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Foxh1 recruits Gsc to negatively regulate *Mixl1* expression during early mouse development

Luisa Izzi[1,6], Cristoforo Silvestri[2,6], Ingo von Both[3], Etienne Labbé[1,6], Lise Zakin[4], Jeffrey L Wrana[3,5] and Liliana Attisano[1,2,6,7,*]

[1]Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada, [2]Institute of Medical Sciences, University of Toronto, Toronto, Ontario, Canada, [3]Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada, [4]Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, CA, USA, [5]Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario, Canada, [6]Terence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Ontario, Canada and [7]Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

***Mixl1* is a member of the Mix/Bix family of paired-like homeodomain proteins and is required for proper axial mesendoderm morphogenesis and endoderm formation during mouse development. Mix/Bix proteins are transcription factors that function in Nodal-like signaling pathways and are themselves regulated by Nodal. Here, we show that Foxh1 forms a DNA-binding complex with Smads to regulate transforming growth factor β (TGFβ)/Nodal-dependent *Mixl1* gene expression. Whereas Foxh1 is commonly described as a transcriptional activator, we observed that *Foxh1*-null embryos exhibit expanded and enhanced *Mixl1* expression during gastrulation, indicating that Foxh1 negatively regulates expression of *Mixl1* during early mouse embryogenesis. We demonstrate that Foxh1 associates with the homeodomain-containing protein Goosecoid (Gsc), which in turn recruits histone deacetylases to repress *Mixl1* gene expression. Ectopic expression of Gsc in embryoid bodies represses endogenous *Mixl1* expression and this effect is dependent on Foxh1. As *Gsc* is itself induced in a Foxh1-dependent manner, we propose that Foxh1 initiates positive and negative transcriptional circuits to refine cell fate decisions during gastrulation.**

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Introduction

During gastrulation, the three germ layers, ectoderm, mesoderm and endoderm, are generated. In mice, this is first apparent with the formation of the primitive streak at the posterior end of the epiblast near the embryonic/extra-embryonic junction (Tam and Behringer, 1997). The primitive streak elongates towards the distal end of the epiblast and cells move through the streak to emerge as mesoderm and endoderm. The organizer or node, which is located at the anterior end of the primitive streak, is a specialized population of cells with axis-inducing abilities that also contributes to the formation of axial mesendoderm, prechordal mesoderm and notochord (Robb and Tam, 2004).

Numerous growth factors regulate gastrulation, including transforming growth factor β (TGFβ) superfamily members such as Nodal and Activin (Schier, 2003). Nodal, like other TGFβ ligands, initiates signaling through its interaction with a heteromeric complex of serine/threonine kinase receptors. It also requires the presence of the EGF-CFC co-receptors, Tdgf1 (Cripto) or Cfc1 (Cryptic) (Attisano and Wrana, 2002; Schier, 2003; Feng and Derynck, 2005). Following activation of the receptor complex, the signal is transmitted to Smad proteins, which translocate to the nucleus and interact with specific transcription factors to regulate the expression of target genes (Attisano and Wrana, 2002; Feng and Derynck, 2005). Loss- and gain-of-function studies in frog, fish and mice have demonstrated that several components of the Nodal signaling pathway are implicated in the establishment of mesoderm and endoderm as well as axial mesendoderm patterning (Schier, 2003).

Several transcription factors are targeted by Nodal signaling and function to regulate gastrulation. For example, *Mix/Bix* paired-like homeobox genes, including *Xenopus Mix.2*, zebrafish *og9x/mezzo* and mouse *Mixl1* act downstream of Nodal-like signaling pathways to regulate both mesoderm and endoderm formation (Chen *et al*, 1997; Hart *et al*, 2002; Poulain and Lepage, 2002). In mice, *Mixl1* is expressed in the primitive streak and emerging mesoderm at the onset of gastrulation and becomes restricted to the posterior primative streak at the early bud stage (Pearce and Evans, 1999; Robb *et al*, 2000). The important role played by *Mixl1* during mouse development is revealed by the numerous defects displayed by *Mixl1*$^{-/-}$ mutant embryos, which include an enlarged primitive streak, abnormal anterior midline structures, absence of heart tube formation and defective gut morphogenesis.

Goosecoid (Gsc) is another paired-like homeodomain-containing transcription factor whose expression is regulated by Nodal-like signaling pathways that functions in gastrulation as well as in axial mesendoderm formation (Blum *et al*, 1992; Kinder *et al*, 2001). In mice, *Gsc* expression marks the onset of gastrulation and is first detected in the primitive streak. As gastrulation proceeds, *Gsc* expression is restricted to the anterior primitive streak and the anterior visceral endoderm (Blum *et al*, 1992; Belo *et al*, 1997). Although its role in early development remains unclear as *Gsc*-null mice present no overt gastrulation or axial midline defects (Rivera-Perez *et al*, 1995; Yamada *et al*, 1995), Gsc can function as a

*Corresponding author. Department of Biochemistry, Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Room 1008, 160 College Street, Toronto, Ontario, Canada M5S 3E1. Tel.: +1 416 946 3129; Fax: +1 416 978 8287;
E-mail: liliana.attisano@utoronto.ca

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transcriptional repressor by directly binding to paired homeodomain-binding sites to repress transcription of its own promoter as well as those of other genes, including *Xenopus brachyury (Xbra)* and *wnt8 (Xwnt8)* (Latinkic *et al*, 1997; Danilov *et al*, 1998; Yao and Kessler, 2001). The N-terminus of Gsc comprises a conserved Goosecoid Engrailed Homology (GEH) domain, and in *Drosophila*, the domain has been shown to interact with Groucho and mediate the repressive activity of Gsc (Jimenez *et al*, 1999).

A key mediator of the Nodal signaling pathway is the forkhead/winged-helix transcription factor, Foxh1 (Attisano *et al*, 2001). Genetic ablation of Foxh1 in mice results in embryonic lethality and a range of defects, including a total lack of embryonic development, aberrant anterior primitive streak patterning, loss of anterior and midline structures, and failure to form definitive endoderm (Hoodless *et al*, 2001; Yamamoto *et al*, 2001). In *Xenopus* and zebrafish, loss of Foxh1 activity results in anterior and axial defects as well as aberrant mesoderm development (Schier, 2003). Thus, loss of Foxh1 activity mimics numerous phenotypes observed in mutants where components of the Nodal signaling pathway have been disrupted (Schier, 2003). In general, Foxh1 binds directly to DNA and cooperates with Smad2/4 complexes to activate Nodal-dependent expression of target genes such as the TGFβ family members, *Nodal* and *Lefty2* and the homeobox factors, *Mix.2, Gsc* and *Pitx2* (Chen *et al*, 1997; Labbé *et al*, 1998; Saijoh *et al*, 2000; Shiratori *et al*, 2001). However, Foxh1-dependent induction of the *Mef2c* gene in the anterior heart field also requires cooperation with Nkx2-5, a heart-specific homeodomain transcription factor (von Both *et al*, 2004). Here, we demonstrate that recruitment of Gsc to the *Mixl1* promoter by Foxh1 represses *Mixl1* expression. Thus, our work reveals that Foxh1 can function either positively or negatively to control target gene expression and we propose that this precise control of gene expression contributes to cell fate determination during gastrulation.

Results

Foxh1 and Smads mediate TGFβ/Activin-dependent transcription of Mixl1

Activin-dependent induction of the *Xenopus Mix.2* gene, a *Mix/Bix* family member, occurs via Foxh1, a DNA-binding forkhead protein, in complex with activated Smads (Chen *et al*, 1997). As expression of *Foxh1* and *Mixl1* appears to overlap during early mouse embryogenesis (Weisberg *et al*, 1998; Hart *et al*, 2002), we sought to determine whether Foxh1 might mediate the TGFβ/Activin-dependent transcriptional regulation of the murine *Mixl1* gene. Examination of human, mouse, rat and rhesus monkey *Mixl1* promoters revealed the presence of a putative Foxh1-binding site (Supplementary Figure 1), suggesting a conserved role for Foxh1 in *Mixl1* transcription. Thus, to examine whether Foxh1 regulates *Mixl1* expression, a 248-bp fragment from the murine *Mixl1* promoter, encompassing the putative Foxh1 site, was subcloned upstream of a luciferase reporter gene (*Mixl1*-luc) and the response to TGFβ was determined in the human hepatocarcinoma cell line, HepG2, which lacks Foxh1 activity (Labbé *et al*, 1998). Co-expression of Foxh1 with the *Mixl1*-luc reporter yielded strong TGFβ-dependent activation of the *Mixl1*-luc reporter (Figure 1A). Direct binding of Foxh1 to the *Mixl1* promoter fragment was confirmed by electrophoretic mobility shift assays (EMSA) using bacterially expressed Foxh1 (Figure 1B). Moreover, Smad2 and Smad4 enhanced both the basal and TGFβ-induced Foxh1-dependent activation of the *Mixl1*-luc reporter (Figure 1C). Although expression of Smad3 and Smad4 also increased the basal Foxh1-mediated activation of the *Mixl1* promoter, TGFβ-dependent responsiveness was lost (Figure 1C).

We next examined whether Foxh1 and Smads cooperate to form a higher-order DNA-binding complex by EMSA. Comparison of DNA-binding complexes from mock-transfected COS-1 cells versus myc-Foxh1-expressing cells revealed the appearance of a slower migrating band in both the presence and absence of the activated Activin type I receptor, ActRIB(TD) (Figure 1D). Co-expression of Smad4 and either Smad2 or Smad3 with Foxh1 resulted in a further decrease in DNA migration, which was most evident in the presence of ActRIB(TD) (Figure 1D). Incubation with antibodies resulted in supershift or loss of DNA-binding complexes, demonstrating the presence Foxh1 and Smads in the TGFβ Responsive Factor (TRF) (Figure 1D). These observations indicate that Foxh1 can bind the *Mixl1* promoter and, on activation of the signaling pathway, forms a DNA-binding complex with Smad2 or Smad3 and Smad4.

To confirm a requirement for Foxh1 binding, two point mutations that prevent Foxh1 binding (Labbé *et al*, 1998) were introduced in the putative Foxh1 site. These mutations abrogated Foxh1 binding to the *Mixl1* promoter (Figure 1E) and abolished TGFβ-dependent signaling (Figure 1F). Smads bind to GC-rich sequences, and thus, to determine the Smad-binding requirements, we generated *Mixl1* promoter constructs harboring either 8 GC to AT point mutations (SBEmut) or a complete deletion of a GC-rich region located downstream of the Foxh1 site (ΔSBE) (Figure 1G). The point mutations reduced, whereas complete deletion abolished, both TGFβ responsiveness and TRF formation on the promoter (Figure 1G and H). Thus, our results, in agreement with previous studies (Hart *et al*, 2005), show that Foxh1 and Smad DNA binding is required for maximal TGFβ-dependent activation of *Mixl1*.

Nodal induces transcriptional activation of Mixl1

Foxh1 expression is confined to early embryogenesis and is thought to be downstream of the Nodal signaling pathway (Schier, 2003). Nodal activates a TGFβ-like pathway by binding Activin receptors and the GPI-linked co-receptors, Cripto or Cryptic (Schier, 2003). To investigate whether Nodal signaling could induce Foxh1-dependent activation of the *Mixl1* promoter, HepG2 cells were transiently transfected with the *Mixl1*-luc reporter and Foxh1 with various combinations of Activin type I and II receptors and the co-receptor, Cripto. Whereas co-transfection of Activin receptor type IB (ActRIB) alone or in the presence of Activin receptor type II (ActRII) mediated induction of the *Mixl1* promoter on Activin treatment, Nodal-dependent activation of *Mixl1* required co-expression of Cripto (Figure 1I), in agreement with a recent study (Hart *et al*, 2005). Taking into account that Nodal plays an essential role in the induction of mesendodermal cell fates (Schier, 2003) and that *Mixl1* is implicated in axial mesendoderm morphogenesis and patterning (Hart *et al*, 2002), Foxh1-dependent induction of *Mixl1* is most likely driven by Nodal signaling during embryogenesis.

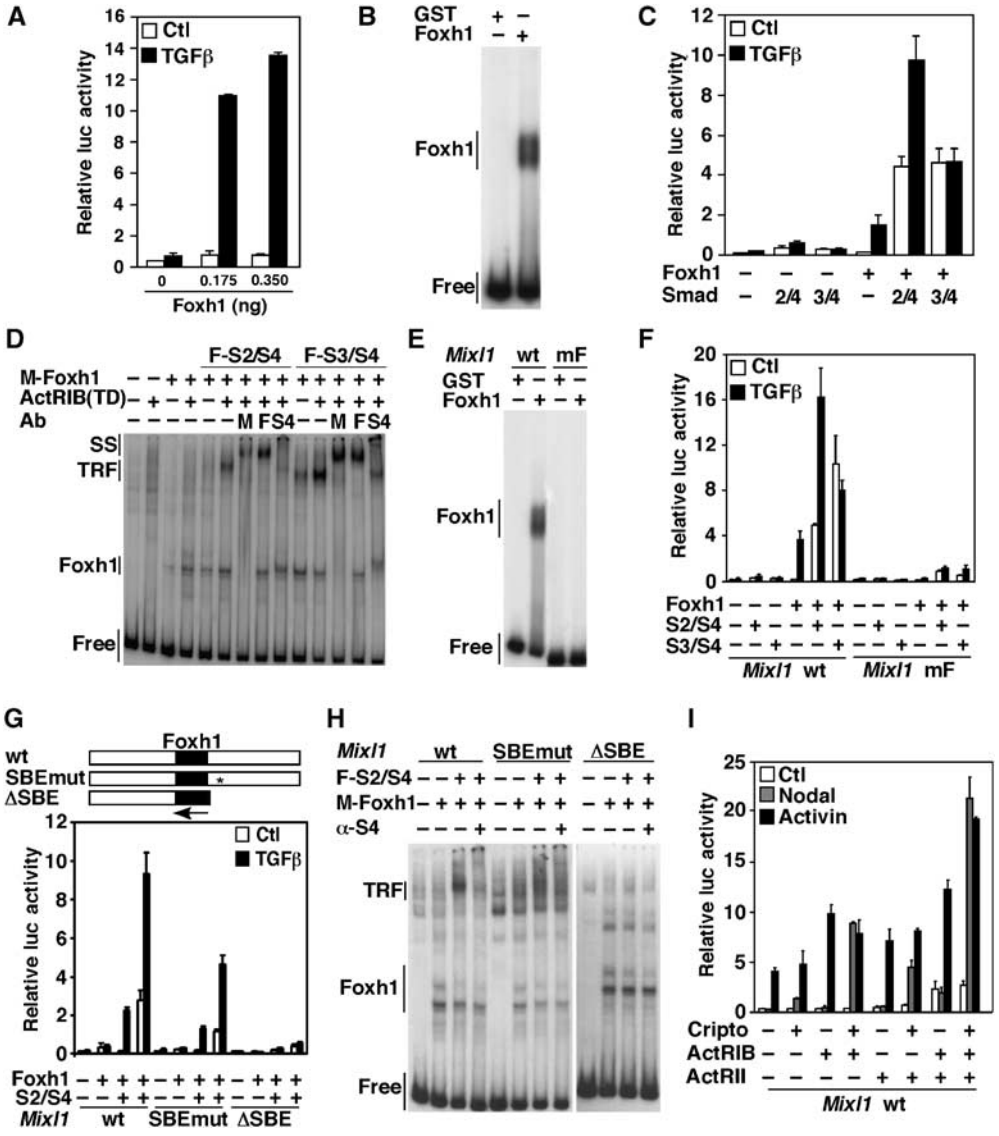

Figure 1 Foxh1 and Smads bind the *Mixl1* promoter and mediate the TGFβ-dependent induction of *Mixl1*. (**A, C, F, G, I**) HepG2 cells were transiently transfected with the *Mixl1*-luc reporter, Foxh1 and Smads (S2, S3, S4), Cripto, ActRIB or ActRII, as indicated. Error bars represent standard deviation of the mean. (**B, D, E, H**) EMSA. A 248-bp *Mixl1* promoter fragment containing a wild-type or mutated Foxh1-binding site was incubated with bacterially expressed proteins (B, E) or crude extracts from COS-1 cells transiently transfected with the indicated DNA (D, H). Protein–DNA complexes were visualized by autoradiography. For supershift assays (D, H), anti-myc (M), anti-Flag (F), and anti-Smad4 (S4) antibodies were added to the reactions.

Foxh1 negatively regulates Mixl1 expression during early mouse embryogenesis

To evaluate the contribution of Foxh1 to the regulation of *Mixl1* transcription *in vivo*, we examined *Mixl1* expression levels in wild-type and *Foxh1* mutant (*Foxh1*$^{-/-}$) embryos. RNA was extracted from genotyped pools of wild-type and *Foxh1*$^{-/-}$ embryos at embryonic day 7.5 (E7.5), and transcript levels were determined by quantitative PCR (QPCR) using primers specific for *Mixl1* transcripts. In RNA extracted from a pool of *Foxh1*$^{-/-}$ embryos, *Mixl1* expression was increased by almost twofold over levels expressed in wild-type embryos (Figure 2A). The level of *Foxh1* expression and the absence of *Foxh1* mRNA in our pool of wild-type and *Foxh1*$^{-/-}$ embryos, respectively, was confirmed by QPCR (Figure 2A).

We next used whole-mount *in situ* hybridization to examine *Mixl1* expression patterns in *Foxh1*$^{-/-}$ mutant embryos. At E7.0, *Mixl1* expression is confined to the nascent primitive streak and emerging mesodermal wings in both wild-type (Figure 2B) (Pearce and Evans, 1999; Robb *et al*, 2000) and *Foxh1*$^{-/-}$embryos (Figure 2B). However, in all *Foxh1*$^{-/-}$ mutant embryos examined, we observed an increase in the intensity of the staining relative to the wild-type embryos (Figure 2B). At E7.5, *Mixl1* staining becomes limited to the primitive streak in wild-type embryos (Figure 2B) (Pearce and Evans, 1999; Robb *et al*, 2000), whereas in *Foxh1*$^{-/-}$ mutant embryos, *Mixl1* expression was expanded anteriorly in the embryonic mesoderm and proximally in the embryonic/extra-embryonic border (Figure 2B). At E8.0, *Mixl1*, which becomes restricted to the posterior primitive streak and the base of the allantois in wild-type embryos (Figure 2B) (Pearce and Evans, 1999; Robb *et al*, 2000), exhibited an expanded expression domain in the caudal end of the primitive streak of *Foxh1*$^{-/-}$ embryos (Figure 2B). Ectopic *Mixl1* staining was

also observed anteriorly in mutant embryos (Figure 2B). Thus, increased *Mixl1* transcript levels are due to both enhanced gene activity and spatial expansion of the *Mixl1* expression domain. Although Foxh1 is best known as transcriptional activator downstream of the Activin/Nodal signaling pathway (Attisano *et al*, 2001), our data suggest that Foxh1 can also act as a negative regulator of target gene expression.

Goosecoid negatively regulates Foxh1-dependent activation of Mixl1

The repressive activity of Foxh1 on endogenous *Mixl1* expression observed in mouse embryos was not recapitulated in HepG2 cells, where Foxh1 promotes activation of the *Mixl1*-luc reporter (Figure 1A). This suggested the possibility that a cofactor present in embryos, but absent in HepG2 cells, was required for the repressive effect. Forkhead and homeodomain-containing proteins have been shown to physically interact to negatively regulate target genes (Foucher *et al*, 2002; Guo *et al*, 2002; Rausa *et al*, 2003). As the expression of *Foxh1*, *Mixl1* and the paired-like homeodomain gene *Gsc* partially overlap during gastrulation (Blum *et al*, 1992; Belo *et al*, 1998; Weisberg *et al*, 1998; Pearce and Evans, 1999; Robb *et al*, 2000) and *Gsc* expression is lost in Foxh1 mutant embryos (Hoodless *et al*, 2001; Yamamoto *et al*, 2001), we next determined whether Gsc might modulate Foxh1-dependent expression of *Mixl1*. For this, HepG2 cells were transfected with Foxh1 and increasing amounts of Gsc and TGFβ-dependent activation of the *Mixl1*-luc reporter was examined. Ectopic expression of Gsc strongly repressed TGFβ-dependent induction of luciferase activity mediated by Foxh1 alone (Figure 3A) or when Smad2 and Smad4 were co-expressed with Foxh1 (Figure 3B). Thus, Gsc functionally interacts with Foxh1 to repress *Mixl1* transcription. To determine whether Gsc modulates the expression of *Mixl1 in vivo*, we next examined the expression of *Mixl1* in E7.5 wild-type and *Gsc*-null embryos by QPCR. In RNA extracted from *Gsc*-null embryos, average *Mixl1* expression was increased by almost twofold over wild-type levels (Figure 3C). Interestingly, the range of *Mixl1* mRNA expression in *Gsc*-null embryos was wider as compared to wild-type embryos. As *Gsc*-null embryos do not display overt defects in early embryogenesis (Rivera-Perez *et al*, 1995; Yamada *et al*, 1995), we speculate that compensatory mechanisms overcome the increased expression of *Mixl1* to allow gastrulation to proceed. As loss of Gsc enhances expression of *Mixl1*, these results suggest that Gsc can negatively regulate *Mixl1* expression *in vivo*.

Physical interaction of Foxh1 with Goosecoid

Gsc can repress transcription of target genes such as *Xbra* and its own promoter by binding directly to DNA at paired homeodomain sites (Danilov *et al*, 1998; Latinkic and

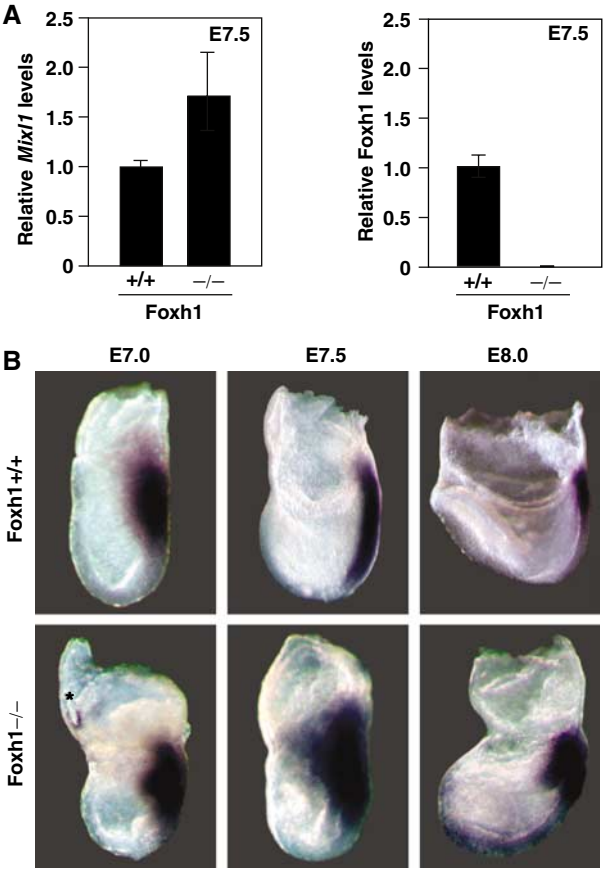

Figure 2 *Mixl1* expression is upregulated in gastrulating *Foxh1*-null embryos. (**A**) RNA was extracted from E7.5 $Foxh1^{+/+}$ and $Foxh1^{-/-}$ embryos. *Mixl1* and *Foxh1* levels were quantified by QPCR and normalized to *Hprt* expression. (**B**) At E7.0 and E7.5, *Mixl1* expression is expanded and upregulated in *Foxh1* mutant (−/−) embryos within the posterior primitive streak and the wings of the nascent mesoderm. At about E8.0, *Mixl1* is ectopically expressed in the endoderm overlying the head process. Images taken from the left. Background staining (*) is noted.

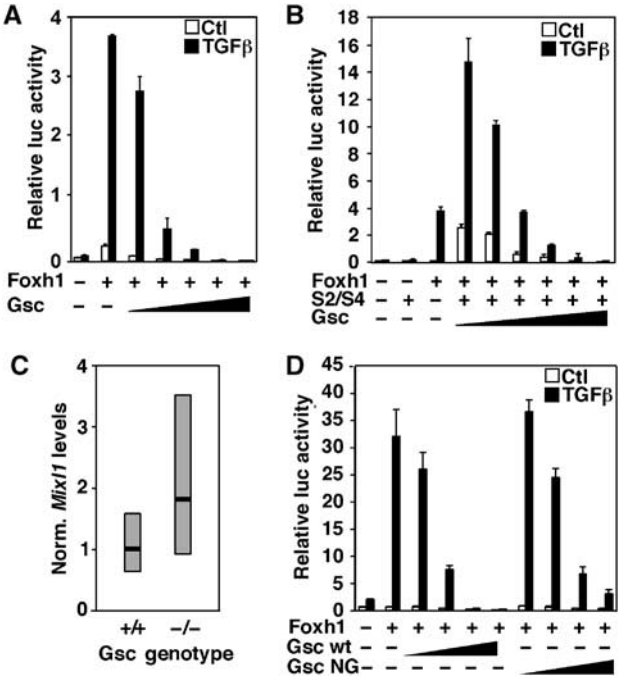

Figure 3 Goosecoid (Gsc) represses Foxh1-mediated induction of the *Mixl1*. (**A, B, D**) HepG2 cells were transiently transfected with the *Mixl1*-luc reporter with combinations of Foxh1, increasing amounts (0.25–250 ng) of Gsc or Gsc NG (N210G). Error bars represent standard deviation of the mean. (**C**) QPCR analysis of *Mixl1* expression in wild-type ($n=5$) and *Gsc*-null ($n=3$) embryos. Expression was normalized to *Gapdh*. Black line represents average and gray box represents range of *Mixl1* expression. Data represent the average of two QPCR experiments.

Smith, 1999). The *Mixl1* promoter does not contain consensus paired homeodomain sites, and the Gsc DNA-binding mutant (Gsc NG; N210G) repressed Foxh1-mediated induction of the *Mixl1* promoter (Figure 3D), indicating that *Mixl1* repression by Gsc occurs independent of its DNA-binding activity.

We next examined whether Gsc might repress transcription by associating with Foxh1. Analysis of immunoprecipitates of cell lysates from COS-1 cells transfected with Flag-Foxh1 and T7-Gsc revealed an interaction between Foxh1 and Gsc (Figure 4A). To determine the region in Foxh1 that mediates the binding to Gsc, we evaluated the ability of two deletion constructs of Flag-Foxh1 to interact with Gsc expressed as a GST fusion. We observed that GST–Gsc bound full-length Flag-Foxh1 and a mutant (ΔC) containing only the forkhead domain, but not Foxh1 lacking the forkhead domain (ΔN, Figure 4B). A similar approach was used to identify the region of Gsc mediating the association with Foxh1. In this case, full-length GST–Gsc and GST–Gsc 1–219 bound Flag-Foxh1, whereas larger deletions lacking the homeodomain did not (Figure 4C). The Gsc homeodomain alone was not sufficient to interact with Flag-Foxh1 (Figure 4C), suggesting that in the context of full-length Gsc, other regions are also necessary for efficient binding to Foxh1. Gsc lacking the homeodomain (Gsc 1–143) did not block Foxh1-mediated induction of the *Mixl1* promoter in luciferase reporter assays (Figure 4D). Thus, our data show that the interaction between Gsc and Foxh1 is mediated by their conserved DNA-binding regions, and that the Gsc homeodomain is required for the repressive activity of Gsc.

Gooseceoid is recruited to the Mixl1 promoter through Foxh1

As Gsc can bind Foxh1, we next examined whether Gsc could be recruited to the *Mixl1* promoter through its interaction with Foxh1 by performing sequential protein–DNA immunoprecipitations. COS-1 cells were transiently transfected with the *Mixl1*-luc reporter together with cDNAs encoding Flag-Foxh1 and T7-Gsc. Protein–DNA complexes were crosslinked by formaldehyde treatment, cell lysates were sequentially immunoprecipitated with anti-Flag and anti-T7 antibodies (Figure 5A) and the amount of immunoprecipitated *Mixl1*-luc reporter was then assessed by QPCR. When Flag-Foxh1 and T7-Gsc were co-expressed, the *Mixl1* promoter fragment was detected (Figure 5A), whereas only basal levels were observed when either protein was expressed alone. In contrast, Gsc 1–143, which does not interact with Foxh1 nor block Foxh1-dependent induction of *Mixl1*, did not bind to the *Mixl1* promoter (Figure 5B). Thus, Gsc can be recruited to the *Mixl1* promoter through its interaction with Foxh1.

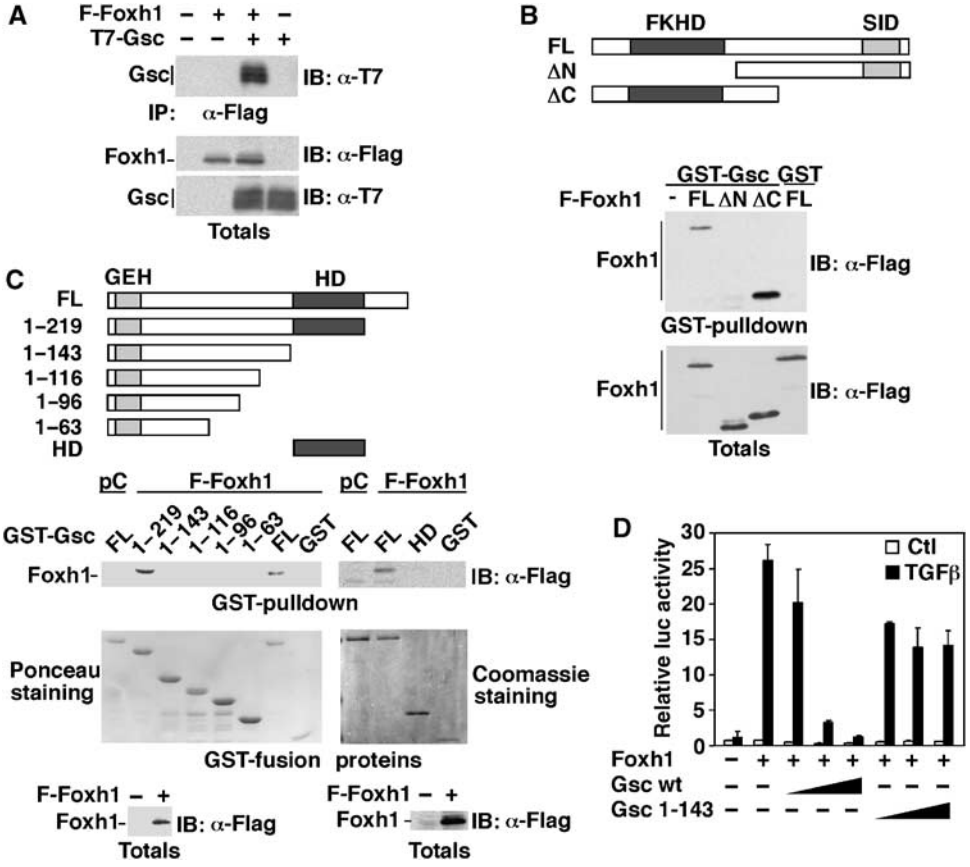

Figure 4 Gsc interacts with Foxh1 and represses Foxh1-mediated activation of the *Mixl1* promoter. (**A**) COS-1 cells were co-transfected with T7-Gsc and Flag-Foxh1 or vector control. Cell lysates were immunoprecipitated (IP) with anti-Flag antibody, and immunoblotted (IB) with anti-T7 antibody. (**B**, **C**) GST pull-down assay was performed by incubating Gsc or Gsc deletion constructs expressed as GST-fusion proteins with lysates from COS-1 cells expressing full-length or deletion mutants of Flag-Foxh1. Bound proteins were detected by immunoblotting with anti-Flag antibody. Equal protein expression was confirmed by immunoblotting or by Ponceau red or Coomassie blue staining as indicated. (**D**) HepG2 cells were transiently transfected with the *Mixl1*-luc reporter, with Foxh1 and increasing amounts (0.25–250 ng) of Gsc or Gsc 1-143 (closed triangles). Error bars represent standard deviation of the mean.

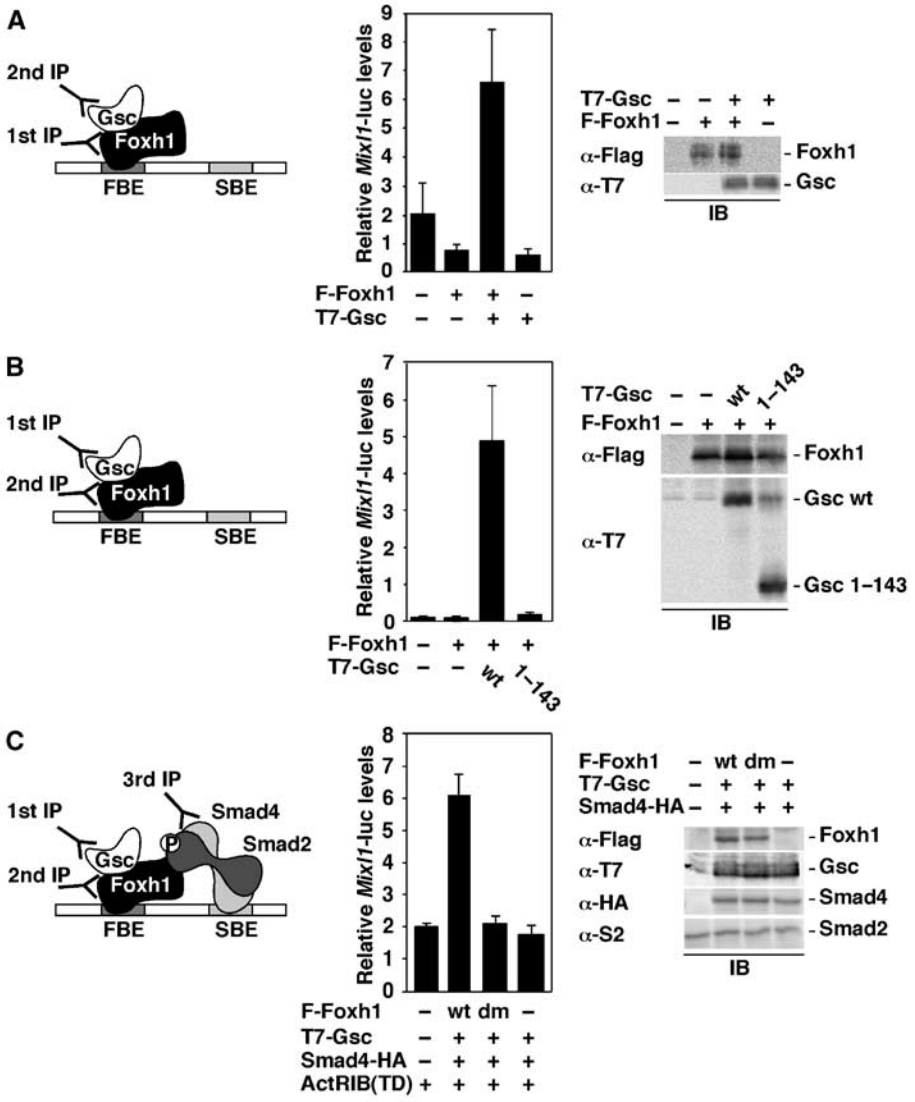

Figure 5 Gsc interacts with Foxh1 and Smads on the *Mixl1* promoter. (**A**–**C**) COS-1 cells were transiently transfected with the *Mixl1*-luc reporter and the indicated cDNAs. Protein–DNA complexes were collected by sequential immunoprecipitations, followed by elution in 1% SDS, using anti-Flag, anti-T7 and anti-HA antibodies as indicated (left panels). Recovered DNA was analyzed by QPCR, and corrected for DNA inputs (middle panels). Protein expression was confirmed by immunoblotting (left panels).

We next evaluated whether Gsc could interact with Foxh1 within the context of a TRF complex by performing a three-step sequential protein–DNA immunoprecipitation. COS-1 cells were transiently transfected with the *Mixl1*-luc reporter along with cDNAs encoding Flag-Foxh1, Smad4-HA, T7-Gsc and ActRIB(TD). Cell lysates were subjected to immunoprecipitation with anti-T7 antibody, followed by elution with 1% SDS, immunoprecipitation of the eluate with anti-Flag antibody, a second elution with 1% SDS and a final immunoprecipitation with anti-HA antibody (Figure 5C). QPCR analysis demonstrated that T7-Gsc bound Flag-Foxh1 within a TRF complex (Figure 5C). In contrast, negligible amounts of *Mixl1* were immunoprecipitated when T7-Gsc was co-expressed with a Foxh1 DNA-binding mutant (Foxh1 dm; R61H, K64N) or with a control plasmid (Figure 5C). Our data show that Foxh1, Smads and Gsc can coexist on the *Mixl1* promoter and suggest that the transcriptional repression mediated by Gsc is unlikely to occur through disruption of the TRF.

The repressive activity of Goosecoid is mediated by histone deacetylases

We next examined whether the conserved GEH repressor domain present at the N-terminus of Gsc is required for the repressive activity. However, Gsc Δ14, which lacks the GEH domain, was able to repress *Mixl1*-luc reporter activity as well as wild-type Gsc (Figure 6A). To investigate whether Gsc might recruit transcriptional corepressors such as HDACs to abrogate transcriptional activation of *Mixl1*, we examined the effect of Trichostatin A (TSA), an inhibitor of HDAC activity on *Mixl1*-luc reporter activity. HepG2 cells, transfected with the *Mixl1*-luc reporter, Foxh1, in the presence or absence of Gsc were incubated with TGFβ and TSA. Treatment with increasing doses of TSA resulted in the enhancement of *Mixl1*-luc activity by up to threefold (Figure 6B), suggesting that recruitment of HDACs by Gsc contributes to the repression of *Mixl1* promoter activity. The association of Gsc with HDACs was then examined using COS-1 cells transfected with T7-Gsc and various Flag-HDACs. Gsc interacted with Class I

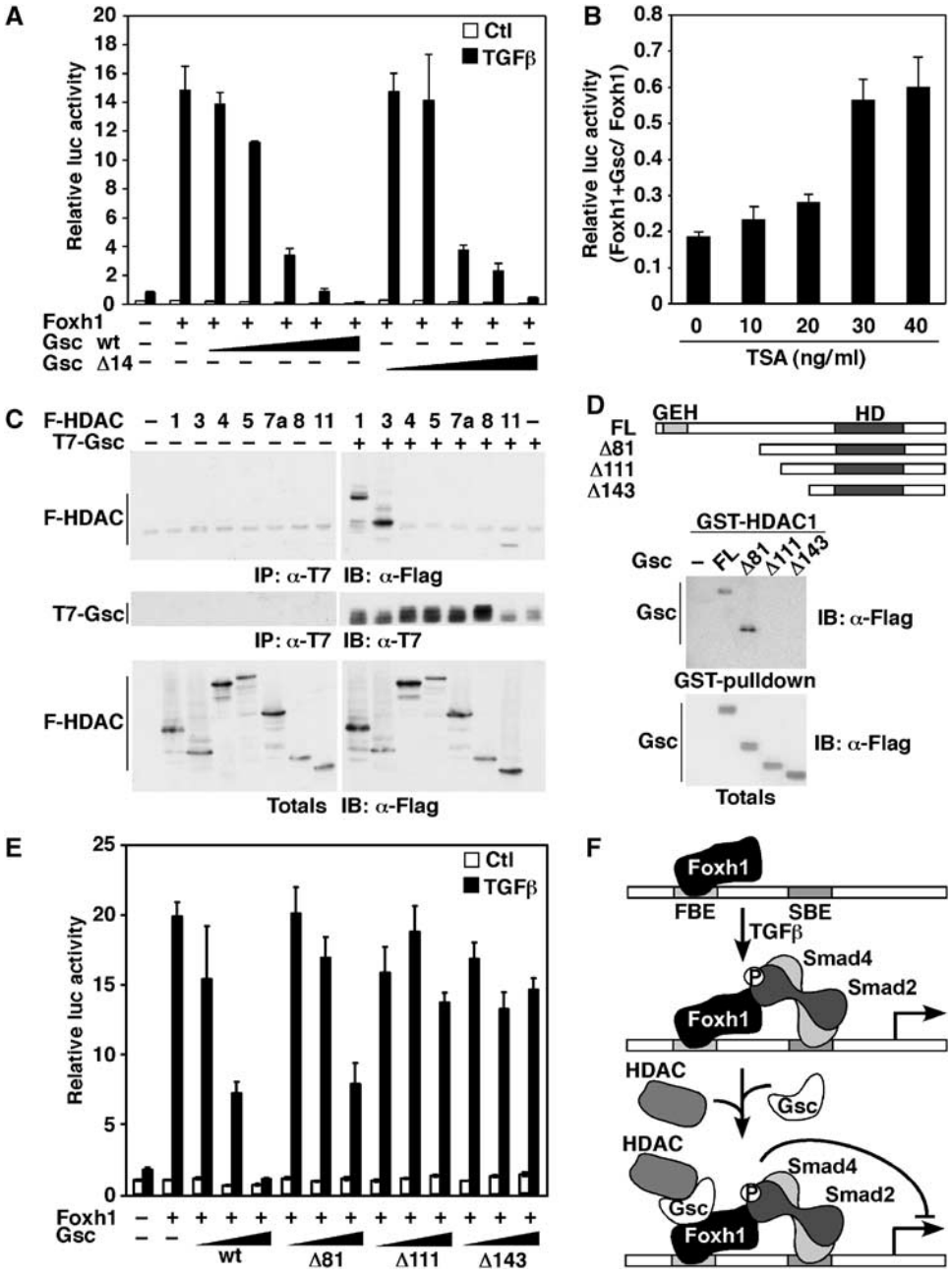

Figure 6 Gsc recruits histone deacetylases (HDACs) to repress *Mixl1* promoter activity. (**A**, **E**) HepG2 cells were transiently transfected with the *Mixl1*-luc reporter, with Foxh1 and increasing amounts (0.25–250 ng) of full-length Gsc, or indicated deletion mutants (closed triangles). Error bars represent standard deviation of the mean. (**B**) HepG2 cells were transiently transfected with the *Mixl1*-luc reporter, Foxh1 and either a control or Gsc expression vector. Cells were incubated overnight with 100 pM TGFβ and the indicated amount of TSA. Luciferase activity from cell lysates was determined and presented as luciferase activity induced by TSA in cells transfected with Gsc relative to luciferase activity induced by TSA in cells transfected with the control plasmid. Error bars represent propagated error on the quotient calculated from standard errors of the mean of numerators and denominators. (**C**) Lysates of COS-1 cells co-transfected with T7-Gsc and the indicated Flag-HDAC constructs or vector control were immunoprecipitated with anti-T7 antibody and immunoblotted with anti-Flag antibody. Protein expression was verified by immunoblotting. (**D**) GST pull-down assay was performed by incubating GST-HDAC1 with lysates from COS-1 cells expressing full-length or deletion mutants of Flag-Gsc. Bound and total protein expression was detected by anti-Flag immunoblotting. (**F**) A model for Foxh1-dependent regulation of *Mixl1*. Foxh1 constitutively binds the *Mixl1* promoter and on TGFβ signaling, Smads are recruited to Foxh1. Gsc binds Foxh1 and recruits HDACs to repress *Mixl1* promoter activity.

HDACs, HDAC1 and HDAC3 and the Class IV HDAC, HDAC11 but not with the others (Figure 6C). To determine the region in Gsc that mediates the interaction with HDACs, we tested the ability of Gsc N-terminal deletion mutants to interact with HDAC1 expressed as a GST-fusion protein (Figure 6D). HDAC1 bound full-length Gsc as well as deletion mutant Δ81 but not deletion mutants Δ111 and Δ143 (Figure 6D). The effect of these deletion mutants on *Mixl1* promoter activation demonstrated that Gsc Δ81 repressed transcription, whereas the larger deletion mutants did not (Figure 6E). Thus, our data show that the interaction between Gsc and HDAC1 is mediated by a region located between residues 81 and 111 of Gsc, and that this region is required for Gsc-mediated repression of the *Mixl1* promoter. Taken together,

our biochemical analysis suggests a model (Figure 6F) in which Gsc associates with Foxh1 that is bound to the *Mixl1* promoter and then functions to recruit histone deacetylases and thereby repress *Mixl1* expression.

Goosecoid-mediated repression of endogenous Mixl1 requires Foxh1

Analyses of *Foxh1*$^{-/-}$ mutant embryos suggested that Foxh1 negatively regulates *Mixl1* expression *in vivo* and our biochemical studies showed that Gsc associates with Foxh1 to mediate the transcriptional repression of *Mixl1*. Thus, we sought to determine whether increased expression of Gsc could repress endogenous *Mixl1* expression and whether this repressive effect of Gsc requires Foxh1. As *Gsc* is itself a Foxh1-regulated gene, the interpretation of *in vivo* data is particularly complex. Therefore, we utilized an *in vitro* assay in which mouse embryonic stem cells are induced to differentiate into embryoid bodies (EBs), a model system that recapitulates gastrulation events, including the generation of all three germ layers (Keller, 2005). For this, wild-type or *Foxh1*$^{-/-}$ mutant ES cell lines stably expressing murine *Gsc* or an empty vector control were generated. Individual lines were isolated and clones overexpressing *Gsc* were identified by QPCR using RNA extracted from ES cell colonies (Figure 7A). Using the hanging drop method (Spector *et al*, 1998), ES cell lines were induced to differentiate by the removal of leukemia-inhibiting factor (LIF) and EBs were collected for up to 7 days post-differentiation. Increased *Gsc* expression in the stable transfectants was maintained throughout the differentiation time course (Figure 7A). Consistent with previous reports, *Mixl1* was transiently induced (Mossman *et al*, 2005; Ng *et al*, 2005; Willey *et al*, 2006), with maximal expression detected at day 5 in both wild type (Figure 7B, top panel) and *Foxh1*$^{-/-}$ control lines (Figure 7B, bottom panel). This concurs with our *in vivo* expression data, demonstrating that activation of *Mixl1* expression occurs independent of Foxh1. However, in EBs derived from *Foxh1*, wild-type ES cells stably overexpressing Gsc, the enhanced expression of *Mixl1* was abrogated (Figure 7B, top panel). In marked contrast, overexpression of Gsc in all four *Foxh1*$^{-/-}$ EBs, resulted in enhanced *Mixl1* expression at either day 5 (for clones 3, 6) or with a short delay on day 6 (for clones 9, 21) (Figure 7B, bottom panel). Thus, in cells lacking Foxh1, Gsc is unable to repress *Mixl1* expression.

To determine whether Gsc is recruited to the endogenous *Mixl1* promoter, we next performed chromatin immunoprecipitation assays (ChIP) using cell lysates from differentiated EBs. Wild-type or *Foxh1*$^{-/-}$ ES cell lines overexpressing Flag-Gsc were induced to differentiate into EBs. After 5 days of differentiation, EBs were collected and protein–DNA complexes were fixed by formaldehyde treatment and immunoprecipitated with either control or anti-Flag antibody. Binding of Flag-Gsc to the region of the *Mixl1* promoter encompassing the Foxh1 site was then assessed by QPCR. Efficient amplification of the target *Mixl1* promoter fragment was detected in *Foxh1* wild-type EBs as compared to EBs derived from Foxh1-null cells (Figure 7C). No amplification of a control DNA fragment, located 2.5 Kb away from the Foxh1-binding site, was detected, although efficient amplification of the DNA fragment was observed in total cell lysates (Figure 7C and data not shown). Thus, our results demonstrate that Gsc is recruited to the endogenous *Mixl1* promoter and that this is dependent on Foxh1. Taken together, our results indicate that Gsc binds to the *Mixl* promoter to downregulate the expression of *Mixl1*, and that this binding and repressive activity requires Foxh1.

Discussion

Foxh1 negatively regulates Mixl1 expression in mouse embryos

The mouse *Mix-like* gene, *Mixl1*, is essential for normal gastrulation and for proper development of the node, notochord, axial mesendoderm, heart and gut (Hart *et al*, 2002). In *Xenopus* and zebrafish, the related *Mix/Bix* family members play pivotal roles in early development, functioning in Nodal-like signaling pathways to induce and specify mesoderm and endoderm (Chen *et al*, 1997; Hart *et al*, 2002; Poulain and Lepage, 2002). Whereas the requirements for induction of *Mix/Bix* genes in frogs and fish have been extensively studied, little is known of the molecular mechanism controlling the expression of the mouse *Mixl1* gene. Our phylogenetic analysis of the *Mixl1* promoter revealed the presence of a conserved DNA-binding site for the forkhead protein Foxh1, a positive transcriptional mediator of Nodal-like signals in early embryos (Attisano *et al*, 2001). However, rather than promoting gene expression, we show that Foxh1 functions as a negative regulator of *Mixl1* transcription and that association of Foxh1 with the homeodomain protein Gsc is required for this repressive effect. Thus, we propose that positive and negative regulation of target gene transcription by Foxh1, a key mediator of Nodal signaling, contributes to refining cell fate decisions in the primitive streak.

Our initial studies (this paper) and those of others (Hart *et al*, 2005) using mammalian tissue culture cells, have shown that Foxh1 binds to the *Mixl1* promoter and can cooperate with Smads to mediate Nodal-dependent signaling. This is consistent with reports defining a requirement for Nodal-like signaling for the induction of *Mix/Bix* family members such as *Xenopus Mix.2*, and *Mix.3/Mixer* (Chen *et al*, 1997; Henry and Melton, 1998) as well as zebrafish *ogx9/mezzo* and *bonnie and clyde (bon)* (Poulain and Lepage, 2002) and more specifically via Foxh1 for *Mix.2* (Chen *et al*, 1997). In contrast, our *in vivo* analysis of embryos lacking Foxh1 revealed prominent expression of *Mixl1*, clearly indicating that in mice Foxh1 is dispensable for induction of *Mixl1* expression. Interestingly, use of morpholinos to knock down maternally expressed FAST1 (i.e., Foxh1) in *Xenopus* oocytes before the onset of zygotic gene expression did not significantly affect *Mix.2* or *Mix.3/Mixer* expression (Kofron *et al*, 2004). Furthermore, reduction of both FAST1 and the *Xenopus*-specific *Foxh1*-related gene, FAST3, in animal caps dissected from morpholino-injected early gastrulae, similarly revealed minimal effects on *Mix.1/Mix.2* expression (Howell *et al*, 2002). Thus, elimination of Foxh1 in either frogs or mice reveals that Foxh1 is not required for the initial induction of *Mix*-like gene expression. Instead, our data indicate that Foxh1 acts to downregulate established *Mixl1* expression. Intriguingly, in the late *Xenopus* blastula, maternal Foxh1 is required to prevent ectopic expression of *Xnr5* and *Xnr6* but not *Xnr3* in the ventral vegetal area (Kofron *et al*, 2004). The mechanism for the inhibitory Foxh1 activity was not determined, but the authors speculate that perhaps these promoters might harbor

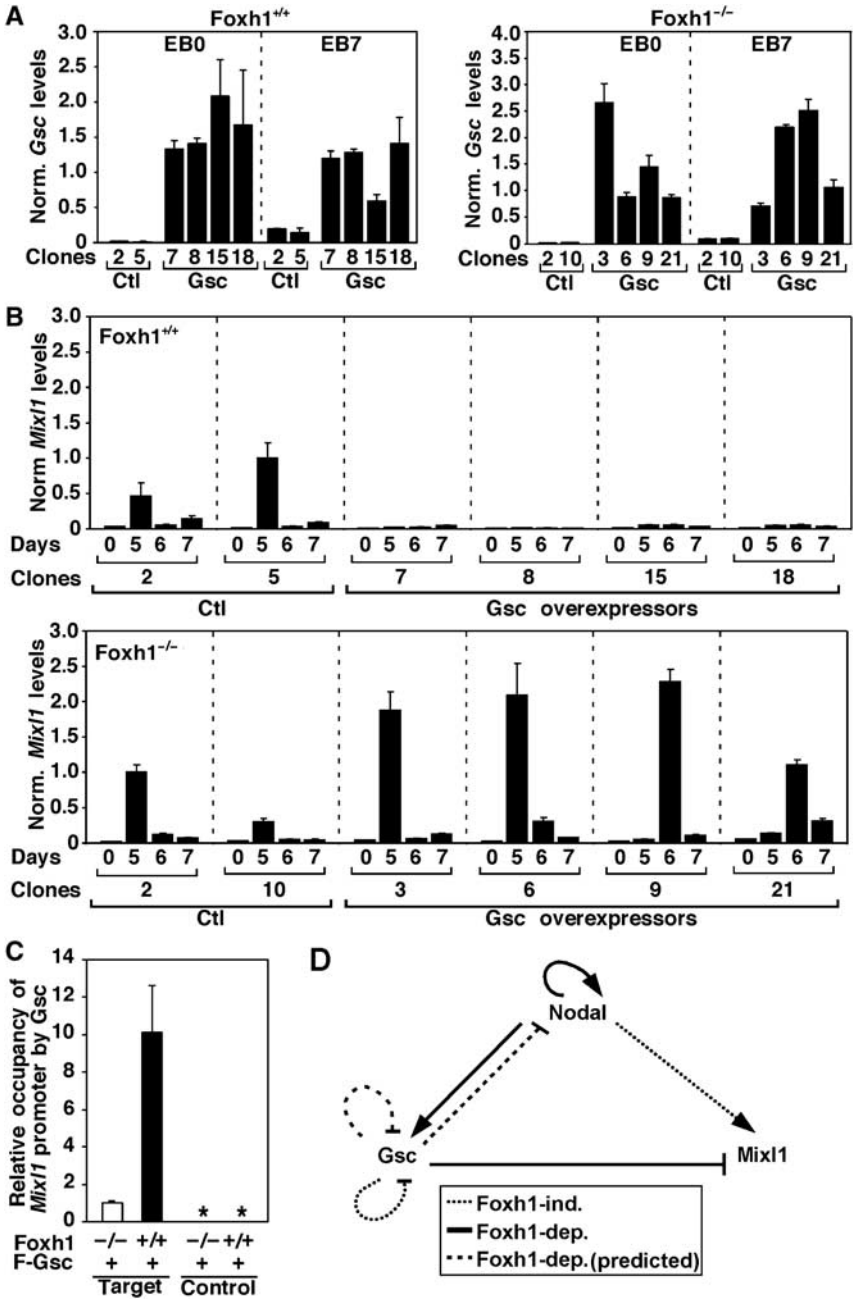

Figure 7 *Mixl1* expression is downregulated in wild-type but *Foxh1*-null EBs overexpressing *Gsc*. Quantitative real–time RT–PCR analysis of *Gsc* (**A**) and *Mixl1* (**B**) expression in control and *Gsc*-overexpressing undifferentiated ES cells (EB0) and EBs cultured for the indicated number of days. Expression was normalized to *Gapdh*. (**C**) EBs were differentiated for 5 days. Protein–DNA complexes were immunoprecipitated with control or anti-Flag antibodies and recovered DNA was analyzed by QPCR with control or target primer pairs. *No amplification detected. (**D**) A model representing positive and negative feedback loops regulating *Mixl1* and *Gsc* expression through Foxh1.

Foxh1-binding sites that function to negatively regulate their expression. Recently, a role for Foxh1 in negatively regulating *flk1* expression in zebrafish was also reported (Choi *et al*, 2007). These observations, together with our data, suggest that Foxh1 may function as a negative regulator of transcription for diverse genes.

Gsc is recruited to Foxh1 and represses Mixl1 expression via histone deacetylases

Negative regulation of certain Foxh1 target genes can occur when the TRF is comprised of Smad3/Smad4 heteromers rather than the positively acting Smad2/Smad4 complex (Labbé *et al*, 1998). In addition, disruption of Foxh1 DNA binding via DRAP1, a corepressor of basal RNA polymerase II transcription, has been reported to prevent Foxh1 target gene expression (Iratni *et al*, 2002). In contrast, our analysis has revealed a distinct mechanism whereby, the homeobox transcription factor Gsc associates with DNA-bound Foxh1 to repress *Mixl1* gene expression. Gsc has been shown to negatively regulate gene expression by binding directly to DNA at specific sites (Danilov *et al*, 1998; Latinkic and Smith, 1999) and by recruiting the corepressor protein Groucho via the N-terminal GEH domain (Jimenez *et al*, 1999). In the case of Mixl, the repressive activity of Gsc is mediated by recruitment

of HDACs. Foxh1 can also recruit the homeodomain-containing protein Nkx2-5 (von Both *et al*, 2004) but, unlike Gsc, recruitment of Nkx2-5 mediates heart-specific induction of *Mef2c* during cardiogenesis (von Both *et al*, 2004). The ability of homeodomain proteins to act as either co-activators or corepressors of forkhead transcription factors has been previously reported (Foucher *et al*, 2002; Kim *et al*, 2003) and may, thus, be a general property of this class of transcription factors.

Nodal regulated gene expression during early development

During early mouse development, Nodal expression is highly dynamic and its activity is tightly regulated. It is first expressed throughout the epiblast and in the visceral endoderm and later is confined to the posterior proximal epiblast, where it marks the site of primitive streak induction (Schier, 2003). Data suggest that at the onset of gastrulation Nodal induces expression of *Gsc* in the primitive streak via Foxh1 (Labbé *et al*, 1998; Hoodless *et al*, 2001; Yamamoto *et al*, 2001), whereas *Mixl1* expression is induced via a Foxh1-independent mechanism (Figure 7D). As gastrulation proceeds, Gsc accumulates in cells fated for the anterior region of the streak, where it is recruited through Foxh1 to the *Mixl1* promoter to repress transcription, thus restricting *Mixl1* expression to the posterior region. Accordingly, in Foxh1 mutant embryos, we observed anterior expansion of *Mixl1* expression. Consistent with this, in *Gsc*-null mice, *Mixl* expression is increased. Thus, Foxh1 functions in a transcriptional regulatory loop that acts through Gsc to negatively regulate *Mixl1.* This mechanism likely reflects part of a much larger transcriptional network that acts to refine gene expression patterning and cell fate decisions in the primitive streak. For instance, the expression of Gsc itself requires Foxh1 (Labbé *et al*, 1998), and thus downregulation of Gsc expression could occur via an autoregulatory loop either by direct interaction with Foxh1 or through direct DNA binding of Gsc to its own promoter as has been previously reported (Danilov *et al*, 1998). Furthermore, Foxh1-dependent enhancer elements present in the first intron of the *Nodal* gene are required for the maintenance and amplification of *Nodal* expression (Saijoh *et al*, 2000). Thus, it will be interesting to investigate whether Foxh1 can also function to negatively regulate *Nodal* expression through the recruitment of Gsc. In *Xenopus,* Nodal-dependent induction has also been reported to occur either through *Mixer* (Germain *et al*, 2000), the *Xenopus* homolog of the human gene Williams–Beuren syndrome critical region 11 (XWBSCR11) (Ring *et al*, 2002) or p53 (Cordenonsi *et al*, 2003), although the role of these modulators has not been examined during early mouse development. The exact mechanisms controlling transcriptional events occurring during mouse gastrulation are likely to have specific spatial, temporal and promoter context requirements and will require further investigation. However, our results indicate that Foxh1, functioning within positive and negative feedback loops may serve to spatially and temporally refine *Gsc* and *Mixl1* activities to ensure appropriate cell fate choices during gastrulation.

Materials and methods

Reporter constructs and transcriptional reporter assays

The *Mixl1* promoter (position −390 to −143 from the translational start site) was amplified by PCR from a mouse *Mixl1* genomic DNA clone (kindly provided by Jonathan J Pearce) and subcloned into the *SacI*/*Bgl*II sites of a modified pGL2-promoter vector (Promega), as described previously (Labbé *et al*, 1998). All mutant *Mixl1*-luc reporters were generated by overlap PCR mutagenesis using primers indicated in Supplementary Table 1. For luciferase assays, HepG2 cells were transiently transfected using the calcium phosphate DNA precipitation method, as described previously (Labbé *et al*, 1998). Unless otherwise indicated, transfections contained 0.083 μg of *Mixl1*-luc reporter, 0.0035 μg of Foxh1, 0.035 μg of each Smad, 0.25–250 ng of Gsc or its derived mutants, 0.1 μg of pCMV-βgal and pCMV5 empty vector to a total of 1 μg per well in a 24-well dish. Trichostatin A (Sigma) in 100% ethanol was added as indicated 18 h before lysis.

Construction of mammalian and bacterial expression vectors

The mature ligand region of Nodal was amplified by PCR and subcloned into a pCMV5 vector containing the Activin pro-region. C-terminal Flag-tagged mouse Cripto was generated by PCR using NIA clone H3029H05 as template. Epitope-tagged Gsc full-length and deletion mutants in pCMV5B were generated by PCR using mouse Gsc cDNA and subcloned in pGEX4T1 for bacterial expression. Flag-Foxh1 ΔN where the first 183 amino acids were deleted was generated by PCR. GST-HDAC1 was generated by PCR using HDAC1 cDNA and subcloned pGEX4T1 for bacterial expression. All primers are listed in Supplementary Table 1.

Gel-shifts, immunoprecipitation, GST pull-downs and immunoblotting

EMSAs were performed as described previously (Labbé *et al*, 1998) except that cell extracts or bacterial fusion proteins were incubated with 30 000 cpm [^{32}P]-labeled DNA before nondenaturing electrophoresis.

For immunoprecipitations and GST pull-downs, COS-1 cells were transfected using the polyethylenimine (PEI; Sigma-Aldrich catalog no. 408727) method. Briefly, for a 100 mm dish, 25 μl of 2 mg/ml of PEI stock was added to 10 μg of DNA made up to 750 μl in serum-free medium. The DNA/PEI solution was vortexed, incubated at room temperature for 5 min and added to cells containing 10 ml of growth medium. Immunoprecipitations, GST pull-downs and immunoblotting were carried out as reported (Labbé *et al*, 1998) using M2 anti-Flag (Sigma) or anti-T7 (Novagen) monoclonal antibodies.

For DNA immunoprecipitations, COS-1 cells were transfected with the appropriate cDNA and *Mixl1*-luc reporter using LipofectAMINE (Invitrogen), and immunoprecipitations were performed as described previously (von Both *et al*, 2004) with the following modifications. For double DNA immunoprecipitation, protein–DNA complexes were immunoprecipitated with 2 μg of Flag antibody or T7 antibody, followed by incubation with protein G Sepharose for 1 h. Protein–DNA complexes were eluted by incubation with 1% SDS, rediluted to a final concentration of 0.1% SDS, and subjected to immunoprecipitation with 2 μg of T7 or Flag antibody overnight. For triple DNA immunoprecipitations, lysates were sequentially immunoprecipitated with 2 μg of T7 antibody for 3 h, followed by an overnight immunoprecipitation with 2 μg of Flag antibody, and a 3-h immunoprecipitation with 2 μg of Y11 rabbit anti-HA antibody (Santa Cruz). Protein–DNA complexes were eluted each time with 1% SDS. For endogenous ChIP, EB cultures were established as described previously (von Both *et al*, 2004) except that ES cells were suspended in 0.05% trypsin and differentiated in bacterial grade Petri-dishes. After 5 days of differentiation, EBs were collected and ChIPs were carried out as described previously (von Both *et al*, 2004), except that EBs were fixed in 1% formaldehyde for 20 min at room temperature and Protein G sepharose beads were blocked with 0.5 mg/ml BSA and 0.2 mg/ml salmon sperm DNA. Levels of *Mixl1* promoter precipitated DNA were analyzed by QPCR using SYBR Green PCR Master Mix (Applied Biosystems) and primers as indicated in Supplementary Table 1. For DNA immunoprecipitation, Ct values were normalized with corresponding DNA input C_t values. Net C_t values were plotted using Comparative C_t method (docs.appliedbiosystems.com/pebiodocs/04303859.pdf). For ChIPs, net C_t values for the anti-Flag immunoprecipitation were corrected with net C_t values of control anti-T7 immunoprecipitation and plotted using the Comparative C_t method.

ES cells and embryoid body differentiation

Flag-tagged Gsc cDNA was subcloned in the episomal expression vector pCAGIP, and this vector was linearized with *Pvu*I and electroporated into the previously described *Foxh1*$^{+/+}$ (no. 9b) and *Foxh1*$^{-/-}$ (no. 3) ES cell lines (Hoodless *et al*, 2001). Individual puromycin-resistant colonies were selected, and *Gsc* mRNA expression was confirmed by quantitative RT–PCR. For *in vitro* differentiation assays, ES cells grown in ES medium (Nagy, 2003) were passaged twice on 0.1% gelatin-coated plates and grown to about 70% confluency. ES cells were trypsinized in 0.05% trypsin, harvested and resuspended in EB medium (ES medium minus LIF). Hanging drop cultures were established as described previously (Spector *et al*, 1998). EBs from 2-day hanging drop cultures were transferred to 24-well ultralow attachement plates at a density of 50 EBs/well. Three days later, EBs were transferred to gelatin-coated six-wells dishes and maintained in EB media until harvesting for RNA extraction.

Quantitative RT–PCR and in situ hybridization analysis of Mixl1 in mouse embryos

Embryonic RNA was isolated using TRIzol reagent (Invitrogen) according to standard procedures from pools of E7.5 embryos genotyped for Foxh1 as described previously (Hoodless *et al*, 2001). *Gsc* mutant embryos were genotyped by PCR as described previously (Belo *et al*, 1998) with DNA extracted from the extraembryonic region of E7.5 embryos. Embryonic RNA from wild-type and *Gsc*-null embryos were isolated using the Nanoprep kit (Stratagene) according to manufacturer's instructions. ES cell and EB RNA were isolated using the RNeasy Mini Kit (Qiagen). RNA samples were treated with DNaseI (Fermentas), primed with oligo p(dT)20 (ACGT Corp.) and reverse transcribed using RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas). QPCR on embryonic samples was performed using SYBR Green PCR Master Mix (Applied Biosystems) and *Mixl1* and *Hprt* primers as indicated in Supplementary Table 1. QPCR on ES and EB samples and wild-type and *Gsc*-null embryos was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) and pre-developed Taqman Gene expression assays (Applied Biosystems) for mouse *Mixl1*, *Gsc* and *Gapdh* using the ABI Prism 7000 or 7900 sequence detection system (Applied Biosystems). Analysis was performed using the Comparative C_t method. Whole-mount *in situ* hybridization analysis of *Mixl1* in mouse embryos was performed as described previously (von Both *et al*, 2004) using a *Mixl1* probe (Pearce and Evans, 1999).

Supplementary data

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

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