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. 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 Lef1/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 Lef1/TCF family members exhibit some functional redundancy (Galceran et al., 1999; Nagayoshi et al., 2008), individual Lef1/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; Roel 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 Lef1/TCF family members seem to be due to their intrinsic properties as transcriptional activators or repressors. Lef1/TCF factors act as repressors when bound to corepressor proteins such as Groucho/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 (Got 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). These results are 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...
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; Limura 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 = 57), 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 2F) 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 2G; 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.
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 SSC 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 SSE and F). By contrast, the pax2.1 stripe in the lateral cells of the pronephric primordium was

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.
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...

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**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), wnt1.1 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.
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.
**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 a transcriptional repressor, we reasoned that cdx4 induction should be suppressed by overexpression of Tcf3. Using the cdx4-luc reporter we found that Tcf3a suppressed basal activity, whereas E4f1 stimulated it, as

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**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 site were precipitated, but exonic amplicon 9 and amplicon c located ~10 Kb downstream of amplicon 6 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 tcfa3a 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 tcfa3a/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 cdx4-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 S’ 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...
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 HIKP2 (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 (Friedieu 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 6xHis peptide were subjected to a second IP with anti-HA antibody; co-precipitated TLE3 was detected by anti-HA blotting. (C) Lnx2b, WT or mutant, enhanced co-precipitation yields of Tcf3 with HDAC1 or TLE3. By contrast, E4f1 overexpression dissociated the corepressors and 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 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
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

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.

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pathway through crosstalk and collaboration (Isaacs et al., 1998; Shimizu et al., 2005; Lengerke et al., 2008; limura 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/TFI1γ, a ligase involved in Smad4 ubiquitination, 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-Remalieh et al., 2010). Most recently, Lacroix et al. (2010) verified a novel role for E4f1 in skin homeostasis 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 hd1 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, H1M7 and TLE3 DNAs for transfection.

Transgenic zebrafish

The PCR amplified 5′ flanking region (~1949 to +217) of cdx4 was subcloned into a pSEAC-40 vector (Kim et al., 2008) between the T012 R t 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 NheI and BglII sites, and the first intron between the BamHI and SalI 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 transplanted 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 1-MER Mammalian Protein Extraction Reagent (Pierce) or in CompleXone 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 (rabbit E4f1, Sigma) and Clontech (mouse Myc). Millipore (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 3×FLAG 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 Tcf5a was used to co-transfected with 5 μg of siRNA. si-E4F1 #1045198, 5'-GGCAAGCGCCUCAAGACUA-3'; si-E4F1 #1045201, 5'-CACAGGUGUUGUGAGAAGUC-3'; si-E4F1 #1045205, 5'-CUUAGAGAUCUCACCG GU-3'. Negative control siRNA (5'-CCUUAGCCCAAAUUUGGUC-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 Tcf5a 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 4°C. After quenching with 125 mM glycine, the cross-linked chromatin was sheared 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):

- laneb amplicon #1 forward primer, 5'-CATGTGGGTGCTAGTC
- laneb amplicon #1 reverse primer, 5'-AAATGCGGATCAGCTCTCA
- TGTTGTTGAGC-3
- TGGTTGTTAATCATAGTCATCA
- laneb amplicon #1 reverse primer, 5'-ATAACAATGGGAACACT
- GCTGATGTAC-3
- laneb amplicon #2 forward primer, 5'-GAGTGGCACACGTCTCTCA
- TGTTGTTGAGC-3
- laneb amplicon #2 reverse primer, 5'-CAGGCAATAGGGAACAGGT
- GTGAGTGCC-3
- laneb amplicon #3 forward primer, 5'-GAGGGCAGACCTTCAAA
- ACCGG-3
- laneb amplicon #3 reverse primer, 5'-GTTCCATCCTGCAATGTCCCA
- ATGCC-3
- laneb amplicon #4 forward primer, 5'-CTTGTAAATGTCTATCGG
- TCCGGG-3
- laneb amplicon #4 reverse primer, 5'-CGTATATATACAGATCTGTATA
- laneb amplicon #5 forward primer, 5'-GAGGACCGGATTCACACT
- AACC-3
- laneb amplicon #5 reverse primer, 5'-GAATGCAATACCTCAAAG
- GCCAAG-3
- laneb amplicon #6 forward primer, 5'-GCCAAACGAGGCTCCTC
- TACCT-3
- laneb amplicon #6 reverse primer, 5'-GAGGCAATACTCAAAAC
- CAAG-3
- laneb amplicon #7 forward primer, 5'-GGATGCGCATATAACCAAA
- GTGCCAC-3
- laneb amplicon #7 reverse primer, 5'-CCCTACTATAAAAACAGATT
- CACATC-3
- laneb amplicon #8 forward primer, 5'-TGAGCTTATGATGCG
- TTGGCAAC-3
- laneb amplicon #8 reverse primer, 5'-TTACCTCTTCTCTGAAAG
- CCCGAC-3
- laneb amplicon #9 forward primer, 5'-CAGTGGAGTTAGTATGGTC
- GACGTC-3
- laneb amplicon #9 reverse primer, 5'-TTGTGAAATACAAACGT
- TACCGG-3
- laneb amplicon #a forward primer, 5'-CAATATTATATGGTAGAC
- AGCC-3
- laneb amplicon #a reverse primer, 5'-GGTGGTGTTAAATCATAGTCAT
- ATGTA-3
- laneb amplicon #b forward primer, 5'-GATAATATTTCTGTGTATT
- ATGTA-3
- laneb amplicon #b reverse primer, 5'-AAGCATTCGATTTGAA
- GGTTGAG-3

For ChIP experiments in cultured cells, 293T cells were transfected with the indicated combination of plasmids and siRNAs: cdx4-lac 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 1:2.4% 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 appropriate stage and fixed in 4% PFA in PBS. Antisense riboprobe were generated with linearized template DNA using appropriate RNA polymerase following the manufacturer’s instructions (Roche). Proteinkinase 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'-CTCTAGCTCCTGCCCAAGCTCC-3'; tmxb splicing MO (Ro and Dawid, 2009), 5'-TTCATTATGTCCATGAC

Conflict of interest
The authors declare that they have no conflict of interest.
References


Modulation of Tcf3 repressor complex composition

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