CHD4/Mi-2β activity is required for the positioning of the mesoderm/neuroectoderm boundary in Xenopus

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Experiments in Xenopus have illustrated the importance of extracellular morphogens for embryonic gene regulation in vertebrates. Much less is known about how induction leads to the correct positioning of boundaries; for example, between germ layers. Here we report that the neuroectoderm/mesoderm boundary is controlled by the chromatin remodeling ATPase CHD4/Mi-2β. Gain and loss of CHD4 function experiments shifted this boundary along the animal–vegetal axis at gastrulation, leading to excess mesoderm formation at the expense of neuroectoderm, or vice versa. This phenotype results from specific alterations in gene transcription, notably of the neural-promoting gene Sip1 and the mesodermal regulatory gene Xbra. We show that CHD4 suppresses Sip1 transcription by direct binding to the 5’ end of the Sip1 gene body. Furthermore, we demonstrate that CHD4 and Sip1 expression levels determine the “ON” threshold for Nodal-dependent but not for eFGF-dependent induction of Xbra transcription. The CHD4/Sip1 epistasis thus constitutes a regulatory module, which balances mesoderm and neuroectoderm formation.

Keywords: CHD4/Mi-2β; Sip1; Xbra; chromatin remodeling; Activin induction threshold; germ layer

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had been blocked by cycloheximide. Under these conditions, abnormal spreading of mesodermal gene expression domains was observed, which was interpreted to reflect the absence of de novo synthesized repressors [Kurth et al. 2005]. Together, these studies illustrate the fundamental importance of repression for the regional induction of gene expression.

Potential repressors of Xbra transcription include the proteins Goosecoid, Otx2, and Mixl1, which bind to homeodomain-binding sites near the Xbra promoter [Latinkic et al. 1997]. This region contains also a single bipartite binding site for the Smad-interacting protein Sip1 [Verschueren et al. 1999; Eissa et al. 2000; Lerchner et al. 2000; Papin et al. 2002]. Sip1 belongs to the 6EF1 family of proteins, which are involved in cell fate decisions in Drosophila and vertebrates [Postigo and Dean 1997]. Mutations in the human Sip1 gene cause a form of Hirschsprung’s disease, associated with mental retardation, microcephaly, and facial abnormalities [Wakamatsu et al. 2001]. Sip1 was originally identified through its ability to interact with receptor-regulated Smad proteins. Gel-shift analyses showed that Sip1 binds to 5’-CACCT-3’ DNA sequences of various promoters, including that of Xbra [Verschueren et al. 1999; Papin et al. 2002]. When overexpressed in Xenopus animal caps, Sip1 displays neural-inducing activity [Eissa et al. 2000; Nitta et al. 2004]. Consistent with this, work in chick embryos has placed Sip1 into a pathway operating during gastrulation, in which FGF indirectly induces the transcription factor Churchill, which induces Sip1, which, in turn, represses mesoderm formation and promotes neurogenesis [Sheng et al. 2003]. In the frog, Xbra and Sip1 are initially coexpressed at the onset of gastrulation, but quickly refine into adjacent expression domains representing mesoderm and neuroectoderm [Papin et al. 2002]. The mechanism by which this separation occurs is not known, but most likely is pivotal for formation and positioning of the boundary between these two germ layers.

Ample evidence supports the notion that the transcriptional activity of genes is mechanistically coupled to their local chromatin structure. The chromatin environment is regulated by two classes of enzymes, which either catalyze covalent modifications of histone tails or hydrolyze ATP to mobilize nucleosomes. Vertebrates contain a set of ~30 nucleosome-stimulated ATPases related to the prototypic SNF2 protein from yeast [Linder et al. 2004]. These ATPases represent the enzymatic core subunits of conserved multiprotein nucleosome remodeling machines [Becker and Hörz 2002]. Recently, several groups have simultaneously described the protein composition of one of these machines, most often referred to as the NuRD complex (for review, see Bowen et al. 2004). This complex shows both remarkable conservation among metazoa as well as heterogeneity at the individual protein subunit level. In addition to its core ATPase CHD4/Mi-2β, which is required for nucleosome sliding/remodeling, the NuRD complex contains histone deacetylases (HDACs) and members of the MBD family of methyl-CpG-binding proteins. By this unique protein composition, NuRD may couple DNA methylation to chromatin remodeling and histone deacetylation [Wade et al. 1999]. Detailed analyses in diverse model organisms and cell culture systems have emphasized the importance of NuRD as a versatile transcriptional repressor complex, which regulates cell type-specific transcriptional programs at the chromatin level [Kehle et al. 1998; von Zelewsky et al. 2000; Unhavaithaya et al. 2002, Fujita et al. 2003, 2004].

The dynamic mRNA expression patterns of the Xenopus orthologs of the mammalian SNF2-like ATPases during early embryogenesis [Linder et al. 2004] have suggested that quantitative and/or qualitative differences in nucleosome-remodeling activities between cells could be of regulatory importance for embryonic gene regulation. In this study, we have tested this assumption by performing gain- and loss-of-function experiments for the Xenopus CHD4-ATPase. Our results place CHD4/Mi-2β activity at the top of a regulatory cascade, which determines the position of the neuroectoderm/mesoderm border along the animal–vegetal axis of the gastrula embryo by controlling specifically the Activin/Nodal input for brachyury transcription.

Results

Interference with CHD4 protein activity affects specific mesodermal and neuroectodermal gene expression domains

Nonuniform expression of the xCHD4 mRNA during frog embryogenesis (see Supplementary Fig. S1 and Linder et al. 2004) suggested that it could be selectively involved in regional gene regulation from early gastrula stages onward. To test this assumption, we used several independent approaches of functional interference with CHD4 protein activity. We created a dominant-negative variant of the CHD4-ATPase (dnCHD4) by point-mutating the lysine residue at position 748 to arginine. The analogous mutation in the SNF2 ATPase maintains the integrity of the yeast SWI/SNF chromatin remodeling complex, but abolishes transcriptional activation of target genes [Richmond and Peterson 1996]. While injection of wild-type CHD4 mRNA (wtCHD4) would be expected to increase CHD4/NuRD activity, overexpression of the dnCHD4 variant should inhibit endogenous CHD4 protein functions by competition. For loss-of-function analysis, we designed a Morpholino oligonucleotide [Heasman et al. 2000], complementary to the translational start site of the CHD4 mRNA (CHD4-Mo) (see Supplementary Fig. S2). This antisense oligonucleotide inhibited endogenous CHD4 mRNA translation both in vitro and in vivo, while an unrelated control Morpholino oligonucleotide had no effect [Supplementary Fig. S2; data not shown].

After injecting the various reagents singly into one blastomere at the two-cell stage, we cultured control and injected embryo populations until gastrula stage, when they were fixed for RNA in situ hybridization. We analyzed a total of 14 genes, which mark specific areas within the developing embryo. These included chordin...
(chd), nodal-related-3 (xnr3), vent2, Sip1, Xbra, Wnt11, MyoD (see Fig. 1), and noggin, goosecoid (gsc), vent-1, mixer, dickkopf, otx-2, and mix1 (data not shown). Among those, the mesodermal markers Xbra, MyoD, and to a lesser extent also Wnt11, were down-regulated in the majority of the CHD4-Mo-injected embryos in several independent experiments (Fig. 1, right column; for phenotypic penetrance, see Supplementary Fig. S3). No perturbation of mesodermal gene expression patterns was observed upon injection of an unrelated control Morpholino (data not shown). In contrast, embryos injected with wtCHD4 mRNA (Fig. 1, middle column; Supplementary Fig. S3) showed a strong, unilateral expansion of Xbra transcripts toward the animal pole. A somewhat milder expansion was also observed for Wnt11 and MyoD.

Injections of dnCHD4 mRNA caused a comparable reduction of the Xbra expression domain on the injected side like the CHD4-Mo, and both phenotypes were rescued by coinjection of wtCHD4 mRNA (see Fig. 2A; Supplementary Fig. S3). We also targeted the Morpholinos, wtCHD4 and dnCHD4 mRNAs specifically to the dorsal [dmz] or ventral marginal zone [vmz] by injection of single blastomeres at the four-cell stage, and stained the embryos for Xbra mRNA (data not shown). Vmz-injected embryos showed normal expression of Xbra, while in dmz-injected embryos we observed a slight reduction in the dorsal-most quadrant of the Xbra expression domain, but only at the highest dose of CHD4-Mo oligo tested (60 ng). Together, these observations indicate that CHD4 controls mainly the dorsolateral aspect of the Xbra expression domain.

The RNA in situ analysis also indicated misexpression of the neural plate marker Sip1. Most embryos injected with wtCHD4 mRNA showed a significant, local reduction of Sip1 transcripts in the prospective neural plate, whereas the Sip1 expression domain was expanded both animaly and laterally in CHD4-Mo-injected embryos (Fig. 1; Supplementary Fig. S3). Coinjection of wtCHD4 mRNA and CHD4-Mo resulted in mostly normal Sip1 expression, indicating that these phenotypes are specific and depend on CHD4 protein abundance (Fig. 2A; Supplementary Fig. S3). The altered Xbra and Sip1 expression domains caused by wtCHD4 overexpression could not be rescued upon the coinjection of a control Morpholino [Fig. 2A; data not shown]. To obtain independent quantitative evidence that CHD4 regulates Sip1 transcription, we overexpressed CHD4 protein in animal cap explants. As little as 0.25 ng of wtCHD4 mRNA was sufficient to suppress Sip1 mRNA levels to ~50% of uninjected control explants, while even 1.0 ng of CHD4 mRNA was not sufficient to silence Sip1 transcription completely (see Fig. 2B).

Most of the tested genes, however, were practically unaffected by these conditions, even though some domains (e.g., chd, vent2) (see Fig. 1) appear distorted near the dorsal midline. Since Xbra and its target gene Wnt11 control cell behavior in the dorsal mesoderm during involution (Kwan and Kirschner 2003), we interpret this distortion to reflect a perturbed midline formation due to Xbra/Wnt11 misexpression, rather than a CHD4-dependent phenotype. Taken together, this analysis indicates a striking selectivity of target genes, which respond to perturbations of CHD4 activity. In summary, Sip1 responded reciprocally to alterations of CHD4 activity, compared with the affected mesodermal genes. Most reports link CHD4/Mi-2β to chromatin-mediated transcriptional silencing (Bowen et al. 2004). Since on one hand Sip1 is known to repress the Xbra promoter (Papin et al. 2002), and on the other hand Xbra has been shown to be sufficient for mesoderm differentiation (Cunliffe

**Figure 1.** Perturbation of CHD4 activity leads to specific alterations in gastrula gene expression. Embryos were unilaterally injected with a dose of 1.0 ng of CHD4 mRNA or 40 ng of CHD4-Mo into one blastomere at the two-cell stage. At gastrula stages, they were fixed, sorted into left-side- or right-side-injected populations by fluorescence of coinjected cGFP (not shown), and used for RNA in situ hybridizations for the marker genes indicated to the left. Displayed are representative embryos from three to five independent experimental repeats, in which the left side serves as an internal control for normal marker gene expression, while the right side shows the expression under the experimental condition (either vegetal or dorso-vegetal views). The injected side is to the right.
and Smith 1992), the embryonic phenotypes suggested an epistasis between CHD4, Sip1, and Xbra, which we decided to investigate further.

CHD4 expression levels define the boundary between neuroectoderm and mesoderm

In normal development, the Sip1 and Xbra expression domains overlap initially in the dorsal marginal zone, but then become quickly separated. At the end of gastrulation, Sip1 is expressed strongly in the neuroectoderm and to a lesser extent in the mesoderm, while Xbra mRNA is restricted to prospective mesoderm (Papin et al. 2002). This raised the issue of whether CHD4 takes part in the process that separates the Sip1 and Xbra domains, or whether CHD4 controls a separate aspect of their regulation.

To address this question, we sectioned gastrula embryos and stained them separately for Xbra or Sip1 mRNA (see Fig. 3A). Attempts to stain embryos simultaneously for both transcripts have failed repeatedly, probably due to the different Sip1 and Xbra mRNA abundances. We found that the Xbra domain was enlarged at the expense of the Sip1 domain in embryos overexpressing CHD4 protein, and vice versa in embryos injected with CHD4-Mo. Injecting wtCHD4 mRNA and CHD4-Mo together resulted mostly in embryos with normal proportions of Xbra and Sip1 expression (Supplementary Fig. S3). However, neither Sip1 nor Xbra mRNAs spread into the underlying endoderm (Fig. 3A). This indicates that CHD4 specifically controls the position of the mesoderm/neuroectoderm boundary along the animal–vegetal axis.

To determine which impact the early imbalance between Xbra and Sip1 expression might have on later development, we examined both the histology and the expression of differentiation markers in sections from unilaterally injected tadpoles (see Fig. 3B,C; Supplementary Fig. S3). Due to the relative morphogenetic movements of mesoderm and neuroectoderm along the rostrocaudal axis, the observed phenotypes were most pronounced in the eye and the trunk somites, respectively. WtCHD4-overexpressing embryos were characterized by reduced or absent eyes (Fig. 3B, top left), accompanied by significantly enlarged myotomes (Fig. 3B, top right). Both phenotypes were rescued by coinjection of dnCHD4 mRNA (Supplementary Fig. S3; data not shown). In contrast, CHD4-Mo-injected embryos displayed a hyperproliferative retina; the somites of these embryos, however, contained loosely packed cells and were locally disorganized and smaller than ipsilateral, uninjected somites (cf. Fig. 3B, bottom panels). All of these embryos contained normal-looking notochords, consistent with the observation that gene expression in the gastrula organizer was unaffected by CHD4 (see above). Furthermore, expression of neural [nrp1] and mesodermal [muscle-actin] differentiation markers was confined to the proper domains, indicating that the affected tissues contained correctly specified cells [Fig. 3C]. In toto, these results suggest that the early alterations of gene expression patterns by the specific perturbation of CHD4 cannot be compensated during development, but have lasting consequences for the embryo.

CHD4 binds to the Sip1 gene

The combined results of Figures 2B and 3A suggest that suppression of Sip1 transcription by CHD4 determines the animal border of the domain, in which Xbra can be
induced. To obtain evidence for a direct regulation of sip1 by CHD4, we performed chromatin immunoprecipitation (ChIP) experiments. The structural organization of the mouse sip1 locus has been described recently (Nelles et al. 2003), including the presence of nine untranslated and alternatively spliced exons (U1–U9), and the nucleotide sequence of two putative promoter regions located upstream of U1 and U4/U5, respectively. Among these elements, cDNA and genomic sip1 DNA sequences from Xenopus (see Materials and Methods for details) showed high sequence similarity for exons U5, E1, and E2, while shorter stretches of clearly conserved DNA sequence extended into the promoter region upstream of U5 (see Fig. 4A). Based on this information, we derived several primer pairs for quantitative PCR analysis of the Xenopus sip1 gene.

The amplicons xU5, xE1, and xE2 cover ~90 kb of the transcribed 5’ portion of the xsip1 gene. Using chromatin fragments from mid-gastrula Xenopus embryos, we precipitated endogenous CHD4 protein and normalized its relative occupancy at these sites to a control amplicon located within the active GAPDH gene. A second control amplicon lies within the promoter region of the xTH/bZIP gene, which is not transcribed during frog embryogenesis until metamorphosis (Furlow and Brown 1999). In three out of three experiments, we found that CHD4 binding was more than threefold enriched at the xE1 amplicon; that is, within the 5’ part of the transcribed gene body (Fig. 4B). These results identify the sip1 gene as a direct target of CHD4 in the embryo at the developmental stage when the boundary between mesoderm and neuroectoderm is formed.

CHD4 controls the dose response of the Xbra promoter to Activin

Our results describe a regulatory pathway in which CHD4 directly suppresses Sip1 transcription to a level that prevents activation of Xbra in the prospective neural plate, but is permissive for its induction in the prospective mesoderm. The Xbra promoter has been shown to be inducible by eFGF and low concentrations of Activin, and to be maintained through an indirect feedback loop, in which Xbra protein induces eFGF expression, which in turn again stimulates Xbra transcription (Latinkic et al. 1997; Casey et al. 1998). The same Activin/FGF-sensitive promoter region contains also a binding site through which Sip1 represses Xbra in the dorsal animal hemisphere (Papin et al. 2002). This detailed insight into brachyury regulation provided us with the opportunity to investigate the interplay of CHD4 and Sip1 activities with growth factor signals.

In a first experimental series, we injected increasing amounts of Activin mRNA into the animal hemisphere
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Figure 4. CHD4 binds to the Sip1 gene. (A) The cartoon depicts the organization of frog and mouse Sip1 gene loci around the first translated exon E1 (AUG indicated by arrow). While exons U5 and E1 are highly conserved in sequence (connected by dashed lines), mouse exons U6–U9 apparently are not conserved in *Xenopus*. Black bars indicate the relative positions of the ChIP amplicons xU5, xE1, and xE2 for *Xenopus*. Not drawn to scale; however, absolute distances between ChIP probes are given in brackets. (B) ChIPs were performed on mid-gastrula *Xenopus* embryos [NF11], using a rat monoclonal antibody mix against xCHD4 protein followed by real-time PCR analysis. They revealed preferential binding of endogenous xCHD4 protein to E1 (n = 3 independent experiments). The relative xCHD4 occupancy was normalized to the xGAPDH amplicon; xTH/bZIP is a silent gene, which becomes activated during metamorphosis. Error bars are the mean standard deviation.

of the four-cell-stage embryo, dissected animal caps, and assessed the resulting *Xbra* mRNA levels by semiquantitative RT–PCR at the early gastrula stage. A minimal dose of 16 pg of *Activin* mRNA was necessary to reproducibly induce *brachyury* transcription over basal levels [Fig. 5A,B, lanes 1–4 each]. This threshold response became significantly altered when we simultaneously manipulated CHD4 activity in the explants. Upon coinjection of wtCHD4 mRNA, *Xbra* was activated already by 1 pg of *Activin* mRNA [Fig. 5A, lanes 5–8]. In contrast, when CHD4-Mo was coinjected, even 24 pg of Activin failed to induce the *Xbra* promoter [Fig. 5B, cf. lanes 4 and 8].

These effects were both selective and specific for several reasons. First, Activin-dependent induction of other genes like *gsc* was unaltered by CHD4 overexpression [Fig. 6B], as was induction of *siamois* and *xnr3* transcription by canonical Wnt signaling [Fig. 6D]. Second, the Activin/Smad signaling pathway remained functional in the presence of CHD4-Mo, since *Xbra* could still be induced by higher Activin amounts [100 pg] [Fig. 5B, lane 9]. Third, the inhibition of *Xbra* induction by CHD4-Mo was overcome by coinjection of wtCHD4 mRNA in a dose-dependent manner [Fig. 6A]. Most notably, however, the normal Activin dose response of the *Xbra* promoter was restored by compensatorial manipulations of *Sip1* protein abundance. Specifically, the sensitization of the *Xbra* promoter caused by CHD4 overexpression was reverted by coinjecting *Sip1* mRNA [Fig. 5A, lanes 9–13], while its CHD4-Mo-dependent desensitization was rescued by inhibiting endogenous *Sip1* protein translation [Fig. 5B, lanes 10–14] with a *Sip1*-specific Morpholino oligonucleotide [Nitta et al. 2004]. Additionally, the desensitizing effect of CHD4-Mo injection on *Xbra* expression could be converted upon the coinjection of CHD4 mRNA [Fig. 6A].

The key observations of these experiments were confirmed by real-time RT–PCR analysis in two additional, independent experiments. Specifically, this included the sensitized induction response of the *Xbra* promoter and its rescue by *Sip1* mRNA [Fig. 5C]. We also show here that *dnCHD4* mRNA efficiently antagonizes the sensitized promoter response induced by wtCHD4 protein. Furthermore, the Xbra induction response to a higher Activin dose was again significantly suppressed by both *dnCHD4* mRNA and CHD4-Mo, and the latter effect was at least partly reversed by Sip1-Mo (Fig. 5D).

Finally, we injected increasing amounts of eGF mRNA, either alone or in combination with CHD4 mRNA or CHD4-Mo [Fig. 6C]. In this case, alterations in CHD4 abundance had no significant effect on the eGF/MAPK-dependent stimulation of the *Xbra* promoter. Together, these results indicate that CHD4 selectively controls, in a Sip1-dependent manner, the “ON” threshold of the *Xbra* promoter for Activin/Nodal-like induction; that is, the primary signal that initiates *brachyury* transcription and mesoderm formation [for review, see Kimelman 2006].

Discussion

This study describes a surprising role for the CHD4/Mi-2β ATPase during germ layer formation; that is, it balances the relative proportions of mesodermal and neuroectodermal territories, which arise through morphogen-mediated inductions [De Robertis and Kuroda 2004; Stern 2005; Heasman 2006]. CHD4 achieves this function by influencing the regulatory interplay of the *Xbra* and *Sip1* genes at the early gastrula stage, which refines the expression domains of these key regulators of mesodermal and neuronal cell fates [Papin et al. 2002; Sheng et al. 2003; Nitta et al. 2004]. Remarkably, CHD4-dependent interference with the Sip1/Xbra module leads to stable, disproportionate differentiation of mesodermal and neural tissues.

With regard to the underlying mechanism, we have shown that CHD4 suppresses *Sip1* transcription both in the embryo and in animal cap explants, through direct interaction targeted at the *Sip1* 5′ gene body. Interestingly, the presence of CHD4 in the vicinity of the *Sip1* promoter modulates the expression of this gene rather than imposing a full transcriptional “OFF” state. This regulatory mode is reminiscent of the paradigm described for the *xHairy2A* gene, where the methyl-CpG-binding protein MeCP2 together with the SMRT corepressor complex modulates transcription in a dynamic manner without complete silencing [Stancheva et al. 2003]. Such regulation may be prominently used in un-
Since most of these genes are regulated by Wnt, FGF, strongly arguing against an unspecific perturbation of normally under the various experimental conditions, the majority of tested genes (12 of 16) were expressed fairly (Figs. 2, 3, 5, 6; Supplementary Fig. S3). In addition, the protein both on the morphological and molecular levels bryos were rescued by coexpression of wild-type CHD4. Secondly, the phenotypes of the CHD4-specific antisense Morpholino strategy, or by reducing endogenous CHD4 protein levels through a lar nature of these alterations is currently unknown, but probably depends on the protein context in which CHD4 probably becomes recruited to the cellular machinery in the formation of the boundary between mesoderm and neuroectoderm. It will be interesting to determine whether CHD4 transcription itself is induced or repressed by growth factor signals, which is suggested by its nonuniform transcription in the embryo (Supplementary Fig. S1, Linder et al. 2004).

The above statements on CHD4 function rest on several lines of independent evidence. First, we observed that an increase in wild-type CHD4 protein levels caused phenotypes that were opposite to those achieved either by reducing endogenous CHD4 protein levels through a CHD4-specific antisense Morpholino strategy, or by overexpressing an ATPase-minus CHD4 protein variant. Secondly, the phenotypes of the CHD4-Mo-injected embryos were rescued by coexpression of wild-type CHD4 protein both on the morphological and molecular levels (Figs. 2, 3, 5, 6, Supplementary Fig. S3). In addition, the majority of tested genes (12 of 16) were expressed fairly normally under the various experimental conditions, strongly arguing against an un Specific perturbation of cellular transcription or of bulk chromatin architecture. Since most of these genes are regulated by Wnt, FGF, BMP, or Nodal signals, we conclude that the major embryonic signaling pathways remained fully functional, and that CHD4 acts in a gene- and signal-specific manner. Finally, we note that similar types of experiments targeted at BRG1, a functionally distinct SNF2-like ATPase found in BAF and PBAF chromatin remodeling complexes, interferes with different developmental processes (Seo et al. 2005; N. Singhal and R. Rupp, unpubl.). The phenotypes reported here are, therefore, specific consequences of the experimental alterations of CHD4 protein abundance.

The qualitatively indistinguishable phenotypes achieved either by Morpholino-mediated knockdown of endogenous CHD4 protein or overexpression of the dnCHD4 variant indicate a requirement for ATP hydrolysis and, thus, most likely involve alterations of the local chromatin structure at the Sip1 locus. The molecular nature of these alterations is currently unknown, but probably depends on the protein context in which CHD4 becomes recruited to the Sip1 gene. CHD4/Mi-2β has been purified biochemically as a component of the high-molecular-weight NuRD complex, which requires the help of other subunits to exert full nucleosome-remodeling activity (Becker and Hörz 2002). Recently, several NuRD complex variants have been purified that differ in their ability to interact with methylated DNA or other proteins (for details, see Bowen et al. 2004; Fujita et al. 2004; Brackertz et al. 2006; Le Guennec et al. 2006). However, CHD4/Mi-2β has also been invoked in gene
Wnt signaling. the induction of and reduced CHD4 protein levels had no significant effect on the moter is not altered by CHD4 sitization of the Xbra 980 GENES & DEVELOPMENT loop helix (bHLH) protein HEB and the histone acetyl-activation, either in a complex with the basic helix–loop–helix [bHLH] protein HEB and the histone acetyltransferase p300 on the CD4 gene during T-cell development (Williams et al. 2004), or in the form of protein supercomplexes containing both NuRD and SWI/SNF subunits (Shimono et al. 2003). Our ChIP results have established Sip1 as a direct CHD4 target (Fig. 4) and suggested an association of CHD4 protein with the actively transcribed Sip1 gene body, because overexpression of CHD4 protein reduced Sip1 mRNA levels in isolated animal caps [Fig. 2B]. Based on this, we propose that a CHD4-containing protein complex—probably NuRD—suppresses Sip1 expression by impeding transcription through nucleosome remodeling and/or HDAC-mediated hypoacetylation. It remains a formidable challenge for future investigations to identify the precise nature of the CHD4 protein complex and how it is recruited to the Sip1 gene, as well as to investigate its mechanism of action at its preferred binding site around exon E1.

Related to our findings, the constitutive NuRD component MBD3 has been shown to be required for pluripotency of murine embryonic stem cells [Kaji et al. 2006]. Interestingly, MBD3−/− ES cells showed defects in gene silencing and were severely compromised in cell fate commitment. Our observations indicate that CHD4 loss of function prevented neither germ layer formation nor cell differentiation. However, MBD3 protein is essential for stable formation of the NuRD complex formation [Kaji et al. 2006], while Morpholino knockdown or overexpression of dnCHD4 will cause only a partial loss-of-function situation. While the apparent discrepancy between the two studies results probably from quantitative differences of NuRD inhibition, it suggests that there may be more functions to be discovered for this conserved chromatin remodeling machine.

Our results also add to the extraordinary complexity of the regulatory machinery underlying gene expression patterns such as the Xbra domain, whose superficially contiguous appearance is shaped by a plethora of positive and negative inputs [see also Heasman 2006; Kimelman 2006; Wardle and Smith 2006]. Elegant experiments in Xenopus had provided insight into how the Sip1 and Xbra domains become segregated during gastrulation, but had also indicated a requirement for additional mechanisms beyond the simple repression of the Xbra gene by Sip1 protein to explain this process [Lerchner et al. 2000; Papin et al. 2002]. Our data add two important regulatory facets to this problem. First, the CHD4-dependent suppression of Sip1 transcription is sufficient to tune the Activin/Nodal-response threshold of the Xbra promoter over a surprisingly broad concentration range in vitro [Fig. 5], and more importantly also under in vivo induction conditions [Figs. 1–3]. Secondly, CHD4 does not significantly interfere with eFGF/MAPK-dependent stimulation of Xbra transcription. Together, this suggests that the CHD4/Sip1 epistasis is involved in restricting the primary, unstable induction of Xbra, but stops operating in presumptive mesodermal cells, in which Xbra has managed to engage the eFGF feedback loop. A major implication of these results is that chromatin remodeling factors such as CHD4/Mi-2 are part of the machinery that translates morphogen signals into spatial territories of gene expression patterns during vertebrate embryogenesis.

Figure 6. Specificity of CHD4-dependent threshold control. The specificity of the Xbra promoter response was further investigated in animal caps. (A) The CHD4-Mo-dependent desensitization of the Xbra promoter can be rescued by wtCHD4 mRNA coinjection. (B) The Activin threshold of the Gsc promoter is not altered by CHD4 overexpression. (C) Elevated or reduced CHD4 protein levels had no significant effect on the eFGF-dependent induction of Xbra transcription (cf. lanes 4, 8, and 12). (D) Neither wtCHD4 nor dnCHD4 mRNA levels affect the induction of Siamois or Xnodal related 3 by Wnt8/canonical Wnt signaling.

Materials and methods

Expression constructs and synthetic mRNAs

The ORF of Xenopus CHD4 was generated by PCR from an EST (BF047668; RZPD) and subcloned via BamHI/XhoI sites into the pCS2+ vector [see Supplementary Fig. S3 for primer sequences]. The dominant-negative CHD4 variant was constructed with a C-terminal domain of xCHD4 (amino acids 1513–1891) was

Table 1. Summary of CHD4-dependent threshold control experiments

| Treatment | wtCHD4 | CHD4-Mo | Activin | eFGF | Xbra | H4 |
|-----------|--------|---------|---------|------|------|---|
| 1         | 1      | -       | 40      | 40   | 40   | 40|
| 2         | 1      | -       | 40      | 40   | 40   | 40|
| 3         | 1      | -       | 40      | 40   | 40   | 40|
| 4         | 1      | -       | 40      | 40   | 40   | 40|
| 5         | 1      | -       | 40      | 40   | 40   | 40|
| 6         | 1      | -       | 40      | 40   | 40   | 40|
| 7         | 1      | -       | 40      | 40   | 40   | 40|
| 8         | 1      | -       | 40      | 40   | 40   | 40|
| 9         | 1      | -       | 40      | 40   | 40   | 40|
| 10        | 1      | -       | 40      | 40   | 40   | 40|
| 11        | 1      | -       | 40      | 40   | 40   | 40|
| 12        | 1      | -       | 40      | 40   | 40   | 40|

Figure 6: Specificity of CHD4-dependent threshold control. The specificity of the Xbra promoter response was further investigated in animal caps. (A) The CHD4-Mo-dependent desensitization of the Xbra promoter can be rescued by wtCHD4 mRNA coinjection. (B) The Activin threshold of the Gsc promoter is not altered by CHD4 overexpression. (C) Elevated or reduced CHD4 protein levels had no significant effect on the eFGF-dependent induction of Xbra transcription. (D) Neither wtCHD4 nor dnCHD4 mRNA levels affect the induction of Siamois or Xnodal related 3 by Wnt8/canonical Wnt signaling.
cloned into the pGEX-4T3 bacterial expression vector (Amersham), expressed in *Escherichia coli*, and purified as described (Linder et al. 1998).

**Morpholino-mediated protein ablation**

We used a 25-mer antisense Morpholino oligonucleotide complementary to the *Xenopus* CHD4 translation start (see Supplementary Fig. S2A). The unrelated, standard control Morpholino supplied by Gene Tools LLC served as a control for specificity [for sequences, see Supplementary Fig. S4]. All Morpholinos were resuspended in distilled water and injected at a dose of 40 ng per embryo, unless stated otherwise. To test the efficiency of CHD4-Mo, we subcloned the first 363 amino acids of CHD4 in-frame upstream of the 6xMyc-tag cassette of the pcS2 + MT6 vector, either with or without the 5’ untranslated region [UTR] complementary to the CHD4-Mo protein. Sip1 mRNA translation was inhibited by the published Morpholino described by Nitta et al. (2004).

**Embryo manipulations and analysis**

Handling, culture, and staging of in vitro fertilized *Xenopus* embryos followed standard procedures (Sive et al. 2000). Microinjections of mRNA or Morpholinos were performed in 5 µL of volume into one blastomere of the two-cell stage for embryonic phenotypes, or with 4 × 2.5 µL into the animal pole of four-cell-stage embryos for preloading of animal cap tissue. CHD4 and Sip1 mRNAs were used at 1.0 ng/embryo, which had been defined as an optimal dose for functional interference without toxic side effects (data not shown). For further analysis, embryos were presorted into left-side- or right-side-injected specimens by coinjected eGFP lineage tracer before fixation.

**Animal cap assay and RT–PCR**

Animal caps were dissected with a gastromaster (yellow tip; Xenotek Engineering) at late blastula (7 h post-fertilization) and cultured in 0.5% MEMFA, 500 µM NaCl and 0.1% SDS (one wash), 500 mM NaCl and 0.1% SDS (one wash), and TE (two washes). The chromatin was sheared to an average size of 300 base pairs (bp) with the Bioruptor (Diagenode) and cleared by centrifugation for 5 min at 1000 rpm. For immunoprecipitation with xCHD4-specific antibodies, 30 µL of DNA/BSA-blocked Protein A-Sepharose bead slurry [Amerham Pharmacia] were precoupled for 60 min with 15 µg of rabbit anti-rat IgG antibodies [Dianova] and mouse anti-rat IgM antibodies [Biozol] in PBS at room temperature. The protein A-Sepharose was subsequently incubated with 10 µL of a mix of different xCHD4-specific monoclonal antibodies, and incubated for 4 h at room temperature. For preclearing, 50 µL of blocked protein A-Sepharose suspension were added to 1 mL of chromatin lysate and rotated for 1 h at 4°C. Lysate aliquots of 100 embryo equivalents were incubated rotating overnight at 4°C with 3 µL of Protein A-Sepharose, which was either preabsorbed with xCHD4-specific antibody mix or with bridging antibodies as a control for unspecific binding. The protein A-Sepharose samples with bound chromatin fragments were pelleted by centrifugation for 1 min at 2000 rpm and washed sequentially by 15 min of rotation at 4°C with 1 mL of buffer A2 containing 0.1% SDS [four washes], 500 mM NaCl and 0.1% SDS [one wash], and TE [two washes]. The chromatin was eluted and the DNA was purified as described (Fujita et al. 2004). The final DNA pellets were dissolved in 50 µL of ddH2O, and 2-µL aliquots were added to each PCR reaction, carried out as duplicates. The *Xenopus* Sip1 Taqman amplicons [for primers and probes, see Supplementary Fig. S4] were based on published cDNA sequence (Eisaki et al. 2000). Exons U5, E1, and E2 were identified by sequence conservation between *Xenopus tropicalis* (genome assembly version 4.1, scaffold 232) and mouse (Nelles et al. 2003) sip1 genomic DNA sequences. The relative occupancy of CHD4 protein at the sip1 locus was calculated by sequential normalization to the input and to the GAPDH control amplicon.

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