FoxH1 represses miR-430 during early embryonic development of zebrafish via non-canonical regulation

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Abstract

Background: FoxH1 is a forkhead transcription factor with conserved key functions in vertebrate mesoderm induction and left-right patterning downstream of the TGF-beta/Nodal signaling pathway. Binding of the forkhead domain (FHD) of FoxH1 to a highly conserved proximal sequence motif was shown to regulate target gene expression.

Results: We identify the conserved microRNA-430 family (miR-430) as a novel target of FoxH1. miR-430 levels are increased in foxH1 mutants, resulting in a reduced expression of transcripts that are targeted by miR-430 for degradation. To determine the underlying mechanism of miR-430 repression, we performed chromatin immunoprecipitation studies and overexpression experiments with mutant as well as constitutive active and repressive forms of FoxH1. Our studies reveal a molecular interaction of FoxH1 with miR-430 loci independent of the FHD. Furthermore, we show that previously described mutant forms of FoxH1 that disrupt DNA binding or that lack the C-terminal Smad Interaction Domain (SID) dominantly interfere with miR-430 repression, but not with the regulation of previously described FoxH1 targets.

Conclusions: We were able to identify the distinct roles of protein domains of FoxH1 in the regulation process of miR-430. We provide evidence that the indirect repression of miR-430 loci depends on the connection to a distal repressive chromosome environment via a non-canonical mode. The widespread distribution of such non-canonical binding sites of FoxH1, found not only in our study, argues against a function restricted to regulating miR-430 and for a more global role of FoxH1 in chromatin folding.

Keywords: Gastrulation, Nodal signaling, FoxH1, miR-430, MZT, Chromatin folding

Background

Gastrulation and hence formation of the three germ layers endoderm, mesoderm, and ectoderm are a key step in development from single-cell to multicellular organism. Among other pathways, dose-dependent signaling by the TGF-beta factor Nodal is central for germ layer induction and patterning. Throughout the vertebrate phylogeny, the loss of Nodal signaling leads to loss of endodermal and mesodermal cell fate [1–3]. A central step in Nodal signaling is the ligand-induced phosphorylation and subsequent nuclear translocation of Smad2. Within the nucleus, Smad2 forms a complex with Smad4 and interacts with different transcription factors, each targeting the SMADs to a different set of target genes ([4–6], reviewed in [7]). The transcription factor FoxH1 was the first SMAD-interacting protein to be identified. Genetic studies established conserved requirements for FoxH1 in mediating Nodal activities related to mesoderm induction and left-right patterning [8–12]. Consistent with the genetic requirements, molecular studies identified several mesoderm-related transcription factors (e.g., tbx6, foxA3, pitx2) and signaling molecules (e.g., FGF8, FGF3, Wnt11) as conserved FoxH1 targets. FoxH1 is further required for modulating Nodal signaling intensity, range, and duration by directly regulating Nodal and Lefty encoding genes, with the latter being a Nodal antagonist [8, 13–16].

FoxH1 binds chromatin via the conserved canonical (CAN) consensus motif AATMCACA. CAN binding sites critical for mediating Nodal signals are further characterized...
by close-by SMAD binding sites (SBS) [8, 16–19]. Currently, it is not entirely clear whether FoxH1 can bind chromatin without the Smad2/3 interaction. Recent work in pluripotent mouse P19 cells showed that binding of FoxH1 to SBS-associated CAN enhancers is strictly Smad2 dependent [20]. However, studies in human embryonic stem cells and very recent findings in frog embryos showed that FoxH1 can be pre-positioned at specific enhancers and that the subsequent interaction with activated Smad2 can induce a release or a switch from repression to activation of associated genes [9, 21].

The FoxH1 protein has two functionally well-defined domains, an N-terminal forhead domain (FHD) for DNA-binding and a C-terminal domain required for interaction with activated SMADs (SID, Smad interaction domain). An additional medially positioned conserved EH1 motif was recently shown to mediate Nodal-independent transcriptional repression via interaction with Groucho/TLE factors [22, 23]. Genetic analyses in zebrafish revealed slightly different phenotypes in foxH1 mutant embryos lacking either a functional FHD (sur/schmalspur) or the SID (mid/midway). In maternal and zygotic (MZ) mutant embryos, both types of foxH1 mutations cause defects in axial mesoderm formation, a loss of ventral neural fates, ventral body curvature, and synophthalmia. However, while axial mesoderm is partially disrupted in MZsur (= foxH1m786) mutants [10, 24], it is missing in MZmid (= foxH1pr1) mutants and the embryos display additional defects in somite patterning. Since MZmid mutants can be rescued to an MZsur like phenotype by injection of sur mutant foxH1m786 mRNA, it was suggested that DNA-binding defects in sur mutants might be partially compensated by the interaction of the FoxH1m786/Smad complex with other DNA-binding proteins [11]. Consistent with this option, canonical FoxH1 binding sites are frequently found in association with binding sites for other Smad2-interacting transcription factors such as Eomes and Mixl1 [12].

Interestingly, previous ChIP analyses revealed that the majority of FoxH1 peaks in fact lack CAN FoxH1 consensus motifs [12, 25]. The molecular nature and functional relevance of these peaks, termed non-canonical (NC) peaks, have not been addressed so far. In this study, we identified the miR-430 loci as targets for NC interaction with FoxH1 and we show that this interaction is relevant for FoxH1 dependent miR-430 repression.

In zebrafish, 415 different miRNAs in 44 families have been found so far ([26], overview in [27]). Among those, three isoforms of miR-430 could be distinguished (a, b, c), which differ in their central and terminal nucleotide sequence, but are homologous in the 3’ region and target recognition site at the 5’ end ([28–30], overview in [27]). The majority of miR-430s are transcribed from a large cluster on chromosome 4 that contains more than 50 copies of miR-430 [31]. Like most vertebrate miRNAs, miR-430 are also transcribed as long primary transcripts (pri-miR-430). These transcripts undergo a maturation process: cleavage into hairpin-structured precursor miRNA (pre-miR-430) by a protein complex of Drosha and its cofactor DGCR8, transport into cytoplasm, generation of a 22-nucleotide long imperfect RNA duplex by Dicer and cofactors, and loading into the RISC (RNA-induced silencing complex) ([31]; overview in [27]). miR-430 family members are highly expressed at the onset of gastrulation (5 h post fertilization, 5hpf) and stay expressed during gastrulation and somitogenesis [32–36]. The early embryonic expression of miR-430 is required for clearance of maternal RNAs at the time when zygotic expression starts (midblastula transition; Additional file 1: Figure S1 [30, 37]). To this end, miR-430 targets hundreds of transcripts for deadenylation and degradation [34]. Furthermore, miR-430 is involved in fine tuning and regulation of Nodal signaling, namely by targeting mRNA encoding the Nodal agonist nodal-related 1 (nrd1; squint) and the antagonist lefty2 [36, 38, 39].

Here we show that NC-binding of FoxH1 at miR-430 loci correlates with a downregulated expression of all three miR-430 subtypes and with a downregulation of miR-430-targets in the MZsur mutant. We further show that the FHD and the SID-containing C-terminus of FoxH1 are both required for miR-430 repression and that mutant forms lacking function of one of these domains dominantly interfere with this activity. Our data demonstrate a novel physiologically relevant requirement for FoxH1 in miR-430 regulation, and they provide a first insight into the mechanism underlying non-canonical gene regulation by FoxH1. The results imply a hypothetical model of indirect FoxH1 activity on miR-430 loci that includes chromatin folding effects.

**Results**

ChIP-seq and microarray revealed non-canonical FoxH1 regulation of miR-430

To gain insight into the function of FoxH1 during early embryonic development, we performed a combination of expression and ChIP-seq analysis on 6hpf epiboly-stage zebrafish embryos. The ChIP analyses [40] revealed 8,342,137 high-quality reads that could be mapped to more than 16,000 peaks ($p < e^{-4}$) in the zebrafish genome (Zv9/danRer7). De novo motif prediction algorithms from two different toolsets (MEME-ChIP and RSAT) [41–43] confirmed the presence of the well-established FoxH1 consensus motif in about 14% (2421; Additional file 2: FoxH1-peaks and Annotation-50 k + 20 k) of these peaks [8, 16–19]. Correlation analysis of FoxH1 peaks with published Smad2 binding regions (SBRs) [44] further confirmed overlap between SBRs and FoxH1 peaks containing the consensus motif (termed CAN-peaks) but not with peaks lacking the motif...
To correlate chromatin binding with requirements for FoxH1-dependent gene regulation, we performed microarray-based transcriptome analyses. Comparison of expression data from 6hpf epiboly-stage wild-type (biological duplicates) embryos with that of MZsur mutants (biological triplicates) revealed a total of 1575 genes with at least twofold changes in expression (Fig. 1b; Additional file 4: Microarray vs. ChIP). To identify potentially direct FoxH1 targets, all regulated genes were analyzed for the presence of FoxH1 peaks within 50 kb upstream and 20 kb downstream of the transcriptional start site (TSS). FoxH1 peaks were found for 551 of the genes with twofold changed regulation in MZsur mutants (Fig. 1c; Additional file 4: Microarray vs. ChIP). From those, 127 genes were associated with Smad2/FoxH1 co-binding, which included most of the previously reported Nodal/FoxH1-activated genes (nmdr1, lft2, pitx2, flh, foxa3, and lhx1a) [3]. Surprisingly, the majority of FoxH1-regulated genes associated with NC-peaks. As FoxH1 has been mainly associated with gene-activating functions, we expected direct targets to be mainly downregulated in the mutants. However, similar numbers of down- and upregulated peak-associated genes in MZsur and MZoop mutants suggested that CAN- and NC-FoxH1 peaks both contributed to Nodal/FoxH1-dependent gene activation and repression (Fig. 1c).

Among NC-peaks, we noted several prominent peaks within the miR-430 “a, b, c” repeat cluster (Additional file 2: FoxH1-peaks and Annotation-50 k + 20 k; Fig. 2a [45]). To determine potential requirements of FoxH1 in miR-430 regulation, we analyzed expression of mature miR-430 isoforms (a, b, c) by using a poly-A tailing approach [47]. Consistent with a general role of FoxH1 in miR-430 repression, all three miR-430 isoforms were significantly increased in gastrula stage MZsur mutant embryos (Fig. 2b; Additional file 5: Individual qPCR values). Increased miR-430 transcription in MZsur as compared to wild type was further confirmed by direct RT-qPCR analyses of selected subsets of pri-miR-430 transcripts for the three isoforms (Fig. 2c; Additional file 5: Individual qPCR values).

To confirm that the repressive effect is specific to FoxH1, we further tested whether injection of foxH1WT mRNA into MZsur embryos would lower pri-miR-430 levels. Consistent with a specific function, the injected foxH1WT mRNA reduced pri-miR-430 levels (Fig. 2c; Additional file 5: Individual qPCR values).

Next, we asked whether miR-430 regulation by FoxH1 is Nodal signaling-dependent. Consistent with a role of Nodal signaling in miR-430 repression 7hpf MZoop mutants (Fig. 2d; Additional file 5: Individual qPCR values) showed increased pri-miR-430 transcript levels. However, this increase was less prominent as compared to MZsur mutants. As a control, we verified that the canonical Nodal target goosecoid (gsc) is reduced in MZoop and MZsur mutants (Fig. 2e; Additional file 5: Individual qPCR values). Together, these data reveal a novel requirement for FoxH1 in pri-miR-430 repression, and they suggest that the sur mutation has Nodal signaling-independent effects on miR-430 regulation.

**Mutations in the FHD and SID of FoxH1 dominantly interfere with miR-430 regulation**

The lack of canonical binding motifs within the miR-430 associated peaks suggests that FoxH1 is not directly interacting with these sites. To further test this, we performed RNA injections with FHD-containing constructs (Additional file 6: Overview of FoxH1 constructs) previously shown to cause robust activation (FHD-VP16) or repression (FHD-EN) on CAN targets (Fig. 3a; Additional file 5: Individual qPCR values) [10, 11]. Analysis of selected CAN-FoxH1 targets in injected embryos showed the expected up- and downregulation by injections of FHD-VP16 and FHD-EN, respectively, and virtually no effect following injection of a FHD-GFP control construct (Additional file 7: Figure S2). In contrast, and consistent with an indirect regulatory effect, all three types of injections (FHD-VP16, FHD-EN and FHD-GFP) caused increased pri-miR-430 levels (Fig. 3a; Additional file 5: Individual qPCR values) with FHD-GFP showing the strongest activity not only in wild type but also in MZsur mutants.

The data suggest that the common FHD-containing N-terminus of FoxH1 in these constructs interferes with pri-miR-430 repression in a dominant negative manner. To clarify which parts of the proteins mediate the dominant negative activities, we tested additional GFP fusion constructs (Fig. 3b; Additional file 5: Individual qPCR values, see also Additional file 6: Overview of FoxH1 constructs). In particular, we addressed functions of the conserved C-terminal region that includes the EH1 motif and of the non-conserved N-terminal part. We found that the 88 most N-terminal amino acids of FoxH1 (5′-FoxH1) were not sufficient to influence pri-miR-430 levels and that the removal of the EH1 motif (FHD(EH1)_GFP) did not prevent interference with pri-miR-430 repression. These data suggest that the FHD, while not directly interacting with the miR-430 loci, is critical for miR-430 regulation. To further test if this critical activity depends on the DNA-binding properties of the FHD, we injected a sur768 mutant variant of FHD-
Fig. 1 (See legend on next page.)
GFP (termed FHDm768-GFP; Additional file 6: Overview of FoxH1 constructs). The single amino acid exchange in FoxH1m768 has previously been shown to specifically prevent binding to FoxH1-consensus motifs [10]. Since FHDm768-GFP had no major effect on the regulation of pri-miR-430 (Fig. 3b; Additional file 5: Individual qPCR values), this suggests that CAN-DNA interaction is important for the dominant negative function of FHD-GFP.

The data imply that SID-depleted fusion proteins with a wild-type FHD bind to an undefined CAN binding site and thereby prevent repressive interactions of this CAN-site with the mir-430 loci. This further suggests that the SID-containing C-terminus of FoxH1 might be required for mediating contact with the mir-430 loci. In this case, overexpression of the FoxH1m768 protein, with an intact SID but defective FHD, should also interfere with pri-miR-430 repression by occupying the mir-430 loci-specific contact site. Consistent with this notion, injection of foxH1m768 mRNA caused twofold increased expression levels of pri-miR-430 in wild type and surprisingly also in MZsur embryos (Fig. 3c; Additional file 5: Individual qPCR values). To directly address whether a FHD-depleted FoxH1 protein is able to interact with the mir-430 loci, we tested a fusion construct between the C-terminal part of FoxH1 and the EN-repressor domain (termed SID-EN; Additional file 6: Overview of FoxH1 constructs). In contrast to foxH1m768 that caused increased pri-miR-430 levels, the injection of SID-EN resulted in strongly reduced pri-miR-430 levels (Fig. 3d; Additional file 5: Individual qPCR values). Since SID-EN lacks known DNA-binding motifs, we reasoned that its interaction with the mir-430 loci might be mediated through interaction with SMAD2/3 proteins. However, downregulation of pri-miR-430 by SID-EN was also seen in MZoep mutant embryos (Fig. 3d; Additional file 5: Individual qPCR values) which lack phosphorylated and thereby nuclear SMAD proteins. These results suggest a SMAD-independent interaction of the SID with the mir-430 loci.

Together, these data suggest that FHD and the SID-containing C-terminus of FoxH1 have unique functions in mediating repressive contact between distal chromatin structures and that injection of FoxH1 proteins lacking one of these domains interfere with this activity in a dominant negative manner.

**Injection of FHD-GFP increased severity of MZsur mutant phenotype**

In previous studies, the injection of foxH1m768 mRNA into MZmid mutants was found to convert the more severe MZmid phenotype into the weaker MZsur phenotype. It was therefore suggested that FoxH1pr1 might be inactive while FoxH1m768 retains residual FoxH1 activities via the intact SID [11]. Our studies show that both types of mutations interfere with mir-430 regulation. Since the loss of the SID in FHD-GFP, resembling FoxH1pr1, has stronger effects on mir-430 regulation as compared to FoxH1m768, we reasoned that the stronger phenotype of MZmid could be associated with the stronger dominant-negative effects of the SID-truncated protein. Consistent with this notion, we found that injection of FHD-GFP into MZsur mutants strongly increased the expressivity of the mutant phenotype. WISH analyses for the axial marker col2a1a in 24hpf embryos confirmed a major effect of FHD-GFP on notochord formation in MZsur mutants (Fig. 4a–d). MZsur mutants lack the floor plate and hypochord and show some variable notochord defects in the tail and anterior trunk [11]. Still, these embryos showed nearly continuous col2a1a staining in the trunk notochord, while more than 60% of FHD-GFP injected MZsur embryos displayed only patchy col2a1a signals. Analyses of foxa2 expression in gastrula stage embryos indicated that FHD-GFP prevents formation of early axial mesoderm in MZsur (Fig. 4e–h). We found that the number of foxa2-positive axial cells was strongly reduced in FHD-GFP injected MZsur as compared to control and un-injected MZsur embryos. Notably, FHD-GFP also caused broadened axial signal in about 50% of the injected control embryos, possibly indicating defects in gastrulation movement. The foxa2 stains also suggested that FHD-GFP has mild effects on endoderm formation (arrowheads in Fig. 4e–h). Further analyses of the endoderm marker sox17 confirmed a slightly reduced amount of endoderm cells, and in addition, they revealed strongly reduced number of forerunner cells in FHD-GFP injected embryos (Fig. 4m–t; Additional file 8: Statistical analysis of forerunner cells).

As the severity of foxH1 mutant phenotype appears to correlate with the level of mir-430 upregulation, we next tested whether an injection of mir-430 blocking dre-mir-430 morpholinos (MOs) [36] is able to attenuate
Fig. 2 FoxH1 regulates miR-430 expression in a negative manner. 

a Genomic organization of the miR-430-containing region on chromosome 4. FoxH1 peaks localize to the miR-430a, b, and c repeat cluster. Enlargements indicate positions of transcripts for miR-430a (red), miR-430b (green) and miR-430c (blue) which were directly extracted from the UCSC browser [45].

b Temporal changes of mature miR-430 levels in MZsur embryos between 4hpf and 9hpf as measured by RT-qPCR relative to wild-type embryos. Starting at 5hpf, levels of mature miR-430 are increased in the mutant.

c The upregulation of pri-miR-430 forms in MZsur mutants can be partly rescued by injection of foxH1WT mRNA. Addition of FoxH1 in wild types does not influence the expression level.

d All three pri-miR-430 isoforms are upregulated in MZsur mutants at the indicated time points. Loss of Nodal signaling in the oep mutant does not significantly change expression levels. All data normalized to respective wild type control.

e Control RT-qPCR shows for MZsur and MZoep mutants a highly significant downregulation of gsc expression as formerly shown [11, 46]. All qPCR data were calculated from biological triplicates except for b (2 biological replicates). Error bars indicate standard error (SEM). Bio-Rad CFX Manager 3.1 software was used to calculate relative normalized expression, standard error, and significance (n. s. p ≥ 0.05; *p < 0.05; **p < 0.01; ***p < 0.001). For individual values, see also Additional file 5: Individual qPCR values.
Fig. 3 (See legend on next page.)
the phenotype of MZsur and FHD-GFP injected MZsur (Fig. 4i–l). However, the morpholino injection had the opposite effect and caused strongly reduced and even absent axial foxa2 signals in MZsur and FHD-GFP injected MZsur, respectively (Fig. 4k, l).

**FoxH1 regulation of mir-430 prevents maternal clearance**

Finally, we asked whether regulation of mir-430 by FoxH1 is functionally relevant in the developing embryo. Based on previous studies, we expected that the increased mir-430 levels in MZsur mutants or RNA-injected embryos would have effects on maternal transcript clearance [34, 47]. To determine functions of FoxH1 in maternal transcript clearance, we analyzed expression of two maternally deposited mir-430 targets, namely cd82b [48, 49] and jade1 [50, 51]. Expression analyses of 4–5hpf embryos by RT-qPCR (Fig. 5a, b; Additional file 5: Individual qPCR values) and WISH (Fig. 5c–f) show that transcripts levels of cd82b and jade1 are significantly lower in MZsur mutants as compared to wild-type embryos and that this phenotype is reduced by injection of foxH1WT mRNA. To exclude a possible direct regulation of these genes by FoxH1 or by canonical FoxH1 targets, we also analyzed expression in FHD-VP16 injected embryos. The similar cd82b and jade1 levels in un-injected and FHD-VP16 injected MZsur embryos emphasize the indirect connection between FoxH1 and cd82b/jade1 and are consistent with the important intermediary role of pri-miR-430 upregulation, as seen following injection of FHD-VP16 (Figs. 3a and 5a, b). In summary, this shows that FoxH1-dependent repression of mir-430 is biologically relevant for the regulation of mir-430 target genes during maternal clearance.

**Discussion**

Here we describe a novel role for the transcription factor FoxH1 in negative regulation of mir-430, and we provide evidence for a regulatory mechanism that is different from the previously described canonical role of FoxH1 in TGF-beta/Nodal signaling.

More than 20 years ago, FoxH1 was identified as the first Smad2-interacting transcription factor mediating transcriptional gene activation downstream of TGF-beta/Nodal signaling. Since then, FoxH1 has become established as a central transcriptional regulator of Nodal-induced mesendoderm induction and left-right patterning. Furthermore, a broad panel of genes were identified that are directly regulated by FoxH1/Smad2 via binding to proximal CAN binding sites [8–19]. Only recently, genome-wide ChiP analyses provided a more complex picture of highly dynamic FoxH1 chromatin occupancy in the early embryo. While these studies confirmed the importance of Nodal/Activin-induced CAN-interactions, they also revealed a much larger number of possibly Nodal/Activin independent NC- DNA interactions of unknown function [12, 25].

Our analyses now provide first evidence for a functional relevance for NC-interactions in gene regulation. Among other NC-targets, we identified the mir-430 cluster as a novel in vivo target that is repressed by NC-FoxH1 interaction. Consistent with a Nodal independent function, we find that MZsur mutants display a stronger up-regulation of mir-430 as compared to Nodal signaling-deficient MZoep mutants (Fig. 2). Functional relevance for pri-miR-430 repression by FoxH1 was confirmed by showing that the increased pri-miR-430 levels in MZsur mutants correlate with a reduction of maternal transcripts that are targeted by miR-430 for de-adenylation and degradation. Our data suggest that the ubiquitous distribution of FoxH1 in the early embryo is required to restrict the level of miR-430 induction at the onset of zygotic gene expression. The patterned zygotic expression of FoxH1 during gastrulation and early somitogenesis [10] further implies that FoxH1 in addition to its role in mediating Nodal signaling might function in spatio-temporal controlled attenuation of miR-430-dependent transcriptional silencing or mRNA clearance. Importantly, miR-430 is dampening not only maternal, but also a large number of zygotically expressed transcripts with various functions in early embryogenesis. Interestingly, this includes lft2 and ndr1, which are both also directly regulated by CAN-FoxH1/Smad2-mediated gene activation [36, 38, 39]. Since both mechanisms for lft2 and ndr1 regulation, CAN-FoxH1-dependent feedback-activation and miR-430-mediated transcript
**Fig. 4** (See legend on next page.)
(See figure on previous page.)

**Fig. 4** FHD-GFP interferes with severity of the MZsur mutant phenotype. **a–d** Wild-type (a, b) and MZsur mutant (c, d) embryos at 24hpf. **col2a1a** in-situ staining in wild-type (un-injected control (a) or injected with FHD-GFP (b)) shows wild-type notochord of expected width (white brackets in enlarged sections a' and b'). In un.injected MZsur embryos the width is reduced (c). Injection of FHD-GFP in MZsur mutants enhances the phenotype (d/d'), note reduced size and additionally discontinuity of staining. **e–l** foxa2 in-situ hybridizations show reduction of axial mesoderm formation in MZsur mutants. Injection of FHD-GFP causes a broadened axial signal in 50% of wild type embryos, but no reduction of axial cells (f), and strengthens the effect in 60% of the MZsur mutants (h). dre-miR-430 morpholinos (MOs) massively reduce axial foxa2 signals in both genotypes (l, k). Co-injection of FHD-GFP and MOs also results in a decreased staining (j, l) when compared to FHD-GFP injection (f, h). Percentage of embryos showing the same phenotype as in the image is given (upper right). **m–t** sox17 in-situ hybridizations show only slight reduction of endoderm after injection of MOs (q–t). Number of forerunner cells (black arrow) is reduced in MZsur control and after MOs injections (o, q, s). FHD-GFP lead to complete loss of forerunner cells (black arrow) in the majority of MZsur embryos (p, t; see also Additional file 8: Statistical analysis of forerunner cells). Numbers of sox17-positive cells seen in dorsal view and standard deviation are given (upper right) as well as number of analyzed embryos (n) (lower right). Size bars: 200 μm. y value was changed to 0.8 in each picture.

**Fig. 5** FoxH1 blocks maternal clearance of cd82b and jade1. **a, b** RT-qPCR analysis of cd82b (a) or jade1 (b) in embryos at 50% epiboly with indicated genetic background. Massive reduction of expression is shown in MZsur mutants which cannot be rescued by injection of FHD-VP16 mRNA but is rescued via injection of foxH1<sup>WT</sup> mRNA. Error bars indicate standard error (SEM) from 2 biological replicates. Calculation of relative normalized expression, standard error, and significance was made with the Bio-Rad CFX Manager 3.1 software (**p < 0.001; n.s. p ≥ 0.05). For individual values, see also Additional file 5: Individual qPCR values. **c–f** WISH for cd82b and jade1 in wild type embryos (c, e) shows staining at sphere stage, but not at shield stage when miR-430 becomes active (c', e'). In MZsur mutants (d, f), weak or no staining is visible for all stages indicating the negative role of FoxH1 in regulating miR-430 activity at early embryonic stages. Size bars 200 μm.
decay, are critical for balancing Nodal signaling, our data hint for a complex multilayered role of FoxH1 in directly and indirectly controlling and mediating Nodal activities in the early embryo. For future studies, it will be important to further characterize direct and indirect activities of FoxH1 on Nodal signaling and to study compensatory interactions between these activities.

MZsur and MZmid embryo phenotypes may not reflect complete loss of FoxH1

Our data suggest that the currently available foxH1 mutants sur and mid both can interfere with gene regulation and that some aspects of the MZsur and MZmid mutant phenotypes might not be seen in a truly null allele. In a previous study, it was shown that the stronger MZmid phenotype is rescued to an MZsur-like phenotype by an injection of foxH1m768 but not by foxH1mid RNA. Since injection of foxH1mid RNA in wild-type embryos caused no obvious defects, it was suggested that FoxH1m768 retains residual activity that is missing in FoxH1mid [11]. Our results confirmed a generally normal morphology of wild-type embryos injected with the foxH1mid-like FHD-GFP mRNA, while they also revealed a broadened notochord in these embryos during gastrulation. Most relevant, we find that injection of FHD-GFP converted MZsur embryos into more severe MZmid-like embryos (Fig. 4). This notion is also consistent with the observed increase of miR-430 levels by FHD-GFP not only in wild type but also in MZsur mutants. Therefore, our data hint for a more complex mechanism in which FoxH1m768 may prevent or attenuate the stronger dominant-negative or possible neomorphic effects of FoxH1mid. Interestingly, we also noticed that FHD-GFP has a stronger effect on miR-430 regulation as compared to FHD-VP16 and FHD-EN (Fig. 3a). Since the corresponding sur-mutant FHDm768-GFP is inactive, we consider FHD-GFP activities as specific to the FHD. Possibly, the robust activation and repression of CAN-FoxH1 targets by FHD-VP16 and FHD-EN, respectively, is able to compensate for aspects seen in FHD-GFP injections.

While we focused on miR-430 regulation to determine functions of the different FoxH1 domains, our combined ChIP and expression data hint for a much broader role for NC-FoxH1 interactions in gene regulation. Consistent with this notion, we propose that the changed miR-430 levels are not the major cause for the phenotypic differences between MZsur and MZmid. In case of a primary cause, the morpholino knock-down of miR-430 should have reduced mesoderm defects in FHD-GFP injected MZsur. Instead, a strong reduction or complete loss of axial mesoderm was observed in these embryos (Fig. 4i–l). Since miR-430s target hundreds of mRNAs, the morpholino injections might cause a dominant phenotype that overrides the expected axial mesoderm rescue by miR-430 reduction. However, in wild-type embryos, injection of dre-miR-430 MOs was shown to reduce Nodal signaling by causing premature and increased translation of Lefty proteins [36], see also Fig. 4i). While the complete loss of axial mesoderm in FHD-GFP and morpholino co-injected MZsur is consistent with a reduction of Nodal signaling by Lefty proteins, the strongly reduced lft1/2 mRNAs levels in MZsur mutants argue against such a mechanism [36]. In this context, the large number of NC-FoxH1 peaks suggest that FHD-GFP, similar to its effect on miR-430 expression, might interfere with other regulators of axial mesoderm formation. Consistent with this notion, our data suggest NC-interaction with Wnt, FGF, and retinoic acid signaling components (Fig. 4; Additional file 2: FoxH1-peaks and Annotation-50 k + 20 k).

In conclusion, these studies show that for a detailed understanding of the early molecular events underlying mesendoderm formation, it is important to have a true null allele for FoxH1. In corresponding mutants, overexpression of distinct FoxH1 variants could be used to separate CAN- and NC-FoxH1 functions and to determine the molecular factors responsible for the phenotypic differences between MZsur and MZmid [10, 11, 24].

A new hypothetical model for FoxH1 regulation of miR-430 locus

The data generated in this study suggest that miR-430 repression by FoxH1 requires interaction of FoxH1 with at least two distinct genomic loci (Figs. 1 and 3). They also suggest that two major protein domains of FoxH1, the FHD and the SID, have specific functions in mediating these interactions and that this activity is independent of the central EH1 domain, which was shown to mediate Nodal-independent gene repression via direct interaction with Groucho/TLE co-repressors (Fig. 3) [22, 23]. As SID-EN is able to repress the miR-430 cluster independent of Nodal-signaling (demonstrated in MZoep mutants; Fig. 3d), our data suggest a SID interaction with chromatin that is independent of activated SMAD2.

Therefore, we propose a novel role of FoxH1 in connecting the miR-430 cluster with a distal regulatory element which then mediates repression per se, or another protein or protein complex does the work, as shown in our hypothetical model (Fig. 6a). Accordingly, we further propose that the currently available mutants dominantly interfere with chromatin scaffolding by association with contact sites either at the distal CAN-motif (Mid) or at the miR-430 cluster (Sur). At the onset of MZT, the inhibitory effect of FoxH1 is overcome by an unknown mechanism. Since Nanog, Pou5f1, and SoxB1 are known positive regulators of miR-430 [47], FoxH1 and the changed chromatin scaffolding may prevent binding of these factors to miR-430 enhancer sites or block translation at
these sites. It is not known whether Nanog/Pou5f1/SoxB1 bind at miR-430 loci from the earliest developmental stages or can only occupy these binding sites once the FoxH1-initiated change in chromatin scaffolding disappears.

In the last decade, it has become clear that NC-DNA interactions of transcription factors are often associated with chromatin looping, bringing distantly located DNA domains closer together ([52], reviewed in [53–56]). Therefore, regulatory elements can influence distal genes not only on the same chromosome but also on different chromosomes [57]. Various examples in model organisms such as Drosophila and mice, as well as human cell culture systems, demonstrate high plasticity in the formation of the so-called Extremely Long-Range Promotor-Promotor Interactions (ELRIs), which are associated with the initiation and/or maintenance of gene activity, even during the earliest developmental stages ([57–62], reviewed in [63]). While the data we present are well explained by an involvement of FoxH1 in chromatin folding, further experiments are necessary to confirm this role. Several methods have been described for studying chromatin loops: 3C and derivatives, ChiA-PET, DNA fluorescent in situ hybridization (overview in [64–66]), and CRISPR/Cas9 guided in vivo chromatin labeling [67, 68]. By using one or a combination of these methods, potential FoxH1-associated loops can be identified. Due to the complexity of the miR-430 loci, identifying relevant chromatin loops might not be as straightforward as for single and well-defined CAN-sites of a given transcriptional factor. However, our data also suggest that NC-FoxH1 activities are not restricted to miR-430. Consistent with a possibly more global role of FoxH1 in early chromatin folding, we find that a large number NC-FoxH1 peaks are associated with genes that are either up- (242/6154 peaks) or downregulated (182/6154 peaks) in MZsur (Fig. 1c). In preliminary ChIP-qPCR studies that were set up to confirm selected NC-interactions of FoxH1, we noticed that enrichment of NC-peaks was variable in 4 and 5.5hpf samples while CAN-peaks were similarly enriched in these samples. In this context, the recently reported high number of dynamic FoxH1 chromatin interactions in early Xenopus development provides a hint for a possible conserved mechanism regulating dynamic interaction of FoxH1 at NC-sites [22, 23].

Conclusions
Overall, our data show that FoxH1, in addition to its established function downstream of TGF-beta/Nodal-signals [8–12], can regulate gene expression via indirect SID-mediated chromatin interaction. By identifying the microRNA-430 family members as functionally relevant targets for non-canonical FoxH1 regulation, our data place FoxH1 upstream of miR-430-regulated processes such as maternal clearance and balancing of Nodal

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**Fig. 6** A proposed model for the role of FoxH1 in the regulation of miR-430 activity. a Wild-type FoxH1 causes chromatin looping, preventing expression of miR-430. In MZsur mutants lacking the FHD, this loop is absent because FoxH1 binds only to the miR-430 cluster. If FoxH1 lacking a functional SID or FHD (due to mutation (m786) or replacement (VP16/GFP/EN)) is injected into wild types, higher expression of miR-430 occurs due to loss of looping and inhibitory regulation. b FoxH1 occupies non-canonical (NC) target sequences at miR-430 loci to induce chromatin scaffolding and prevent pri-miR-430 expression. With the onset of gastrulation, FoxH1 leaves the site, allowing miR-430 to become active and repress its targets (T1/T2).
signaling. In addition, the widespread distribution of NC binding sites of FoxH1, found not only in our study, but also in a recent ChIP analysis performed in Xenopus [22], hint for a more global role of FoxH1 in early embryonic chromatin folding. Therefore, this new knowledge of NC-FoxH1 functions provides us with important new insights into the molecular and epigenetic mechanisms underlying early zygotic gene regulation.

**Methods**

**Zebrafish lines**

The following lines were used: MZsur\(^{m768/m768}\) [69], MZoep\(^{m134/m134}\) [70], and Tü (Tübingen) as wild-type control. Embryos were raised at 28.5°C and staged to hours post-fertilization or percentage of epiboly as described [71].

**mRNA injections**

foxH1\(^{WT}\), eGFP-foxH1, FHD-VP16, FHD-GFP, FHD-EN (all described in [10]), FHD\(^{ΔEH1-GFP}\), foxH1\(^{m768}\), foxH1\(^{m768-GFP}\), 5′/foxH1, and S1D-EN were synthesized with the SP6 mMESSAGEmMACHINE in vitro transcription kit following the manufacturer’s protocol (Life Technologies, AM1340). Embryos were injected at 1–2-cell stage with 2 nl (~90 pg) of each mRNA.

**Morpholino injections**

Morpholinos against dre-miR-430a, b, and c were a kind gift from Caroline S. Hill [36]. Stock solutions of 2 mM were diluted for injection at a 1:12 ratio in water. Two nanoliters of a mixture of the three MOs was injected in embryos at the 1–2-cell stage.

**Chromatin immunoprecipitation assay (ChIP)**

We used MZsur\(^{m768/m768}\) embryos for the assay to exclude occupation of FoxH1 binding sites by endogenous FoxH1 proteins.

ChIP experiments were performed as described [72, 73] with minor changes. In total, 6000 foxH1 mutant embryos (MZsur\(^{m768/m768}\)) were injected each at 1–2-cell stage with 8 ng/μl (~30 pg/embryo) of eGFP-foxH1 mRNA, encoding full-length FoxH1 with an N-terminal eGFP tag. Embryos were collected and cross-linked at 6hpf. Chromatin was extracted and sheared into 100–200 bp fragments with the Bioruptor Sonicator (Diagenode, Belgium).

Anti-eGFP antibody (Torrey Pine Biolabs, 0715119, Protein A purified rabbit IgG) was pre-blocked and added (10 μg/1000 embryos) to the cross-linked samples and incubated overnight at 4°C. The next day, Dynabeads (Life Technologies, 10007D) were added to IP down protein-DNA complexes. Validated ChIP-DNA samples were amplified following the protocol of LinDa [74, 75]. The concentration and fragment size of final amplification products were analyzed on a Bioanalyzer High Sensitivity DNA chip.

**High-throughput sequencing and data analysis**

LinDa-amplified ChIP-DNAs were sequenced on an Illumina HiSeq2000 at BGI (https://www.bgi.com/global/sequencing/services/epigenetics/chip-seq/) and produced a total of 24,932,016 raw clean 50 bp single-end reads. These reads were then processed and filtered to remove low-quality reads and artifacts (e.g., residual LinDa adapters) resulting in 12,430,636 high-quality reads which were mapped to the zebrafish genome assembly Zv9/ danRer7 using the BWA (v0.6.2) short read mapper [76].

**Peak calling**

Read alignments were first filtered using samtools to only retain uniquely mapping reads by removing all reads that map to multiple positions with equal alignment score. Then, peaks were called using MACS 1.4.2 [77] setting the \( p \) values cutoff to \( 1 \times 10^{-5} \) and disallowing duplicate tags (reads) at the same position to avoid amplification biases.

This produced 23,724 raw peaks, of which 8446 had a \( p \) value <\( 1 \times 10^{-5} \). These peaks were further filtered such that they did not overlap by more than 50% of their length with a non-UTR Ensembl exon and that they did not contain the following simple repeats: CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT, AGAGAGAGA [GA] AGAGAGAGAGAGA, GAGAGAAA. This finally resulted in 16,908 peaks.

**FoxH1 motif de novo analysis**

The FoxH1 consensus binding motif was obtained by using meme-chip from the MEME suite [41] and the RSAT suite [42]. Sequences from peak regions that did not overlap with repeat-masked regions or genomic regions annotated as exons in the Ensembl database [78] but overlapped with conserved regions in fish and were within 5.5 kb of a TSS, were used as input sequences for motif prediction.

**Gene expression microarray**

Total RNA was extracted and purified from wild-type, MZsur and MZoep embryos using Qiagen RNeasy Mini Kit (74104). Three biological replicates were used for MZsur/MZoep and two for wild type, all of them showed RIN value > 8 when analyzed with Agilent 2100 Bioanalyzer. Agilent’s Zebrafish Oligo Microarrays (V2), P/N G2519F (Agilent Microarray Design ID 019161) were hybridized according to the manufacturer’s protocol. Raw data were analyzed using the Ag4x44PreProcess-Package (“R” package v1.22.0.) and the LIMMA-Package [79, 80]. Background correction mode was “half”; for normalization, the “quantile” option was used. The
“Mean Signal” from the AFE software was used as signal, the “BG Median Signal” as background. Data quality was confirmed using MA plots (data not shown).

**FoxH1 target gene annotations**

Genes were annotated as potential direct targets of FoxH1 by running our in-house developed peak annotation tool adapted from [81] and written in Perl. Briefly, the tool annotates ChIPseq peaks applying the following association rules: each RefSeq gene (Zv9/danRen7) was assigned a basal regulatory domain 5 kb upstream and 1 kb downstream from the TSS. This basal domain could also overlap with basal domains from other genes. The basal domains were then extended in both directions to the nearest non-overlapping basal domain but no more than 50 kb upstream and 20 kb downstream from the TSS. Peaks within such regulatory domains (basal and extended) were associated with the corresponding genes. Genes associated with FoxH1 motif-containing peaks were defined as the “canonical FoxH1 regulated” genes.

**Analysis of FoxH1-SMADs direct targets**

The Smad2 SBRs were obtained from [44]. The original data based on Zv7/danRen5 was transposed to Zv9/danRen7. The transposed SBRs were then re-annotated and associated with genes. Association rules described above (−5 kb−TSS−+1 kb+extension−50 kb−TSS−+20 kb) were applied. These SBRs were then centered at CAN-FoxH1 peaks, and their distribution was plotted in 250 bp bins at the given distance to FoxH1 peaks. For building the random profiles, we generated the same number of random peaks as real peaks and analyzed them correspondingly.

**Genome-scale identification of potential FoxH1/SMAD targets**

The sequences around CAN-Foxh1 peaks were scanned with known SMAD consensus binding motifs derived from Genomatix matbase (www.genomatix.de) and SMADs/FoxH1 pairs in which a SMAD motif (V$Smad.01, V$Smad3.01, and V$Smad4.01) was located no more than 100 bp 5-prime of the FoxH1 motif were extracted and annotated.

**Mature miRNA extraction and RT-qPCR**

The total RNA was extracted and purified by miRNeasy Kit (Qiagen, 217184) from 30 embryos. cDNA synthesis and qPCR were performed following a previously published protocol [82]. The oligo 5′-GCAGGTCAGTT TTTTTTTTTTTTCTACCCC-3′ was used for cDNA synthesis by Superscript III kit (Life Technologies, 18080-051). The miR-103 was used for sample normalization in mature microRNA RT-qPCR. Primer data can be found in Additional file 9: qPCR primer.

**RNA isolation and cDNA synthesis for pri-miRNA**

TRizol (Life Technologies, 15596026) was used to prepare total RNAs following the manufacturer’s protocol. After DNA digestion by RNase-free DNeasy, total RNA was cleaned up by EtOH purification. One microgram RNA was used as template for reverse transcription using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, # K1641).

**Quantitative real-time PCR for pri-miRNA**

For each probe, 10 μl of 1:10 diluted cDNA was mixed with 4 μl HOT FIREPol Eva Green Mix (Solis Biodyne, 08-31-00001), 5.5 μl water, and 0.5 μl of one of the primer mixes in Additional file 9: qPCR primer (100 pmol/μl). Following protocol was used: initial step, 95 °C for 15 min; 40 cycles: 95 °C 15 s, 60 °C 30s, 72 °C 20 s.

The data analysis is described elsewhere [83, 84]. If not indicated, differentially wild-type control was used for normalization. Significance was calculated using the program Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, USA) (n. s. p ≥ 0.05; *p < 0.05; **p < 0.01; ***p < 0.001). Microsoft Excel and CoralDraw were used for graphic composition.

**Whole mount in situ hybridization**

For whole-mount in-situ hybridization, cd82b-, jade1-, col2a1a-, foxa2-, and sox17-mRNA were used for generating digoxigenin-UTP (Roche Applied Science, 11175025910) labeled antisense probe. Staining followed a recently described protocol [85] using NBT/BCIP as dye substrate. For reducing variability, all wild-type or MZ mutant embryos at different stages were stained in the same tube; probe and staining solutions were made for all and distributed to the single tubes.

For photo documentation, embryos were cleared in glycerol and imaged using a Leica CTR6000 microscope equipped with a Leica DFC 300FX camera (Leica Microsystems GmbH; Wetzlar, Germany). CoralDraw was used for assembling figures. Single dorsal view images were used for quantification of sox17-positive cells.

**Additional files**

- Additional file 1: Figure S1. miR-430 expression starts at midblastula transition. (PDF 76 kb)
- Additional file 2: FoxH1-peaks and Annotation-50k+20k. (XLSX 1476 kb)
- Additional file 3: SBR to FoxH1 peaks. (XLSX 1476 kb)
- Additional file 4: Microarray vs. ChIP. (XLSX 778 kb)
- Additional file 5: Individual qPCR values. (XLSX 90 kb)
- Additional file 6: Overview of FoxH1 constructs. (PDF 241 kb)
- Additional file 7: Figure S2. Regulation of CAN-target pitx2 and fgf3 by different constructs. (PDF 303 kb)
- Additional file 8: Statistical analysis of forerunner cells. (XLSX 10 kb)
- Additional file 9: qPCR primer. (XLSX 9 kb)
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Authors’ contributions
DM, PF, HC, and FP conceived and designed the experiments. PF, HC, and FP performed the experiments. PF, HC, FP, DR, RK, and DM analyzed the data. PF and DM wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article, its additional files, and publicly available repositories. Following additional files are included:

Additional file 1. pdf: Figure S1.
Additional file 2. xlsx: FoxH1-peaks and Annotation-50k+20k
Additional file 3. xlsx: SBR to FoxH1 peaks
Additional file 4. xlsx: FoxH1 peaks and Annotation-50k+20k
Additional file 5. xlsx: Macrons vs ChIP
Additional file 6. pdf: Overview of FoxH1 constructs
Additional file 7. pdf: Figure S2. Regulation of CAN-target ptx2 and fgf3 by different constructs.
Additional file 8. xlsx: Statistical analysis of forerunner cells
Additional file 9. xlsx: qPCR primer
Following datasets are available at public repository:

ChIP data on Gene Expression Omnibus (GSE133990).

All data generated or analyzed during this study are included in this published article, its additional files, and publicly available repositories.

Availability of data and materials
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Ethics approval and consent to participate
Maintenance and handling of zebrafish followed the approved applications for animal experiments at the Bundesministerium für Wissenschaft, Forschung und Wirtschaft (BMWF) of the Republic of Austria. BMWF-66.008/0019-I/3b/2013, BMWF-66.008/0009-WF/II/3b/2014, BMWF-66.008/0022-WF/II/3b/2016.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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