FOXA1 is a transcriptional activator of Odf2/Cenexin and regulates primary ciliation

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Primary cilia are sensory organelles essential for embryonic and postnatal development, and tissue homeostasis in adulthood. They are generated in a cell cycle-dependent manner and found on most cells of the body. Although cilia formation is intensively investigated virtually nothing is known about the transcriptional regulation of primary ciliation. We used here Odf2/Cenexin, encoding a protein of the mother centriole and the basal body that is mandatory for primary cilia formation, as the target gene for the identification of transcriptional activators. We identified a consensus binding site for Fox transcription factors (TFs) in its promoter region and focused here on the Fox family. We found transcriptional activation of Odf2 neither by FOXO TFs nor by the core TF for multiciliation, FOXJ1. However, we identified FOXA1 as a transcriptional activator of Odf2 by reporter gene assays and qRT-PCR, and showed by qWB that Foxa1 knockdown caused a decrease in ODF2 and CP110 proteins. We verified the binding sequence of FOXA1 in the Odf2 promoter by ChIP. Finally, we demonstrated that knockdown of FOXA1 affected primary cilia formation. We, thus, showed for the first time, that FOXA1 regulates primary ciliation by transcriptional activation of ciliary genes.

Cilia are microtubule-based structures protruding from the cell surface into the environment. They are assembled at the distal part of the basal body, which is the former mother centriole. Basal bodies are attached to the cell membrane via their distal appendages thus exposing the ciliary axoneme outward. The core axonemal structure of all eukaryotic cilia and flagella consists of nine doublet microtubules arranged in a ring. This organization is known as a 9 + 0 structure and is found in immotile cilia. The presence of two additional singlet microtubules and further accessory structures, such as e.g., dynein arms, characterizes motile cilia that are present in multitudes on the surface of specialized epithelial cells, such as those in the airway, the brain ventricles, and oviducts. Their coordinated movement enables the transport of fluids or cargos across the cell surface. In contrast, immotile cilia are present only once and are hence named primary or mono-cilia. Furthermore, primary cilia are found on the surface of nearly all cells of the body. Primary cilia are essential sensory organelles, acting as mechano- and chemo-sensors and transducing signals in response to fluid flow in kidneys, mechanical load in bone, or ligand-activated receptors. Primary cilia are thus crucial for embryonic and postnatal development, and tissue homeostasis in adulthood. Dysfunctional primary cilia are causative for a large number of severe heritable diseases and syndromes, collectively classified as ciliopathies, as kidney and liver diseases, neural tube defects, and defects in left–right asymmetry.

The generation of primary cilia is coordinated with the cell cycle and, vice versa, primary cilia might also affect the progression of the cell cycle and cell proliferation. The formation of primary cilia starts in the G1-phase of the cell cycle and they are disassembled before mitosis. However, primary cilia are predominantly found in cell-cycle arrested cells that have withdrawn from the cell cycle in the early G1 phase and entered a quiescent state (G0). In differentiated cells, quiescence is the terminal stage. However, when reversible, cells can be induced to re-enter the cell cycle in response to growth signals. Reversible quiescence characterises tissue-resident stem cells that function in replenishing tissue loss throughout life but is also found in non-stem cells, e.g. in endothelial cells. Extrinsic and intrinsic factors regulate the cell cycle culminating in the activation of cyclin-dependent kinases, CDKs, by the binding to their regulatory cyclins that together drive the cell through the cell cycle. The differential expression of cyclins throughout the cell cycle is mandatory for cell cycle progression. Furthermore, cyclin-dependent kinase inhibitors, CKIs, antagonize cell cycle progression. Extrinsic factors, as nutrients and growth factors, activate the mitogen-induced signaling pathway (MAPK pathway) and the serine/
threonine-protein kinase B (PI3K/AKT) pathway eventually inducing transcription of cyclin D that binds and activates CDK4/6 thus initiating progression from G1 to S phase and cell proliferation1. On the other hand, low nutrient levels favour cell cycle arrest by increased AMP/ATP and ADP/ATP ratios which activate the energy-sensing AMP-activated protein kinase, AMPK. In between genes activated by AMPK is FOXO3a (also named FOXO3 or FOXO2)17. FOXO proteins are regulators of the cell cycle, metabolism, and apoptosis, and are inhibited by AKT-mediated phosphorylation18–20. Notably, FOXO3a inhibits mitochondrial gene expression, activates transcription of antioxidant enzymes, and regulates cell cycle progression21–24. Activation of FOXO3a, thus, correlates with cell cycle arrest and the formation of primary cilia. The FOXO proteins belong to the large family of forkhead transcription factors (TFs) characterized by a winged-helix DNA-binding domain of ~110 amino acids (aa), termed the 'forkhead box' serving as the epo-nym for the whole Fox family25–27. The founding member fork head (fkh) was first identified in Drosophila as a region-specific homeotic gene required for the formation of terminal embryonic structures28,29. Soon thereafter, related genes were identified in diverse organisms ranging from yeast to humans. FOX TFs are evolutionary conserved and are required for a wide variety of biological functions in development and differentiation, and tissue homeostasis30. They have been categorized by sequence similarities into subclasses A to E31. The hepatocyte nuclear factor 3 forkhead homolog 4, HFH4, now renamed into FOXJ1, is essential for the formation of motile cilia. Homozygous Foxj1-deficient mice displayed randomized left–right asymmetry and a complete loss of motile multi-cilia in epithelial cells whereas sensory mono-cilia were present32,33. FOXJ1 is an essential component of the transcription factor network controlling the formation of motile cilia by regulating the expression of essential ciliary genes34–37. However, while a substantial insight into the transcriptional regulation of motile cilia has already been accumulated our knowledge about the transcriptional regulation of sensory mono-cilia is inconceivable. FOX TFs are essential for diverse biological functions, including cell cycle regulation, thus raising the question if they are also involved in primary cilia formation. A first hint came from the ChIP-seq dataset from the ENCODE Transcription Factor Targets Datasets that lists several ciliary genes as FOXA1 target genes, including Odf238. ODF2/Cenexin, which is a sub-distal appendage protein that marks the mother centriole and its derivative, the basal body, is mandatory for cilia formation39–41. Odf2-deficient cells miss the primary cilium and Odf2+/− mice die during preimplantation42,43. Furthermore, the initiation of ciliogenesis depends on a crucial amount of ODF2 present at the mother centriole44. The tight regulation of Odf2, therefore, seems to be mandatory for the generation of primary cilia. We have carried out a candidate gene approach here, focusing on Odf2, which is essential for cilia formation, to investigate whether FOX TFs activate transcription of Odf2 eventually promoting primary cilia formation. We have chosen the mouse fibroblast cell line NIH3T3 as an established cellular model for the investigation of primary cilia. Our results show that FOXA1 binds to the Odf2 promoter and activates transcription to eventually regulate primary ciliogenesis. Results ODF2 is essential for primary cilia formation. To validate the relevance of ODF2 for the formation of primary cilia, Odf2 knockdown via transfection with either a short-hairpin plasmid (sh3) or siRNA was performed45. The plasmid K07 or the scrambled non-target siRNA served as controls for sh3- or siRNA-mediated knockdown, respectively. For rescue, the human Cenexin plasmid (hCenexin46), was co-transfected with either sh3 or Odf2 siRNA. Additionally, to identify transfected cells, the plasmid encoding histone H4 fused to Egfp (H4:GFP) was always co-transfected. Cells were fixed 24 h post-transfection and primary cilia immunologically decorated for the ciliary marker ARL13B (Fig. 1A). Primary cilia were manually counted by visual inspection and scanning through all focal planes taking into consideration only H4::GFP positive cells. We found a clear reduction in primary cilia when Odf2 was knocked down by either the sh3-plasmid or siRNA when compared to the controls (K07-plasmid or scrambled non-target siRNA, respectively) (Fig. 1B). We have counted ~17% primary cilia in K07-transfected cells as compared to only 5% in sh3-transfected cells (p = 0.00114 **), and ~15% in the siRNA control cells versus 7% in Odf2 siRNA transfected cells (p = 0.022857 *). In contrast, the rescue experiment caused an increase in primary cilia to ~13% (sh3 + hCenexin, p = 0.000989 +++ to sh3), and ~21% (Odf2 siRNA + hCenexin, p = 0.005358 ++ to Odf2 siRNA). Our results, thus, confirmed ODF2 as being essential for primary cilia formation. FOXA1 is a transcriptional activator of the Odf2 promoter. We previously reported the characterization of the mouse Odf2 promoter and identified C/EBPa and the stress-activated JNK-pathway as transcriptional activators37. Here, we identified a putative binding site for the forkhead-box TFs at position ~1775 of the Odf2 promoter resembling the consensus binding sequence for FOXO (TTG/AGTTTAC or GTAAA(T/C)AA;48), or FOXA1 (T(G/A)TT(T/G)AC49). To verify whether TFs of the FOX-family are involved in transcriptional regulation of ciliary genes and formation of primary cilia, we first investigated the impact of forkhead-box TFs on the activation of the Odf2 promoter, which controls expression of the firefly luciferase reporter gene (2.2-pGL3)47. The reporter vector 2.2-pGL3 comprised 1.8 kb upstream of the transcriptional start site of the mouse Odf2 gene together with 358 bp of the transcribed region (~1805/+358) positioned upstream of the firefly luciferase coding sequence. The reporter vector 2.2-pGL3 was co-transfected with the internal control vector phRL-SV40, which strongly expresses the Renilla luciferase under the SV40 promoter, and either without co-expression of TFs in the control probe or with, to investigate the effect of TFs on the activity of the Odf2 promoter. The relative activity of the reporter vector was first calculated by dividing the light signals of the firefly luciferase by the light signals of the Renilla luciferase used as an internal control. The relative activity was then related to the average of the relative activity of the control probe, i.e. the reporter vector 2.2-pGL3 without co-expression of any TF, which was set to 1, finally giving the fold change in expression. When compared to the activity of the control probe, we observed a significant increase in reporter gene activity in cycling cells by Foxa1 co-trans-
fection \((p=0.001565^{**})\), but not by co-transfection of Foxo1, Foxo3a, Foxj1, or co-transfection of both, Foxj1 and Rfx3 (Fig. 2A). Additionally, our previous results reporting transcriptional activation by C/EBPa, and the JNK-pathway (MEKK1 + cJUN) were verified. The co-transfection of Foxa1 with either C/ebpα, Mekk1, cJun or combinations of these factors revealed that FOXA1 together with C/EBPα has no additive effect on the reporter activity compared to C/EBPα. FOXA1 together with MEKK1 significantly increased the reporter gene activity \((p=0.026645^{*})\), but without further increase when compared to MEKK1. FOXA1 together with cJUN increased the reporter gene activity to a similar extent as MEKK1-activated cJUN, and significantly \((p=2.84466 \times 10^{-5^{****}})\) when compared to FOXA1. The co-transfection of Mekk1, cJun, and Foxa1 caused a further significant upregulation of the reporter activity compared to cJun \((p=0.004115^{**})\), cJUN + MEKK1 \((p=0.0002307^{***})\), and FOXA1 \((p=3.37212 \times 10^{-6^{****}})\). These data indicated a positive interaction between FOXA1 and cJUN in the transcriptional activation of Odf2 that is reinforced by MEKK1-mediated activation of cJUN. According to Student’s T-test, the co-expression of all four factors, MEKK1, cJUN, C/EBPα, and FOXA1 did not significantly increase the reporter gene activity compared to the co-expression of these factors but without FOXA1. The combination of FOXA1 with cJUN and C/EBPα did not cause an up-regulation of reporter gene activity as compared to FOXA1 alone.

Since cilia formation is stimulated by serum starvation-induced cellular quiescence simultaneously with enhanced Odf2 promoter activity\(^47\), we wondered whether FOX TFs are also activated by serum starvation. The
FOXA1 is a transcriptional activator of the Odf2-promoter. Co-expression of transcriptional activators, FOXO1, FOXO3A, FOXA1, FOXJ1, RFX3, C/EBPα, MEKK1, or cJUN with the firefly luciferase reporter under control of the Odf2 promoter, 2.2-pGL3, indicated FOXA1 as a transcriptional activator in cycling cells (A) and serum-starved cells (B), and suggested interaction between FOXA1 and C/EBPα. The firefly luciferase activity was related to the Renilla luciferase activity (encoded by phRL) as internal control and the fold changes of activity were calculated using the average of the relative luminescence of the control (2.2-pGL3/phRL) as 100% (or 1). Measurements of reporter gene activities were always performed in biological triplicates per experiment using a total of n biological replicates in cycling cells: n = 26 (control, 2.2-pGL3), n = 24 (FOXO3A), n = 33 (FOXO1), n = 36 (FOXA1), n = 9 (FOXJ1), n = 6 (FOXO1 + RFX3), n = 15 (C/EBPα), n = 9 (C/EBPα + FOXA1), n = 6 (C/EBPα + MEKK1), n = 9 (MEKK1), n = 9 (MEKK1 + FOXA1), n = 9 (FOXJ1 + cJUN), n = 6 (cJUN), n = 18 (cJUN + MEKK1), n = 12 (cJUN + MEKK1 + FOXA1), n = 6 (MEKK1 + cJUN + C/EBPα), n = 6 (C/EBPα + FOXA1), n = 6 (cJUN + C/EBPα + FOXA1), and in serum-starved cells: n = 18 (control, 2.2-pGL3), n = 18 (FOXO3A), n = 18 (FOXO1), n = 3 (FOXO1 ADA), n = 18 (FOXA1), n = 9 (FOXJ1), n = 3 (FOXJ1 + RFX3), n = 9 (C/EBPα), n = 3 (C/EBPα + FOXA1), n = 6 (MEKK1), n = 6 (MEKK1 + FOXA1), n = 6 (MEKK1 + cJUN), n = 3 (cJUN + MEKK1 + FOXA1). Student’s T-test two-tailed, homoscedastic p < 0.05 *, p < 0.01 **, p < 0.001 ***, p < 0.0001 ****. Calculation of T-test to the control (2.2-pGL3/phRL) * or to FOXa1 co-transfection 

To further verify FOXA1 as a transcriptional activator of the Odf2 promoter, the reporter vector 2.2-pGL3 was co-transfected with the Foxa1 expression plasmid, and either the negative control siRNA duplex, or one of the Foxa1 siRNA duplexes A, B, or C. The activities of the firefly luciferase reporter and the Renilla luciferase as the internal control were measured either 24 h post-transfection (cycling cells) or after 48 h cultivation in serum starvation medium (cell cycle-arrested cells). Calculation of the relative luminescence revealed an approximately 2.5-fold increase of the reporter gene activity when expression of FOXA1 was enforced compared to control cells in which only the reporter 2.2-pGL3 and the internal control phRL were co-transfected (Fig. 3). Co-transfection of the negative control siRNA did not significantly change the expression of the reporter vector. However, we observed reduced activity of the reporter vector when Foxa1 siRNA duplexes B or C were co-transfected while siRNA A was not effective. Though, significant reductions in reporter gene activity (p*) were observed exclusively for the Foxa1 siRNA duplex C in both, cycling cells (p = 0.03953205*) as well as in quiescent cells (p = 0.0142835*). Since the Foxa1-mediated activation of the Odf2 promoter can be efficiently repressed by Foxa1 siRNA duplexes these data demonstrate the importance of FOXA1 for the transcriptional activation of Odf2, as well as the specificity of Foxa1 siRNA duplexes for the knockdown of FOXA1.

FOXA1 binds to the Odf2 promoter. Reporter gene assays were performed to narrow down the binding site of FOXA1 in the Odf2 promoter. Fragments of the Odf2 promoter, cloned upstream of the firefly reporter gene in pGL3 as described in Pletz et al. 17 (Fig. 4B), were investigated for their responsiveness to the FOXA1 transcription factor. Here, FOXA1-induced transcriptional activation was related to the basal level of the respective reporter construct without Foxa1 co-transfection. Strong induction of transcription was observed for the promoter region −1282 to −1805 in clone 7.6 (~70× when co-transfected with Foxa1 as compared to the control without FOXA1 overexpression), for the region −1368 to −1805 in clone 22.1 (~16×), and the region −94 to −22 (~4×) (Fig. 4A), indicating that the FOXA1-binding site most likely is located in the region between −1282 and −1805 of the Odf2-promoter.

A sequence (TGTTTAC) with similarity to the FOXA1 consensus binding sequence (T/G)A(T/T)T(G/A)AC9 was identified in the Odf2 promoter at position −1768 to −1775 upstream of the transcription start site. To investigate the binding of FOXA1 to this sequence we performed chromatin immunoprecipitation using the Odf2-promoter reporter vector 2.2-pGL3 as bait and the anti-FOXA1 antibody as the fishing agent. Furthermore, ChIP was additionally performed in cells co-transfected with the Foxa1 expression plasmid. Enrichment of the binding sequence was investigated by qPCR using primers that flank the supposed binding site and obtained Ct-values were adjusted to the input (ΔCt), and the control ΔCt-values (ΔΔCt). We found ~26× enrichment of the binding site in cells co-transfected with the Foxa1 expression plasmid (p < 0.01*), and ~4× enrichment when the endogenous FOXA1 was precipitated (p = 0.137344, Student’s T-test two-tailed, homoscedastic) (Fig. 4C). No enrichment was found for the sequence of the putative DREAM binding site (not shown) that we identified at positions +27 to +33 related to the transcription start site indicating the specificity of FOXA1-binding. Although precipitation of the endogenous FOXA1 did not reveal significant enrichment of the target sequence.
compared to the control IgGs, co-expression of FOXA1 together with the Odf2-reporter 2.2-pGL3 as bait resulted in a significant enrichment of the target sequence. These results indicated that the binding site of FOXA1 in the Odf2 promoter is represented by the sequence TGT TTA C situated at position −1768 to −1775 upstream of the transcription start site.

Expression of Fox transcription factors in NIH3T3 cells. Our data indicated FOXA1 as an activator of Odf2 transcription in NIH3T3 fibroblasts. However, it was unknown whether Foxa1 or any other TF of the Fox-family is indeed expressed in NIH3T3 cells. We, therefore, verified the expression of Fox TFs by RT-PCR using cDNA prepared from NIH3T3 cells. Although no products were present after the first RT-PCR round, a nested PCR reaction using the first RT-PCR reaction as the template, revealed products of the expected lengths for all Fox TFs investigated (Fig. 5A). The correct amplifications were verified by sequencing PCR products. Thus, all four transcription factors investigated, Foxa1, Foxo1, Foxo3a, and Foxj1, were expressed in NIH3T3 cells albeit at a low level. Furthermore, the endogenous FOXA1 was detected immunologically inside the nuclei showing a speckled appearance that colocalized with nucleoli (Fig. 5B, a and c).

Efficient down-regulation of FOXA1 by Foxa1 siRNA. We have demonstrated that Foxa1 siRNA duplexes inhibit FOXA1-mediated activation of the Odf2 promoter in reporter gene assays. To further prove that this effect is caused by siRNA-mediated knockdown of FOXA1, quantitative analyses were performed. Because of the low-level expression of Foxa1, we used FOXA1::GFP overexpression by co-transfection of Foxa1::gfp with either the control siRNA, or one out of the three Foxa1 siRNA duplexes A, B, or C. Proteins were analysed by quantitative Western blotting using anti-FOXA1 antibodies, and anti-α-Tubulin antibodies (Fig. 6A, anti-FOXA1 in green, anti-α-Tubulin in red). Signal intensities of FOXA1::GFP at ~77 kDa (Fig. 6A asterisk) and α-Tubulin were quantified and their ratios calculated for each lane. The obtained relative intensities were related to the average relative intensity of the control siRNA samples giving the fold change of expression. We observed an efficient reduction of FOXA1::GFP by Foxa1 siRNA C (in cycling cells: \( p = 0.039532^* \), in serum-starved cells: \( p = 0.014283^* \), Student’s T-test, two-tailed, homoscedastic). Three biological replicates each.
Foxa1 siRNA-mediated knockdown affects the endogenous expression of Foxa1, Odf2/ODF2, and CP110. Having proven an efficient reduction of FOXA1 protein by Foxa1 siRNA-mediated knockdown, we asked whether knockdown of Foxa1 could also be detected at the endogenous transcript level and whether a knockdown of FOXA1 would be accompanied by a reduction of ODF2 proteins. NIH3T3 cells were, thus, transfected with the Foxa1 siRNA duplexes and oDF2 proteins. NIH3T3 cells were, thus, transfected with the Foxa1 siRNA duplexes and ODF2 promoters were investigated for their activation by FOXA1. FOXA1-mediated transcriptional activation was always related to that of the respective vector, without FOXA1 co-expression. The strongest activation was observed for the reporter vectors 7.6 (~ 70x), 22.1 (~ 16x), and 7.1 (~ 4x) indicating that the FOXA1-binding site is positioned in the region ~1282 to ~1805. Three biological replicates. (B) Scheme of the Odf2 promoter fragments cloned upstream of the firefly reporter gene (e1 = exon1, i1 = intron1). The consensus binding site (cbs) for FOX TFs is located at position ~1768 to ~1775 (*). (C) Chromatin immune-precipitation by FOXA1 and qPCR of the binding-site sequence revealed significant enrichment. NIH3T3 cells were transfected with the Odf2 promoter reporter vector 2.2-pGL3 as bait. Co-transfection of the Foxa1 expression plasmid (Foxa1 + 2.2-pGL3/anti-FOXA1) resulted in an ~26× enrichment of the FOXA1-binding site compared to the control IgGs (Foxa1 + 2.2-pGL3/control IgG) (**, p = 0.007838). Precipitation of the endogenous FOXA1 by anti-FOXA1 antibodies (2.2-pGL3/anti-FOXA1) caused an ~4× enrichment of the binding sequence compared to the control (2.2-pGL3/control IgG) that is, however, not significant (p = 0.13744).
FOXA1 was knocked down by Foxa1 siRNA C ($p = 0.000631^{***}$) (Fig. 7B, C; $p^{***}$). Additionally, FOXA1 knockdown caused also a significant reduction of CP110, which has been annotated as a target gene of FOXA1$^{38}$ (Fig. 7D, E $p = 0.002232^{**}$).

**FOXA1 is necessary for primary cilia formation.** Since the amount of ODF2 is crucial for primary cilia generation we asked whether FOXA1 is involved in cilia formation. NIH3T3 cells were transfected with either one of the three different siRNA duplexes (A, B, or C) or the scrambled negative control siRNA duplex. 24 h post-transfection the medium was exchanged for serum starvation medium to induce cilia formation, and cells were cultivated for another 24 h or 48 h. Primary cilia were then immunologically decorated for ARL13B and manually counted (Fig. 8A). siRNA-mediated knockdown of FOXA1 caused a reduction of primary cilia to ~ 0.8 × when cultivated in serum starvation medium for 24 h ($p < 0.05^+$ or $p < 0.01^{++}$). Cultivation in serum starvation medium for 48 h caused a reduction of primary cilia to ~ 0.8 × by Foxa1 siRNA A ($p < 0.05^*$) and ~ 0.5 × in Foxa1 siRNA B or C transfected cells (both $p < 0.01^{**}$) (Fig. 8B). The Foxa1 siRNA A has been turned out once more to be the least effective thus corroborating the data of the reporter gene assays (Fig. 3) and the Western blots (Fig. 6). However, it has to be kept in mind that the siRNA-mediated knockdown effects in reporter gene assays and Western blots were based on the reduction of the co-expressed Foxa1-plasmid which most likely accounts for the somehow differing results. Our results thus indicate that FOXA1 is mandatory for the formation of primary cilia.

**Co-immune precipitation revealed no direct interaction between FOXA1 and cJUN.** Our data demonstrated that FOXA1 is a transcriptional activator of Odf2. Furthermore, reporter gene assays suggested a positive interaction between FOXA1 and cJUN. To verify, we transfected cells with expression plasmids either Foxa1::gfp or Foxa1::gfp and Mekk1, followed by capturing of FOXA1::GFP using immobilized anti-GFP antibodies. Although FOXA1::GFP (of ~ 77 kDa) was successfully captured, demonstrated by its presence in the eluate of the bead-bound fraction, neither cJUN nor MEKK1-phosphorylated cJUN of either 36–39 kDa or 42–45 kDa, respectively, were co-precipitated (Fig. 9A). Thus, neither the unphosphorylated (Fig. 9A, B) nor the phosphorylated cJUN (Fig. 9A) were found to directly interact with FOXA1, despite FOXA1::GFP and cJUN colocalised in the nuclei of NIH3T3 cells (Fig. 9C).

**Discussion**

Primary cilia are essential sensory organelles present on nearly all cells of the body. They are built in a cell cycle-dependent manner and are mainly found in quiescent cells. Generation of primary cilia depends on a crucial amount of ODF2/Cenexin, a basal body protein mandatory for cilia formation and viability$^{42–44}$. Transcription of Odf2 is cell cycle-dependent with upregulation in serum-starved cells and thus correlated with primary cilia formation$^{42}$. We have previously identified TFs controlling the transcription of Odf2$^{45,50}$. However, although few TFs have been identified to be involved in primary ciliation, as RFX3, the TF network regulating the formation of primary cilia has still to be figured out$^{51}$. Motile ciliogenesis is controlled by the master regulator FOXJ1
in cooperation with RFX factors\textsuperscript{35,52,55}. 

Odf2 was not annotated as a direct target gene of FOXJ1 and neither RFX3 nor FOXJ1 TFs activate transcription of Odf2 in NIH3T3 cells\textsuperscript{37,47}. However, Odf2 was down-regulated in RFX3-deficient ependymal cells of the mouse and possesses RFX3-binding sites identified by ChIP indicating that expression of Odf2 is regulated by RFX3 in multi-ciliogenesis\textsuperscript{54}. Furthermore, opposed to multiciliogenesis primary ciliogenesis is FOXJ1-independent\textsuperscript{55}.

FOXJ1 belongs to the large family of evolutionarily conserved forkhead-box (Fox) TFs that have diverse functions in development and differentiation. FOX TFs are characterised by the conserved DNA-binding domain of ~110aa, denoted as fork head domain, first identified in FKH and the rat hepatocyte-enriched transcription factor HNF-3A\textsuperscript{26,27}. Currently, more than 44 genes were annotated in both, mice and humans, and categorized into subclasses A to S. FOX proteins are essential TFs as the deletion of just one Fox gene very often leads to lethality, and mutations in Fox genes are associated with developmental disorders or diseases\textsuperscript{31,56}. FOXJ1, alias HFH4, is the master regulator for motile cilia formation and is, therefore, essential for the execution of the specialized functions of epithelial cells harbouring motile cilia\textsuperscript{32,33}.

The FOXO-proteins and FOXM1 function in cell cycle control. FOXM1 is a key regulator of both the G1/S phase and G2/M phase transition and is essential for proper mitotic progression\textsuperscript{57}. Furthermore, FOXM1 is a component of the DREAM complex that inhibits transcription of target genes for cell proliferation in the quiescent state but promotes expression during the cell cycle\textsuperscript{58}. The DREAM complex contacts DNA via the cell cycle genes homology region (CHR), which most commonly comprises the sequence TTTGAA\textsuperscript{59}. The FOXO proteins are inhibitors of the cell cycle and regulate metabolism and lifespan. Like FOXM1 their activity is controlled by post-translational modifications. AKT-mediated phosphorylation of FOXO proteins caused their inactivation by translocation into the cytoplasm where they are stabilized by interaction with 14-3-3 scaffolding proteins\textsuperscript{20}. FOXO proteins regulate cell proliferation via up-regulation of the cell cycle inhibitor p27\textsuperscript{KiP1} and downregulation

Figure 6. Knockdown of FOXA1::GFP by Foxa1 siRNA duplexes. (A) Western blots demonstrating the reduction in FOXA1::GFP by Foxa1 siRNA. Foxa1::gfp was co-transfected with either control siRNA (control) or one of the Foxa1 siRNA duplexes A, B, or C (Foxa1 siA, B, or C). 48 h post-transfection cells were harvested, and the cell lysates were analysed by Western blotting. Detection of FOXA1::GFP (green, ~77 kDa, asterisk) and α-Tubulin (red) on the same blot. The original blots are presented in Supplementary Figure S2. (B) Efficient reduction of FOXA1::GFP by Foxa1 siRNA-mediated knockdown. The quantity of FOXA1::GFP was related to the quantity of α-Tubulin in the same lane and the fold changes in the relative quantities calculated to the average of the relative quantity in control siRNA transfected cells. Three biological replicates for each RNA duplex. Student’s T-test two-tailed, homoscedastic: siRNA A p = 0.412464, siRNA B p = 0.004772**, siRNA C p = 0.002733**.
Figure 7. Decreased expression of Foxa1, Odf2/ODF2, and CP110 by siRNA-mediated Foxa1 knockdown. (A) Knockdown of Foxa1 and Odf2 transcripts by Foxa1 siRNA. NIH3T3 cells were transfected with either the scrambled control siRNA (control siRNA) or the Foxa1 siRNA (Foxa1 siRNA) and transcription of Foxa1 and Odf2 quantified by RT-PCR. The relative expression of Foxa1 or Odf2 was calculated to both housekeeping genes, Gapdh and Hprt, by ΔCt. Their relative expression following siRNA-mediated depletion of Foxa1 was compared to the control siRNA using the ΔΔCt-method and calculated as 2−ΔΔCt. Significant reduced expression of Foxa1 (p = 0.02727533*) and Odf2 (p = 0.00062325**) related to the control siRNA. qRT-PCR was performed with at least three biological replicates, each measured in triplicate. Student’s T-test two-tailed, homoscedastic. (B) Western blot showing expression of ODF2 in NIH3T3 cells transfected with either the scrambled control siRNA (control siRNA, biological replicates #1 to #3) or the Foxa1 siRNA C (biological replicates #1 to #6). Cells were harvested 48 h post-transfection and cultivation in serum-deprived medium. Detection of ODF2 (~100 kDa) and β-Actin (~42 kDa) simultaneously on the same blot using the fluorescent-labeled secondary antibody anti-rabbit IgGCW800. An unspecific band was observed > 100 kDa. (C) Foxa1 siRNA caused a reduction in ODF2 protein. The relative quantity of ODF2 was obtained by calculating the ratio between the quantity of ODF2 and β-Actin in each lane, and relating the relative quantities to the average of the relative quantity obtained in control siRNA-transfected cells. Significant reduction of ODF2 expression by Foxa1 siRNA C (p = 0.000631***). Six biological replicates each and a total of n loadings: control n = 7, siRNA B n = 7, siRNA C n = 9. (D) Western blot showing expression of CP110 in control siRNA, and Foxa1 siRNA C transfected cells. (control siRNA: 4 biological replicates, Foxa1 siRNA: 5 biological replicates). CP110 (<130 kDa) and β-Actin (~40 kDa) were both detected simultaneously on the same blot (in green). (E) FOXA1 knockdown caused a decreased quantity of CP110 (p = 0.002232**). The relative quantity of CP110 was calculated as described for ODF2 quantification using the quantity of β-Actin as internal standard. For quantification six biological replicates for both, the control and siRNA were used and a total of n loadings were quantified: control n = 7, siRNA n = 8. Always Student’s T-test two-tailed, homoscedastic. The original blots are presented in Supplementary Figure S2.
of cyclin D1\(^{60,61}\). Their function as cell cycle inhibitors suggested a causal relationship to the transcriptional regulation of primary cilia formation, which are mainly found in quiescent cells. As a candidate approach, we focused on the sub-distal appendage protein and marker of the mother centriole and the basal body, ODF2/Centrin, because it is mandatory for cilia formation and transcriptionally upregulated in serum-starved, cell-cycle arrested cells.\(^{42,44,60}\) A consensus binding sequence for the forkhead-box TFs FOXO and FOXA1 was identified in the promoter region of the mouse Odf2 gene.\(^{46,49}\) However, neither FOXO1 nor its constitutively active form FOXO1ADA, nor FOXO3A activated transcription of Odf2 in reporter gene assays. These data indicated that FOXO TFs are most likely not transcriptional activators of Odf2 and largely rule them out as regulators of ciliation. A knockdown will therefore most likely have no effect on cilia formation. Instead, we found significant repression of Odf2 expression by FOXA1 and cJUN. Thus, the binding of FOXA1 to the Odf2 promoter might enable concurrent binding of cJUN corroboring its function as a pioneer factor.

The mammalian FOXA TFs were first identified in the rat liver and hence named hepatocyte nuclear factor 3 (HNF3) \(\alpha\), \(\beta\), and \(\gamma\), respectively FOXA1, 2, and 3.\(^{35}\) Expression of FOXA1, FOXA2, and FOXA3 during development and in adult tissues exhibit overlapping but also distinct patterns.\(^{31}\) Furthermore, FOXA1 is more widely expressed in adult tissues than FOXA2.\(^{31}\) Foxa1-deficient mice survive until after birth but die between postnatal day 2 and P12 due to hypoglycemia and defects in kidney function.\(^{63,64}\) FOXA1 is essential to the known tasks of FOXA TFs, our data add the novel function in the regulation of primary cilia formation (Fig. 8). We have shown that FOXA1 binds to its consensus sequence in the mouse Odf2 promoter and activates the expression of Odf2/ODF2. Knockdown of FOXA1 not only downregulated Odf2 transcripts and ODF2...
proteins but also inhibited primary cilia formation. We found a significant reduction of Foxa1 (to ~0.5x) as well as Odf2 transcripts (to ~0.6x) after transfection of Foxa1 siRNA that correlated with a 0.6-fold decrease in ODF2 proteins. Since Foxa1 siRNA caused only a knockdown of FOXA1 but could not completely abolish it, FOXA1 is still present and, in addition, could also be compensated by FOXA2 that contributed to the continued expression of ODF2. Foxa2 is also transcribed in NIH3T3 cells as was observed by nested RT-PCR (Supplementary Fig. S1) but due to a lack of validated siRNAs for the mouse FOXA2, its impact on primary ciliation could not be investigated. Reduced expression of ODF2 by FOXA1 knockdown provoked a decline of ciliated cells up to 0.55–0.75x
Figure 10. FOXA1 is a transcriptional activator of *Odf2*, which ultimately promotes cilia formation. FOXA1 binds to the sequence TGT TTA C at positions –1768 to –1775 of the *Odf2* promoter and promotes transcription of *Odf2*. The JNK-pathway via cJUN, and C/EBPα are also involved in the transcriptional activation of *Odf2*, although their binding sites have not been determined. Positive feedback was found between cJUN and FOXA1 in the transcriptional activation of the *Odf2* promoter, although a direct interaction could not be detected. According to the ChIP-seq dataset from the ENCODE Transcription Factor Targets Datasets, FOXA1 targets many more essential ciliary genes, including *CP110*, which we have shown is indeed regulated by FOXA1. The finally generated ODF2 protein, which is mandatory for the generation of primary cilia, eventually promotes cilia formation together with other ciliary proteins. The structure of the ODF2 protein (*Mus musculus*) was predicted by AlphaFold (AlphaFold DB version 2022-11-01, created with the AlphaFold Monomer v2.0 pipeline, licence CC-BY-4.0), and made available by EMBL-EBI (Wellcome Genome Campus, Hinxton, UK). The primary cilia were detected by immunological decoration of both, acetylated α-Tubulin (green) and ARL13B (red), shown here as merged image (yellow), and the nucleus stained with DAPI (blue) (inset).

Methods

Cell culture and immune-cytology. The mouse fibroblast line NIH3T3 was obtained from DSMZ (ACC59) and cultivated in Dulbecco's Modified Eagle's Medium (DMEM; GlutaMax™ with high glucose concentration (4.5 g/l); ThermoFisher Scientific, #10566), supplemented with 10% (v/v) fetal calf serum (FCS), 1000 U/ml penicillin and 1,000 µg/ml streptomycin at 37 °C and 5% CO₂. Primary cilia were induced by cultivation in the above medium supplemented with 0.5% FCS (serum starvation medium) for 24 to 48 h.

For immune-cytology, cells were reseeded at a density of 12 × 10⁶ cells per well of a 6-well plate on glass coverslips. Cells were fixed in 3.7% paraformaldehyde (PFA) for 20 min at 4 °C, permeabilized with 0.3% Triton X-100 in PBS (phosphate-buffered saline) for 10 min at room temperature, followed by blocking non-specific binding sites by incubation in PBS containing 1% bovine serum albumin (BSA) and 0.5% Tween-20 for at least 1 h. Samples were incubated with the primary antibodies anti-acetylated α-Tubulin (clone 6-11B-1; Santa Cruz Biotechnology, Inc., #sc-23950, diluted 1:50), anti-ARL13B (Proteintech, #17711-1-AP, diluted 1:400), anti-FOX1 (Proteintech 20411-1-AP, diluted 1:200), anti-ODF2 (ESAP15572, antibodies-online, diluted 1:100), anti-α-Tubulin (Oncogene, #Ab-1, diluted 1:100) at 4 °C overnight. Secondary antibodies used are goat anti-mouse-IgG-DyLight 488 (#35503, ThermoScientific), goat anti-mouse-IgG-AlexaFluor555 (#A21242, Lot 948498, Invitrogen/Mol. Probes), and goat anti-rabbit-MFP590 (#MFP-A1037, Molecular Probes, Eugene). DNA was counterstained with DAPI. Images were taken by confocal microscopy (LSM 980, Zeiss) and processed using Adobe Photoshop 7.0. Primary cilia were manually counted by visual inspection and scanning through all focal tissues64. FOXA1 and FOXA2 TFs contribute to the maintenance of epithelial cell identity and their deregulated expression is associated with cancer formation76,77. FOXA1/2 are important for Sonic hedgehog (SHH) signalling by restricting the expression of the transcriptional mediators, Gli1 and Gli276,78,79. The hedgehog pathway is essential for vertebrate development, and aberrant activation of the pathway is associated with cancer formation80. Furthermore, the hedgehog signal transduction pathway is strictly dependent on the primary cilium81. The primary cilium is involved in both the promotion and inhibition of tumorigenesis, as has also been described for deregulated FOXA expression, but is generally absent in cancer cells82,83. The data, thus, indicated a link between the hedgehog signal transduction pathway and the primary cilium.
Approximately 500 cells for each replicate were scored for the presence of a primary cilium. The total counts are given in the results as n, comprising all replicates.

**Transfection of cells.** Plasmid DNA or siRNA was transfected using EndoFectin™ Max Transfection Reagent following the manufacturer’s instructions (GeneCopoeia #EF014). The mouse Foxa1 gene (NM_008259) tagged with Myc-DDK in pCMV6-Entry was obtained from Origene (MR225487). The coding region of Foxa1 was cloned in-frame to Efgp in pEGFP-N1 (Clontech).

Foxa1 knockdown was achieved using three unique 27mer siRNA duplexes (Origene, SR415184A, SR415184B, SR415184C, all used at a concentration of 10 nM), and a universal scrambled negative control siRNA duplex (Origene, SR30004; final concentration 25 nM).

For Odf2 knockdown, Odf2 siRNA (stealth siRNA ODF2MSS207236; Life Technologies; final concentration 40 nM) and control siRNA (siGenome Non-targeting siRNA #1; ThermoFisher Scientific Biosciences) were used. Additionally, the short hairpin constructs sh3 (specifically targeting sequence gacctctcagagga of mouse Odf2/Cenexin[46] or K07 (Origene), which functions as control while lacking homology with any known mRNA, were used. For rescue, the expression plasmid encoding human Cenexin (hCenexin[46]) was co-transfected, and to identify transfected cells, human histone H4[44] fused to egfp was also co-transfected.

**Expression analyses by reverse-transcribed PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR).** Total RNA was prepared usingpeqGOLD RNPure™ (PeqLab, Erlangen, Germany) following the recommendations of the manufacturer, and treated with Ambion™ TURBO DNA-free™ DNase (Ambion #AM2238). The absence of genomic DNA was validated via amplification using Gapdh or Hprt, followed by cDNA synthesis (Maxima First Strand cDNA Synthesis Kit; ThermoFisher Scientific, #K1641). The following primers were used for expression analyses (RT-PCR): Hprt (mHPRT-for2 gggactctttgggcttggc/mHPRT-rev2 gggagagcaccatcaacagt), Gapdh (mGapdhf caccacaaccagtctac/mGapdhr cggatactgggggagag), Foxa1 (for first PCR: Foxa1-for4 tattaccgccagaacacg/Cfox1-rev4 acgggtctggaatacagttg (expected size: 793 bp), and for nested PCR: Foxa1-for1 ccttcaacagattctgcgtgcc/Foxa1-rev2 gagaaggagtgatgaaagga (525 bp)), Foxo1 (for first PCR: Foxo1-for1 gctctcactaatgaggggaggtgcc/Foxox1-rev1 cattttctgatcttcctgcc (631 bp)), Foxo3a (for first PCR: Foxo3a-for1 ctttcagccctcagagt/Foxox3a-rev1 ggtctgctgctgcgtcgctag (752 bp) and for nested PCR: Foxo3a-for2 ggctcatctgctcttcagtc/Foxo3a-rev2 gagaaggagtgatgaaagga (525 bp), and for Foxo1 (for first PCR: Foxj-for1 ctttaccaacgctgcgtgcc/Foxox1-rev1 gatgtgcagaaagtgatg (1027 bp) and for nested PCR: Foxj-for2 caactctgctcttcagtc/Gfoxj-rev2 caagacttctcagagtg (674 bp).

The quantitative real-time PCR (qRT-PCR) was performed on CFX96TM Real-Time System (Bio-Rad) using BlazeTaq SYBR Green qPCR mix 2.0 (GeneCopoeia, Rockville, MD). Primer efficiency was validated for all primer pairs and the specificity of the amplification reaction was verified by melting curve analyses. The following primer pairs were used for qRT-PCR: Foxa1-for3 (gacgccaagacctagtggcagc)/Foxa1-rev3 (gtggcttgctgcgtggcagc), Odf2-na-f4 (acatgggaagagcctctctct)/Odf2-na-r4 (gcgacacccattcctgtgcgt), and mHPRT-for2/mHPRT-rev2 and mGapdhf/mGapdhr as above. Three technical replicates were used for each analysis. The relative expression was calculated by 2−ΔΔCt method. For the T-test, the averages of the relative expression of the technical replicates were used.

**Reporter gene assay.** NIH3T3 cells were seeded at a density of 1×10⁵ cells per well of a 12 well plate. 24 h later cells were co-transfected with the reporter vector (1 µg/well), the internal control vector phRL-SV40 (Promega, Madison, USA; 10–100 ng/well), and expression plasmids encoding transcription factors (each at 100 ng/well). As reporter vector either 2.2-pGL3 or one of the truncated Odf2-promoter vectors (#1, 22.1, 7.6, 7.1, 1.5, A1, 0.5) were used, which in part of the promoter region was cloned upstream of the firefly luciferase reporter pGL3[9]. The Odf2 promoter constructs comprise the following promoter regions: 2.2 (-1805/+358), 7.1 (-1805/+94), 7.6 (-1805/+1282), 22.1 (-1805/+1368), #1 (-1675/+1), 1.5 (-797/+1), A1 (-797/+358), and 0.5 (-94/+255)[9]. The following expression plasmids encoding transcription factors were used: Foxa1 (Origene, MR225487), Foxa1 (addgene 12148), Foxa1 A4 (addgene 12143, constitutively active, containing three point mutations Thr24Ala, Ser253Asp, Ser316Ala), Foxo3a (addgene 1787), Foxj1 (Steven Brody, St. Louis), Rfx3 (Walter Reith, Genf), C/Eβpa (addgene 12550), and cjun (pFA2-cjun, encoding the transactivation domain of aa 1–223) and Mekk1 (MAP3K1; pFC-MEKK, aa 380–672) both from Stratagene (PathDetect cjun trans-Reporting System; Stratagene, La Jolla, USA).

To investigate the effect of FOXA1 knockdown on reporter gene activity, the Foxa1 expression plasmid (Origene, MR225487) (100 ng/well), and either one of the Foxa1 siRNA duplexes (Origene, SR415184A, SR415184B, or SR415184C, all used at a final concentration of 20 nM), or a universal scrambled negative control siRNA duplex (Origene, SR30004; final concentration 25 nM) were co-transfected. Cells were either cultivated in standard medium for 24 h post-transfection (cycling cells), or the medium was exchanged for serum starvation medium 24 h post-transfection, and cells cultivated for another 48 h (serum-starved cells). The Dual-Glo Luciferase Assay System (Promega, USA) was used for measuring firefly and Renilla luciferase activity using the Centro LB 960 luminometer (Berthold Technologies, Germany). Fold changes were calculated based on the relative luminescence (firefly luminescence/Renilla luminescence). Each experiment was performed in triplicates and repeated up to six times.

**Co-immune-precipitation and Western blotting.** Cells were transfected with the expression plasmids either Foxa1::Efgp or Foxa1::Gfp and Mekk1 (MAP3K1; pFC-MEKK, aa 380–672). 24 h post-transfection cells
Control IgG (ΔΔCt). The Ct-values obtained by either FOXA1- or control IgG-precipitation were adjusted to their flanking a putative DREAM binding-site of sequence TTT GAA found at position + 27 to + 33 related to the enrichment of the binding site sequence was calculated by adjustment to the validated first. The Ct-values obtained by either FOXA1- or control IgG-precipitation were adjusted to their flanking a putative DREAM binding-site of sequence TTT GAA found at position + 27 to + 33 related to the 

The supernatant was incubated with GFP Trap Magnetic Agarose (chromotek, gtma-20) for 1 h at 4 °C on a rotating wheel. Beads were, thereafter, washed 4-times with 500 µl RIPA-buffer each, and 10% of each wash solution stored for later analysis. Bead-bound proteins were eluted by boiling in SDS-sample buffer containing β-mercaptoethanol for 5 min.

Proteins were separated on denaturing SDS-gels and transferred to Hybond ECL. Blot membranes were blocked for 1 h in 5% dry milk in TBST (10 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.05% Tween20), and incubated with the primary antibodies (rabbit anti-FOXA1, self-made, and mouse monoclonal anti-β-JUN, protein-tech 66313-1-Ig) overnight at 4 °C. For quantitative Western blots anti-ODF2 (ESAP15572, antibodies-online, diluted 1:1000), and anti-CP110 (Proteintech #12780-1-AP, diluted 1:3000) antibodies were used, and as the internal standard anti-β-Actin antibody (proteintech #20536-1-AP). Primary antibodies were detected with the fluorescent-labelled secondary antibodies IRDye800CW goat anti-mouse IgG (LI-COR, #925-32210), and IRDye680RD goat anti-rabbit IgG (LI-COR, #925-68071), or with IRDye800CW goat anti-rabbit IgG (LI-COR, #925-32211) and IRDye680RD goat anti-mouse IgG (LI-COR, #925-68070). Images were captured with LI-COR Odyssey CLX and analysed using Image Studio Lite (LI-COR). Quantification was performed by calculating the ratio between the target protein and the internal standard (β-Actin) in the same lane. The images were then related to the average relative quantity in the control, giving the fold change of target protein expression. 

**Chromatin-immune-precipitation.** NIH3T3 were transfected either with the Foxa1 expression plasmid and the Odf2 reporter vector 2.2-pGL3 or with 2.2-pGL3 exclusively to investigate the binding of the endogenous FOXA1. The chromatin-immune-precipitation (ChIP) protocol was a modification of Denissov et al. Briefly, cells were crosslinked with 1.1% formaldehyde in PBS (phosphate-buffered saline) containing 0.07 mM EDTA, 0.035 mM EGTA, and 3.5 mM Hepes for 30 min at room temperature, quenched by adding glycine to 0.125 M final concentration for 5 min at room temperature, and afterward washed twice with cold PBS. Cells were lysed in Hepes-buffer B (20 mM Hepes, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100) for 10 min at 4 °C, followed by scraping, and cell collection by centrifugation. Cells were washed once in cold Hepes-buffer C (50 mM Hepes, 1 mM EDTA, 0.5 mM EGTA, 0.15 M NaCl), and finally resuspended in incubation-buffer (20 mM Hepes, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100 with proteinase inhibitor mix; ProteoBlock Protein-erase inhibitor Cocktail, Fermentas R1321). Probes were sonified using 12 cycles (30 s on/30 s off) in the Bioruptor (Diagenode), and clarified by centrifugation.

Protein A Dynabeads (Dynal Biotech, #10001) were pre-cleared and pre-blocked before use for immunoprecipitation. Beads were washed twice in incubation buffer, and once in incubation buffer containing 1 mg/ml BSA (bovine serum albumin), and incubated in incubation buffer containing 2 mg/ml BSA and 100 µg/ml sheared salmon sperm DNA overnight at 4 °C. The next day, beads were washed twice in incubation buffer containing 1 mg/ml BSA and incubated with the sheared chromatin for 1 h at 4 °C for pre-clearing. After magnetic beads collection, 10% of the supernatant was removed and stored as the input sample, and the remaining supernatant was transferred into new tubes and incubated with the antibody, either anti-FOXA1 antibody (2 µg/150 µl of chromatin; Proteintech #20411-1-AP) or rabbit IgG as control (4 µg/150 µl of chromatin; Diagenode #C154110206), together with fresh pre-cleared and pre-blocked beads at 4 °C overnight on a rotating wheel. The next day, beads were collected and washed twice in wash buffer 1 (20 mM Hepes, 1 mM EDTA, 0.5 mM EGTA, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate (NaDOC)), once in wash buffer 2 (20 mM Hepes, 1 mM EDTA, 0.5 mM EGTA, 0.5 M NaCl, 1% Triton X-100, 0.1% SDS, 0.1% NaDOC), once in wash buffer 3 (20 mM Hepes, 1 mM EDTA, 0.5 mM EGTA, 0.25% LiCl, 0.5% NP-40), and twice in wash-buffer 4 (20 mM Hepes, 1 mM EDTA, 0.5 mM EGTA, 1% sodium deoxycholate, 0.1% SDS, 0.1% sodium deoxycholate (NaDOC)). Beads and input samples were then incubated with 0.2 µg/µl RNase A in 10 mM Tris–HCl, pH 8.0, for 30 min at 37 °C followed by the addition of 20 µg Proteinase K and incubated in lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% Triton X-100) for 1 h at 65 °C overnight by constant agitation. DNA were extracted using Nucleospin Gel and PCR Cleanup (Machery–Nagel, #740609-250). Binding site occupancy was investigated by qPCR using Foxa1 primers flanking the consensus binding site in the Odf2 promoter (Foxa1-7.6-for gactctgagatatgatgag / Foxa1-7.6-rev gctctgagatatgattggc), or primers flanking a putative DREAM binding-site of sequence TTTGAAA found at position + 27 to + 33 related to the transcription start (DREAM-E1-for cttcatgcccaagattgg / DREAM-E1-rev ggcactgtccgctccctg). Primers were validated first. The Ct-values obtained by either FOXA1- or control IgG-precipitation were adjusted to their respective input Ct (ΔΔCt), and the enrichment of the binding site sequence was calculated by adjustment to the control IgG (ΔΔCt).

**Statistical analyses.** Data were processed and analysed using Excel. The box in the boxplots represents the 25–75th percentile. The median is given as a line, the mean by a cross. The whiskers show the minimum and maximum values inside the range given by Q1-1.5 × interquartile range (IQR) and Q3 + 1.5 × IQR. Data were analysed by Student’s T-test, p < 0.05*, p < 0.01**, p < 0.001***, p < 0.0001****.

**Data availability** There are no datasets generated during and/or analysed during the current study.

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References

1. Hoyer-Fender, S. Primary and motile cilia: their Ultrastructure and Ciliogenesis. In Cilia and Nervous System Development and Function (eds Tucker, K. L. & Caspary, T.) 1–53 (Springer, Netherlands, Dordrecht, 2013).

2. Satir, P. & Christensen, T. Overview of structure and function of mammalian cilia. Annu. Rev. Physiol. 69, 377–400 (2007).

3. Fisch, C. & Dupuis-Williams, P. Ultrastructure of cilia and flagella – back to the future! Biol. Cell. 103, 249–270 (2011).

4. Reiter, J. & Leroux, M. R. Genes and molecular pathways underpinning ciliopathies. Nat. Rev. Mol. Cell Biol. 18, 533–547 (2017).

5. Quarmby, L. M. & Parker, J. D. K. Cilia and the cell cycle. J. Cell Biol. 169, 707–710 (2005).

6. Kim, S. K. & Tsikas, L. Cilia and cell cycle re-entry. Cell Cycle 10, 2683–2690 (2011).

7. Plotnikova, O. V., Pugacheva, E. N. & Golemis, E. A. Primary cilia and the cell cycle. Methods Cell Biol. 94, 137–160 (2009).

8. Wheatley, D. N. Cilia in cell-cultured fibroblasts. III. Relationship between mitotic activity and cilium frequency in mouse 3T6 fibroblasts. J. Anat. 110, 367–382 (1917).

9. Tucker, R. W., Pardee, A. B. & Fujiwara, K. Centriole ciliation is related to quiescence and DNA synthesis in 3T3 cells. J. Cell Biol. 60, 2683–2690 (1973).

10. Malumbres, M. Cyclin-dependent kinases: a family portrait. Nat. Cell Biol. 11, 1275–1276 (2009).

11. Reiter, J. & Leroux, M. R. Genes and molecular pathways underpinning ciliopathies. Nat. Rev. Mol. Cell Biol. 18, 533–547 (2017).

12. Hoyer-Fender, S. Primary and motile cilia: their Ultrastructure and Ciliogenesis. In Cilia and Nervous System Development and Function (eds Tucker, K. L. & Caspary, T.) 1–53 (Springer, Netherlands, Dordrecht, 2013).

13. Fisch, C. & Dupuis-Williams, P. Ultrastructure of cilia and flagella – back to the future! Biol. Cell. 103, 249–270 (2011).

14. Malumbres, M. Cyclin-dependent kinases: a family portrait. Nat. Cell Biol. 11, 1275–1276 (2009).

15. Liu, S. & Kaldia, P. Cdk, cilia and CKEs: roles beyond cell cycle regulation. Development 140, 3079–3093. https://doi.org/10.1242/dev.091744 (2013).

16. Malumbres, M. Cyclin-dependent kinases. Genome Biol. 15, 122 (2014).

17. Wang, Z. Regulation of cell cycle progression by growth factor-induced cell signaling. MDPI Cells 10, 3327. https://doi.org/10.3390/cells10123327 (2021).

18. Johnson, D. G. & Walker, C. L. Cylinders and cell cycle checkpoints. Annu. Rev. Pharmacol. Toxicol. 39, 295–312 (1999).

19. Sanchez, I. & Drylacht, B. D. New insights into cdcks, cdks, and cell cycle control. Sem. Cell Dev. Biol. 16, 311–321 (2005).

20. Carter, M. E. & Brunet, A. FOXO transcription factors: Quick guide. Curr. Biol. 17, R114 (2007).

21. Kops, G. J. P. L. et al. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell Biol. 22, 2025–2036 (2002).

22. Greer, E. L. & Brunet, A. FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene 24, 7410–7425 (2005).

23. Tailon, G., Dobson, M. & Ramakrishnan, G. FoxO transcription factors: regulation by AKT and 14-3-3 proteins. Biochim. Biophys. Acta. 1813, 1938–1945 (2011).

24. Kops, G. J. P. L. et al. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature 419, 316–321 (2002).

25. Tothova, Z. et al. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell 128, 325–339. https://doi.org/10.1016/j.cell.2007.01.003 (2007).

26. Zhang, Y. et al. Regulation of cell cycle progression by forkhead transcription factor FOXO3 through its binding partner DNA replication factor Cdt1. Proc. Natl. Acad. Sci. USA 109, 5717–5722 (2012).

27. Lai, E. & et al. FOXA1, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev. 4, 1427–1436 (1990).

28. Weigel, D. & Jackle, H. The fork head Domain: A novel DNA binding motif of eukaryotic transcriptional factors. Cell 63, 455–456 (1990).

29. Lai, E., Pizziozio, V. R., Tao, W., Chen, W. S. & Darnell, J. E. Jr. Hepatocyte nuclear factor nuclear factor 3α belongs to a gene family in mammals that is homologous to the Drosophila homeotic gene fork head. Genes Dev. 5, 416–427 (1991).

30. Jürgens, G. & Weigel, D. Terminal versus segmental development in the Drosophila embryo: the role of the homoeotic gene fork head. Roux’s Arch. Dev. Biol. 197, 345–354 (1988).

31. Weigel, D., Jürgens, G., Küttner, F., Seifert, E. & Jackle, H. The homoeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. Cell 57, 645–658 (1989).

32. Hoyer-Fender, S. et al. Normal development of forkhead transcription factor (FOXO) family members in mice reveals its functional diversification. Proc. Natl. Acad. Sci. USA 109, 2975–2980. https://doi.org/10.1073/pnas.0400931010 (2004).

33. Colson, M. L. & Kaestner, K. H. Fox transcription factors: from development to disease. Development 143, 4558–4570 (2016).

34. Chen, J., Knowles, H. J., Hebert, J. L. & Hackett, B. P. Mutation of the mouse hepatocyte nuclear factor/forkhead homolog 4 gene results in an absence of cilia and random left-right asymmetry. J. Clin. Invest. 102, 1077–1082 (1998).

35. Anderson, C. T. & Stearns, T. Centriole age underlies asynchronous primary cilium growth in mammalian cells. Curr. Biol. 19, 1498–1502 (2009).

36. Yang, K., Tylkowski, M. A., Huber, D., Tapia Conterras, C. & Hoyer-Fender, S. ODF2/Cenexin maintains centrosome cohesion by restricting β-catenin accumulation. J. Cell Sci. 131, jcs220954. https://doi.org/10.1242/jcs.220954 (2018).

37. Soung, N. K. et al. Requirement of bCenexin for proper mitotic functions of pole-like kinase 1 at the centrosomes. Mol. Cell Biol. 26, 8316–8335 (2006).
47. Plata, N. et al. Transcriptional activation of Otf2/Cexuin by cell cycle arrest and the stress activated signaling pathway (NK pathway). Biochim. Biophys. Acta 1833, 1338–1346 (2013).
48. Hedrick, S. M., Michelin, R. H., Doedens, A. L., Goldrath, A. W. & Stone, E. L. FOXO transcription factors throughout T cell biology. Nat. Rev. Immunol. 12, 649–661 (2012).
49. Bochkis, I. M. et al. Genome-wide location analysis reveals distinct transcriptional circuitry by paralogous regulators FOXA1 and FOXA2. PLoS Genet. 8(6), e1002770. https://doi.org/10.1371/journal.pgen.1002770 (2012).
50. Dylkowski, M. A., Yang, K., Hoyer-Fender, S. & Stoykova, A. Pax6 controls centriole maturation in cortical progenitors through Otf2. Cell Mol. Life Sci. 72, 1795–1809. https://doi.org/10.1007/s00018-014-1766-1 (2014).
51. Ait-Lounis, A. et al. Novel function of the ciliogenic transcription factor RXF3 in development of the endocrine pancreas. Diabetes 56, 950–959 (2007).
52. You, Y. et al. Role of Fox-box factor Fox1 in differentiation of ciliated airway epithelial cells. Am. J. Physiol. 286, L650–L657 (2004).
53. El Zein, L. et al. RXF3 governs growth and beating efficiency of motile cilia in mouse and controls genes involved in human ciliopathies. J. Cell Sci. 122, 3180–3189 (2009).
54. Lemeille, S. et al. Interplay of RXF transcription factors 1, 2 and 3 in motile ciliogenesis. Nucleic Acids Res. 48, 9019–9036 (2020).
55. Jain, R. et al. Temporal relationship between primary and motile ciliogenesis in airway epithelial cells. Am. J. Respir. Cell Mol. Biol. 43, 731–739. https://doi.org/10.1165/rcmb.2009-03280C (2010).
56. Hannenhalli, S. & Kaestner, K. H. The evolution of Fox genes and their role in development and disease. Nat. Rev. Genet. 10, 233–240 (2009).
57. Laoukili, J. et al. FOXM1 is required for execution of the mitotic program and chromosome stability. Nat. Cell Biol. 7, 126–136 (2005).
58. Chen, X. et al. The forkhead transcription factor FOXM1 controls cell cycle-dependent gene expression through an atypical chromatin binding mechanism. Mol. Cell Biol. 33, 227–236 (2013).
59. Müller, G. A. et al. The CHR site: definition and genome-wide identification of a cell cycle transcriptional element. Nucleic Acids Res. https://doi.org/10.1093/nar/kuu696 (2014).
60. Medema, R. H., Kops, G. J., Bos, J. I. & Burgering, B. M. AFX-like forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p72kip1. Nature 404, 782–787 (2000).
61. Schmidt, M. et al. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D1. Mol. Cell Biol. 22, 7842–7852 (2002).
62. Costa, R. H., Grayson, D. R. & Darnell, J. E. Jr. Multiple hepatocyte-enriched nuclear factors function in the regulation of tran/sre-fam and alpha 1-antitrypsin genes. Mol. Cell Biol. 9, 1415–1425 (1989).
63. Behr, R. et al. Mild nephrogenic diabetes insipidus caused by Foxa deficiency. J. Biol. Chem. 2769, 41936–41941 (2004).
64. Friedman, J. R. & Kaestner, K. H. The Foxa family of transcription factors in development and metabolism. Cell Mol. Life Sci. 63, 2317–2328 (2006).
65. Weinstein, D. C. et al. The winged-helix transcription factor HNF-3 beta is required for nodule development in the mouse embryo. Cell 78, 575–588 (1994).
66. Ang, S. L. & Rossant, J. HNF-3B is essential for node and nodule formation in mouse development. Cell 78, 561–574 (1994).
67. Matyromatiskis, Y. E. et al. Foxa1 and Foxa2 positively and negatively regulate Shh signalling to specify ventral midbrain progenitor identity. Mech. Dev. 128, 90–103 (2011).
68. Behr, R., Sackett, S. D., Bochkis, I. M., Le, P. P. & Kaestner, K. H. Impaired male fertility and atrophy of seminiferous tubules caused by a deficiency in Foxa1 and Foxa2. Am. J. Physiol. Cell Mol. Life Sci. 306, 636–645 (2007).
69. Kawashima et al. FOXM1 and FOXA2 are required for the development of a functional testis in mice. Proc. Nat. Acad. Sci. USA 107, 8562–8566 (2010).
70. Carvajal, J. M. et al. Bookmarking by specific and nonspecific binding of Foxa1 pioneer factor to mitotic chromosomes. Genes Dev. 27, 251–260 (2013).
71. Clark, K. L., Halay, E. D., Lai, E. & Burley, S. K. Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles the DNA-binding domain of the mammalian HNF-3 beta. J. Biol. Chem. 272, 1795–1805 (1997).
72. van Dam, T. J. P., Hnepen, R. G., Hnepen, M. A. & Bus. H. The SYSCILIA gold standard (SCGSv1) of known ciliary genes and components and its applications within a systems biology consortium. Cilia 3, 1795–1809. https://doi.org/10.1007/s00018-014-1766-1 (2014).
73. Zaret, K. S. et al. The forkhead transcription factor Foxa1 and linker histone H1. Cold Spring Harb. Symp. Quant. Biol. 75, 219–226 (2010).
74. van Dam, T. J. P. et al. FOXA2 and FOXA1 are required for cilia formation and ciliogenesis in mouse embryos. Development 143, 1833–1837 (2016).
75. Bernardo, G. M. & Keri, R. A. FOXA1: a transcription factor with parallel functions in development and cancer. Biosci. Rep. 32, 113–130. https://doi.org/10.1042/BSR20110046 (2012).
76. Epstein, D. J., McMahon, A. P. & Joyner, A. L. Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and—-independent mechanisms. Development 126, 281–292 (1999).
77. Filosa, S. et al. Goosecoid and HNF-3beta genetically interact to regulate neural tube patterning during mouse embryogenesis. Development 124, 2843–2854 (1997).
78. Pasca di Magliano, M. & Hebrok, M. Hedgehog signalling in cancer formation and maintenance. Nat. Rev. Cancer. 3, 903–911 (2003).
79. Bangs, F. & Anderson, K. V. Primary cilium and mammalian ciliogenesis. Cold Spring Harb. Perspect. Biol. 9, a028175 (2017).
80. Higgin, M., Obadi, I. & McMorrow, T. Primary cilium and their role in cancer. Oncol. Lett. 17, 3041–3047 (2019).
81. Fabbri, L., Bost, F. & Mazure, N. M. Primary cilia in cancer hallmarks. Int. J. Mol. Sci. 20, 1336. https://doi.org/10.3390/ijms20061336 (2019).
82. Albig, W. & Doenecke, D. The human histone gene cluster at the D6S105 locus. Hum. Genet. 101, 284–294 (1997).
83. Denissov, S. et al. Identification of novel functional TBP-binding sites and general factor repertoires. EMBO J. 26, 944–954 (2007).

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Author contributions
Material preparation, data collection, and analyses were performed by C.C.C., A.B., M.C., M.O., and S.H.-F. S.H.-F. designed the experiments, wrote the manuscript, and prepared the figures. All authors commented on previous
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