDAC1 and anti-TLE antibodies after depleting endogenously expressed *E4F1*. *cdx4-luc* plasmid and Tcf3a were co-transfected with or without *E4F1* siRNA. Note that ChIP yields increased significantly in *E4F1*-siRNA-treated cells. q-PCR was carried out at least in triplicate, and standard error of the mean is shown in the panels. A proposed mechanism is shown in (**D**). In this model, a complex composed of Tcf3–Gro/TLE–HDAC1–Lnx2b represses the *cdx4* gene; E4f1 dissociates the complex, preserving Tcf3 binding to the DNA. (**E**) Expression of *wnt3a* and *wnt8.1* is much more restricted in the caudal domain at the bud stage than *cdx4*. A proposed role for E4f1 derepression in achieving this pattern is discussed in the text.

on the *cdx4* promoter, leading to a relief of Tcf3 repression. Recently, Hikasa *et al* (2010) reported studies on Tcf3-mediated regulation of the *vent2* and also the *cdx4* gene in Xenopus, showing that Tcf3 phosphorylation leads to its removal from a cognate binding site in the *vent2* gene. As E4f1-mediated derepression of *cdx4* in zebrafish occurs while Tcf3 remains bound to the promoter, these two mechanisms are distinct, and their possible interaction remains to be analysed.

Four Lef/Tcf factors are expressed during zebrafish embryonic development (Dorsky *et al*, 1999; Kim *et al*, 2000; Young *et al*, 2002; Veien *et al*, 2005). Since some functional redundancy has been reported among these factors (Galceran *et al*, 1999; Nagayoshi *et al*, 2008; Nguyen *et al*, 2009), it seemed possible that Lef/Tcf factors in addition to Tcf3 are involved in translating the Wnt signal to caudal tissue specification by regulating *cdx4* expression. Among these factors, Tcf4 seems to be ruled out as it is not expressed in the caudal domain (Young *et al*, 2002). In addition, even though *lef1* and *tcf7* transcripts can be detected in the caudal region, combined inhibition of Lef1/Tcf7 function caused pectoral fin defects but not tail agenesis (Nagayoshi *et al*, 2008). Thus, our evidence combined with previous reports strongly supports the view that the transcriptional repressor activity of Tcf3 is the key activity to mediate Wnt signalling in caudal development through the regulation of *cdx* and *hox* gene expression (Kim *et al*, 2000; Dorsky *et al*, 2003; Merrill *et al*, 2004). Several different signals control the Cdx-Hox

pathway through crosstalk and collaboration (Isaacs *et al*, 1998; Shimizu *et al*, 2005; Lengerke *et al*, 2008; Iimura *et al*, 2009; Mallo *et al*, 2009; Young and Deschamps, 2009). In our proposed mechanism, E4f1 and Lnx2b have a role in fine-tuning Tcf3-mediated repression of *cdx4* by affecting the composition of the repressor complex, with Lnx2b enhancing and E4f1 disrupting the complex (Figure 7D).

Biological role of E4f1 antagonism of Tcf3 repression

The biological significance of the proposed mechanism can be illustrated with Figure 7E. Wnt 3a and Wnt 8.1 are expressed in a tight domain at the caudal tip of the embryo, as reported previously (Lekven *et al*, 2001; Thorpe *et al*, 2005). While Wnt factors can diffuse (Yan and Lin, 2009), the effective ligand concentration will become low at a distance from the source, and fine differences in levels might lead to imprecise boundaries of *cdx4* activation at the animal edge of the gradient. The presence of a constitutive derepressing factor, E4f1, may contribute to the robustness of the induction of *cdx4* in this region of the embryo. This interpretation is consistent with our observation that inhibition of E4f1 expression combined with attenuation of Wnt signalling leads to a gradual diminution of the domain of *cdx4* expression (Figure 2).

The E3 ubiquitin ligase Lnx2b appears to have a modest refining role in the interactions that lead to Tcf3-mediated repression and derepression. Lnx2b stabilizes the repressor complex, but does not strongly resist dissociation of the complex by E4f1 (Figure 6C). This is reflected in a measurable but weak inhibition of E4f1-dependent *cdx4-luc* reporter activity by Lnx2b (Figure 3D–F). These experiments did, however, uncover the interesting fact that Lnx2b acts as a scaffold protein in this situation, independently of its E3 ubiquitin ligase activity. The ability to serve as a binding platform is not a unique property of Lnx2b. For instance, ectodermin/TIF1γ, a ligase involved in Smad4 ubiquitylation, acts as a scaffold protein for recruiting positive elongation factors to erythroid genes to promote transcription (Bai *et al*, 2010). Recently, Honda *et al* (2010) reported that PDZRN3 (LNX3) negatively regulates Wnt/β-catenin signalling by reducing LRP6 phosphorylation through an unknown mechanism. It will be intriguing to test whether LNX3 and Lnx2b regulation of the Wnt pathway exhibit overlapping modalities.

The role of Tcf3 is widespread in different cells and tissues. It will be of particular interest to test whether the expression of pathogenic Wnt target genes is modulated by E4F1 and LNX proteins in cells or tissues in which Tcf3 expression is tightly regulated, for instance in skin epithelia, hair follicles and embryonic stem cells (Jiang *et al*, 2008; Nguyen *et al*, 2009; Abu-Remaileh *et al*, 2010). Most recently, Lacroix *et al* (2010) verified a novel role for E4f1 in skin homoeostasis and epidermal stem cell maintenance by generating skin-specific E4f1 conditional knockout mice. The possible involvement of E4F1 in pathogenesis by dysregulation of Wnt signalling during skin agenesis through the transient hyperplasia of epidermal stem cell could be addressed in further studies.

Materials and methods

Fish embryos

Embryos were obtained from natural spawning of WT (AB*) or *MZ hdl* mutant lines (Kim *et al*, 2000).

Cell culture and transfection

293T cells were grown in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Cells were transfected using FuGeneHD (Roche). After 24–48 h, cells were harvested and assayed. We used zebrafish *lnx2b*, *tcf3* and *β-catenin*, and human *E4F1*, *HDAC1* and *TLE3* DNAs for transfection.

Transgenic zebrafish

The PCR amplified 5′ flanking region (−1494 to +217) of *cdx4* was subcloned into a pSCAC-40 vector (Kim *et al*, 2008) between the Tol2 Rt arm and the *egfp* reporter gene. The PCR amplified first intron (1637 bp) of *cdx4* was subcloned into the above construct. To generate transgenic animals, 10 pg of plasmid was co-injected with 50 pg of *in vitro* transcribed mRNA encoding transposase into one-cell stage zebrafish embryos (Kawakami *et al*, 2004).

Luciferase assay

The *cdx4* genomic DNA used for transgenesis above was subcloned into pGL3-Basic Vector (Promega). The 5′ flanking region was inserted between the *Nhe*I and *Bgl*II sites, and the first intron between the *Bam*HI and *Sal*I sites. 293T cells that were 50% confluent were transfected with 5 ng of pRL-SV40, 100 ng *cdx4-luc* and 100 ng of other indicated plasmids. Luciferase assays were performed using Dual-Luciferase Reporter Assay System (Promega). Injected embryos (6.5 or 11 h.p.f.) or transfected 293T cells (24 h after transfection) were lysed in Passive Lysis Buffer (Promega). Each luciferase activity was measured at least three times, and significance was assessed by the Student's *t*-test.

IP and western blotting

IP was performed by mixing lysates of transfected cells with 20 μl of Protein G Sepharose 4 Fast Flow (GE Healthcare) in M-PER Mammalian Protein Extraction Reagent (Pierce) or in CompLysis Protein Extraction Reagent (SignaGen) with Complete protease inhibitor cocktail (Roche; hereafter CPIC). The total volume of lysate mixture was adjusted to 1 ml with NP-40 IP buffer (150 mM NaCl, 1 mM DTT, 10 mM Tris–Cl pH 7.5, 0.2% NP-40, CPIC). Precipitates were washed four times with 1 ml ice-cold NP-40 IP buffer, and were analysed by western blotting. After separation in Nu-PAGE 4–12% Bis-Tris Gel (Invitrogen) and transfer onto PVDF membrane, the membrane was immersed in blocking solution (4% skim milk or 2% BSA in TBS with 0.05% Tween-20) at room temperature for 30 min, incubated with primary antibodies diluted in blocking solution for 1 h, followed by incubation with HRP-conjugated secondary antibody (Jackson, 1:5000) for 1 h, all at room temperature. The detection was performed using the ECL detection system (Pierce). The antibodies were supplied from Roche (rat HA), Sigma (mouse Flag M2), Clontech (mouse Myc), Millipore (rabbit Myc; rabbit HDAC1 used for Two-Step IP in Figure 6B and ChIP in Figure 7C), Vector (rabbit IgG), Santa Cruz Biotechnology (goat HDAC1 N-19, used for IP in Figure 6E), Cell Signaling Technology (rabbit TLE1/2/3/4 for ChIP in Figure 7C), Proteintech Group, Inc. (rabbit HDAC1, used for immunoblotting), Abcam (rabbit HA used for ChIP in Figure 7B), Bethyl Laboratories Inc. (rabbit E4f1) and Calbiochem (mouse T7, mouse α-tubulin). Anti-rabbit Tcf3a antibody that was used for ChIP was a gift of Dr Dorsky (Gribble *et al*, 2009).

Two-Step IP

Plasmid mixtures of HDAC1, HA–TLE3, Flag-Lnx2b, Flag-Lnx2b Mu (Ro and Dawid, 2009) and Flag-Mock vector were transfected into 293T cells. After 48 h, the cell lysates were incubated with Protein G Sepharose beads and anti-Flag M2 antibody for 6 h at 4°C. The beads were washed with ice-cold NP-40 IP buffer three times, and proteins were eluted with 300 μl modified NP-40 IP buffer (250 mM NaCl, 1 mM DTT, 10 mM Tris–Cl pH 7.5, 0.2% NP-40, CPIC and 3xFLAG peptide (300 μg/ml, Sigma)) for 2 h at 4°C. The eluted products were adjusted to 1 ml with salt diluted NP-40 IP buffer (final NaCl concentration 150 mM). After adding anti-HDAC1 antibody and Protein G Sepharose beads, the samples were incubated for 6 h at 4°C with gentle agitation, and then washed with ice-cold NP-40 IP buffer three times. Immunoblotting was performed as above.

siRNA

Cultured 293T cells in 60 mm dish were used for siRNA transfection. In all, 1 μg of plasmid encoding Tcf3a was co-transfected with 5 μg of siRNA. si-*E4F1* #1045198, 5′-GGCAAGCGCUACAAGACUA-3′; si-*E4F1* #1045201, 5′-CACAGUGUUGGUGGAGUUC-3′; si-*E4F1* #1045205, 5′-CUAUAGAGACUUCACCCGU-3′. Negative control siRNA (5′-CCUACGCCACCAAUUUCGU-3′, Bioneer Inc.) was used for normalization of transfection amount. X-tremeGENE siRNA Transfection Reagent (Roche) was used according to the manufacturer's instructions.

Chromatin immunoprecipitation

ChIP was performed as described previously with small modification (Nelson *et al*, 2006), using polyclonal Tcf3a antibody, epitope tag antibodies or control IgG. Two hundred dechorionated embryos (10-somite stage) were fixed with 2.2% paraformaldehyde (PFA) dissolved in PBS for 15 min at RT. After quenching with 125 mM Glycine, the cross-linked chromatin was sheered to ~300 bp length using the Bioruptor Next Gen (Diagenode). Following IP, PCR (26–40 cycles) using AccuPower PCR PreMix (Bioneer Inc.) or q-PCR using LightCycler 480 (Roche) was performed. The following primer sets were used for amplifying the indicated amplicons (~200 bp):

lnx2b amplicon #1 forward primer, 5′-CATTGTTTGGTGCATGC CCCAACTCTG-3′;
lnx2b amplicon #1 reverse primer, 5′-ATAACAATGGGAACCT GCTGTAGTGTAC-3′;
lnx2b amplicon #2 forward primer, 5′-GAGTTGCACACGTTCTCA TGTTGGTGAGC-3′;
lnx2b amplicon #2 reverse primer, 5′-CAGGCAATTAAGGCAGGT GTGGATCACCG-3′;
tcf3a amplicon #1 forward primer, 5′-GAGAGCCCGCATTTCCATA AACCGG-3′;
tcf3a amplicon #1 reverse primer, 5′-GTCCCATCTGCATAGTACCC ATGCC-3′;
tcf3a amplicon #2 forward primer, 5′-CTTGTAAAATGTTATCACG TGTGCC-3′;
tcf3a amplicon #2 reverse primer, 5′-CTATAATTACAAAGTAA TCTTTATAGC-3′;
tcf3a amplicon #3 forward primer, 5′-GTGATTATTAATTTTACAT TGAC-3′;
tcf3a amplicon #3 reverse primer, 5′-GCACAATATAACAGGAAAT ATTATC-3′;
tcf3a amplicon #4 forward primer, 5′-GAATGCAAATCTAAA GGTTTTGAAG-3′;
tcf3a amplicon #4 reverse primer, 5′-CACTGGGATCCAAGGCCCG ACTC-3′;
tcf3a amplicon #5 forward primer, 5′-CTCAGGTTTTGAGGGTTA TCTGTC-3′;
tcf3a amplicon #5 reverse primer, 5′-TTTACAGTTAGGACGTGTG ACTCTG-3′;
tcf3a amplicon #6 forward primer, 5′-TCAGGCGGCGACTTCTTTC AACACC-3′;
tcf3a amplicon #6 reverse primer, 5′-AAAAGCACAACTGAACACA CAAAG-3′;
tcf3a amplicon #7 forward primer, 5′-GGATGCATATCAAACAA GTGCAGC-3′;
tcf3a amplicon #7 reverse primer, 5′-GCCTACTATAAACCAAGAT-CACATC-3′;
tcf3a amplicon #8 forward primer, 5′-TGGACTTATGTATGCC TTTCGCAAC-3′;
tcf3a amplicon #8 reverse primer, 5′-TTACCTGTCTCTCTGAAAG CCCGAG-3′;
tcf3a amplicon #9 forward primer, 5′-CAGGTACGGAGTTTCATGG GACGTG-3′;
tcf3a amplicon #9 reverse primer, 5′-TTGCTGAAATCAAACGTTA TACCCG-3′.
tcf3a amplicon #a forward primer, 5′-CAATTGAATTTTTGGTAAT AGCC-3′;
tcf3a amplicon #a reverse primer, 5′-GTTGTGTTAATCATAGTCA ATGTA-3′;
tcf3a amplicon #b forward primer, 5′-GATAATATTTCCTGTTAT ATTGTG-3′;
tcf3a amplicon #b reverse primer, 5′-AAGCATTCGATTTGAAA GGTGTAG-3′;
tcf3a amplicon #c forward primer, 5′-TGCTGTAGTGAAGACTGCA TTAGTCC-3′;
tcf3a amplicon #c reverse primer, 5′-GTATATATACAGATCTGTATA AAAAGAC-3′.

For ChIP experiments in cultured cells, 293T cells were transfected with the indicated combination of DNAs and siRNAs: *cdx4-luc* plasmid, *cdx4* promoter and first intron DNA fragments amplified by PCR, pCS2+/Tcf3a, pCS2+/MT-Tcf3a (gift of Dr Chitnis), pCS2+/HA–TLE3, pCS2+/E4F1, control siRNA and *E4F1* siRNA. Empty vector was used as a negative control (Mock). After 24 h transfection, the cells were fixed in 1.42% PFA in PBS for 12 min and quenched with 125 mM glycine. Sheared cross-linked chromatin was immunoprecipitated with 2–5 μg of the indicated antibodies. After extensive washing with ChIP-IP buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.5% NP-40, 1% Triton X-100), the precipitated DNA fragments were eluted and then analysed using the LightCycler 480 (Roche).

Whole mount in situ hybridization

The embryos were collected at the appropriated stage and fixed in 4% PFA in PBS. Antisense riboprobes were generated with linearized template DNA using appropriate RNA polymerase following the manufacture's instructions (Roche). Proteinase K treatment (10 μg/ml) was performed for 1 to 10 min depending on the stages of embryos. The hybridized probes were detected using pre-absorbed anti-digoxigenin-AP Fab fragments (Roche) diluted (1:2000) in blocking solution (PBS, 0.1% Tween-20, 5% sheep serum, 0.2% Blocking reagent (Roche)). After staining, the embryos were fixed and examined under the microscope.

Microinjection

cDNAs were subcloned into the pCS2+. mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion Inc.). RNAs, plasmids or MOs were injected into the yolk of 1–4 cell stage of embryos. MOs were supplied from Gene Tools, LLC. In all, 1 ng of p53 MO was co-injected with other MOs to inhibit possible off-target effects when we analysed the embryos after 24 h.p.f. (Robu *et al*, 2007). The following represents the sequence of MOs used in our assay; *e4f1* ATG MO, 5′-CTTCAGTCATGTCGTTCCAAGCCTC-3′; *lnx2b* splicing MO (Ro and Dawid, 2009), 5′-GTAAGTGATGCAA TACCATCTTCGC-3′; *wnt3a* MO, 5′-GTTAGGCTTAAACTGACACGCA CAC-3′; *wnt8.1* MO (Lekven *et al*, 2001), 5′-ACGCAAAAATCTGG CAAGGGTTCAT-3′; *wnt8.2* MO (Lekven *et al*, 2001); 5′-GCCCAACG GAAGAAGTAAGCCATTA-3′; *cdx1a* MO (Shimizu *et al*, 2005), 5′-GT CCAGCAGGTAGCTCACGGACATT-3′; *cdx4* MO (Davidson *et al*, 2003), 5′-CGTACATGATTTGGAAGAAACCCCT-3′; *tcf3b* MO (Dorsky *et al*, 2003), 5′-CGCCTCCGTTAAGCTGCGGCATGTT-3′; *p53* MO, 5′-GCGCCATTGCTTTGCAAGAATTG-3′.

LiCl treatment

Mid-gastrula (60% epiboly) embryos were raised in fish water containing 0.2 M LiCl for 40 min at 28.5°C. After rinsing several times with fresh fish water, embryos were incubated to the desired stage.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: HR and IBD conceived the project, designed the experiments, analysed the data and wrote the manuscript. HR carried out all experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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