

The LIM-only coactivator FHL2 modulates WT1 transcriptional activity during gonadal differentiation

Xiaojuan Du^a, Philip Hublitz^a, Thomas Günther^a, Dagmar Wilhelm^b,
Christoph Englert^b, Roland Schüle^{a,*}

^aUniversitäts-Frauenklinik und Zentrum für Klinische Forschung, Klinikum der Universität Freiburg, Breisacherstrasse 66, 79106 Freiburg, Germany

^bInstitute of Toxicology and Genetics, Forschungszentrum Karlsruhe, P.O. Box 3640, 76021 Karlsruhe, Germany

Received 5 February 2002; received in revised form 14 May 2002; accepted 4 June 2002

Abstract

An essential step during sex determination is the maintenance of the Müllerian duct in females and its regression in males caused by the expression of Müllerian inhibiting substance (MIS). In testes, the Wilms' tumor suppressor and the orphan nuclear receptor SF1 cooperatively bind to the promoter and activate transcription of MIS. In the ovaries, on the other hand, the orphan nuclear receptor DAX1 binds to SF1, inhibits transactivation by WT1/SF1 and thereby suppresses the induction of MIS expression. In addition, WT1 itself is responsible for the upregulation of DAX1 transcription. So far, little is known on which protein–protein interactions or cofactors elicit the spatiotemporal control of WT1-mediated transcription. Here we demonstrate coexpression of the LIM-only coactivator FHL2 and WT1. FHL2 and WT1 functionally interact both in vitro and in vivo. The importance of this interaction is revealed by the ability of FHL2 to potentiate the synergistic induction of MIS gene expression by WT1/SF1. Moreover, FHL2 coactivates transactivation of the DAX1 promoter by WT1. Hence, we present FHL2 as a novel transcriptional coactivator of WT1. The ability to modulate both DAX1 and MIS expression might allow FHL2 to act in the molecular fine tuning of WT1-dependent control mechanisms in the reproductive organs.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: WT1; Transcriptional regulation; FHL2; Coactivator; Nuclear hormone receptor

1. Introduction

In mammalian ontogeny, the gonadal system has the potential to differentiate either into testes or ovaries. The fate of the bipotential gonad is mainly controlled by the presence or absence of the transcription factor SRY (sex-determining region on the Y chromosome) [1]. In absence of SRY, the gonads develop into ovaries and the Müllerian ducts develop into oviducts, uterus, and upper end of the vagina. If the Y chromosome is present, SRY is expressed during a short developmental period, in mouse between E10.5 and E12.5, and directs the gonads to form testes. Once the testicular cords are formed, they start to produce the Müllerian inhibiting substance (MIS), a transforming

growth factor β -like hormone. MIS is the key regulator for the active regression of the Müllerian ducts, further establishing the male specific pathway. Mice with XY karyotype and deficiency of MIS develop as pseudohermaphrodites [2], while in fetal XX rats, addition of exogenous MIS results in partial sex reversion [3]. Therefore, the regulation of MIS expression is a central event in sex determination.

Characterization and analysis of the promoter region of MIS identified an extended consensus half site that is bound by SF1 [4–6]. A mutation of this site abolished SF1 binding and reduced MIS expression although MIS transcription is correctly initiated and the reduced level of MIS protein is still sufficient for the regression of the Müllerian duct [4,7]. Recent findings demonstrated that SF1 associates with WT1 through the DNA binding domain (DBD) of SF1 and that the association results in a synergistic activation of MIS gene expression depending on a bipartite WT1/SF1-binding site [8].

The transcription factor WT1 can function either as a transcriptional activator or as a repressor depending on the

* Corresponding author. Tel.: +49-761-2706310; fax: +49-761-2706311.

E-mail address: schuele@frk.ukl.uni-freiburg.de (R. Schüle).

cell type and promoter context analyzed [9–11]. Abnormalities in the WT1 gene have been shown to be associated with the development of kidney tumors (referred to as Wilms' tumor) and Denys–Drash syndrome, characterized by streak gonads and pseudohermaphroditism [12,13]. Mice with disruption of WT1 display an arrest of kidney and gonadal development in early developmental stages [14]. WT1 is alternatively spliced at exons 5 and 9, respectively, thus resulting in four isoforms [15]. Exon 5 encodes a 17 amino acid segment in the N-terminus of WT1 whereas exon 9 codes for the variable three amino acid insertion (KTS) between zinc fingers III and IV. The KTS-insertion modulates the DNA-binding properties of the WT1 isoforms. WT1(–KTS) binds with high affinity to GC-rich response elements, whereas WT1(+KTS) preferentially associates with general splicing factors and is incorporated into spliceosomes [9,16–18].

The C-terminus of WT1 harbors the zinc-finger type DBD. The N-terminal region of WT1 contains a transactivation domain, spanning the amino acids 182 to 250, and a repression domain from amino acids 85 to 124 [19]. Besides MIS in testis, a number of potential WT1 target genes have been isolated. Among them, the gene coding for the orphan nuclear receptor DAX1 (dosage-sensitive sex-reversal, adrenal hypoplasia congenita, on the X-chromosome, gene 1) is upregulated by WT1 during ovarian differentiation [20]. In ovaries, DAX1 directly interacts with SF1 and abolishes MIS expression by antagonizing the transcriptional synergism between WT1 and SF1 [21,22].

Therefore, in sex differentiation, WT1 has different functions in male and female. WT1 is needed for the upregulation of MIS in males, whereas in females, it has the opposite function by upregulating DAX1 gene expression. However, the mechanism by which WT1 regulates gene expression in either testis or ovary is not fully understood, and little is known about bona fide coactivators of WT1.

Recently, we identified the four-and-half LIM-domain protein FHL2 as a coactivator for the androgen receptor [23,44]. In this report, we show that FHL2 is expressed in the developing embryonic testes and ovaries and that FHL2 expression is overlapping with that of WT1. Both genes are concomitantly expressed with SF1 and MIS in testis and with DAX1 in ovaries (reviewed in Ref. [24]). Further analyses reveal direct interaction between FHL2 and WT1 in vivo and in vitro. Accordingly, FHL2 functions as a coactivator of WT1 by either potentiating the synergistic induction of MIS by WT1/SF1 or increasing DAX1 expression through transcriptional activation of WT1. Taken together, our data suggest that FHL2 functions as a novel coactivator of WT1-dependent transcription. Thus, the analysis of FHL2 sheds new light to the yet ill-defined regulatory functions of WT1 in the fine-tuning of genes important in reproductive organ differentiation.

2. Materials and methods

2.1. Recombinant plasmids

MIS-RE1-LUC was generated by inserting two copies of the WT1/SF1-response element (5'-GATCCCACTGTCCCCAAGGTCAA-3'; [4]) at the *Bam*HI site of the minimal promoter plasmid pTATA-LUC [25]. The mDAX1-LUC reporter [20], the expression plasmids for hFHL2 and the respective deletion mutants [23], the expression plasmids for mSF1 [26] and for hWT1 lacking Exon 5 and the KTS insertion, further abbreviated as WT1(–/–) (pcDNA3-WT1 and pCWL0) [27], were described previously. pCMX-Flag_{3x} was constructed by inserting two copies of the Flag-epitope (MDWLHDDG) at the *Eco*RI site of pCMX-Flag [26]. pCMX-Flag_{3x}-WT1 and pGBKT7-WT1 were constructed by inserting the WT1 cDNA at the *Eco*RI sites of pCMX-Flag_{3x} or pGBKT7 (Clontech). The cDNA fragments for the WT1 expression plasmids pCMX-K-ATG-WT1_{1–429}, pCMX-K-ATG-WT1_{1–298}, pCMX-K-ATG-WT1_{298–449}, pCMX-K-ATG-WT1_{1–182}, pCMX-K-ATG-WT1_{182–298}, and pGBKT7-WT1–FIAD_{182–298} were produced by PCR amplification and cloned at the *Eco*RI site of the corresponding expression plasmid (numbers indicate the first and the last amino acid of WT1). pBSSK-mFHL2 was constructed by inserting the mFHL2 cDNA at the *Eco*RI site of pBluescript SK (Stratagene), pBSK-hWT1 was described by Herzer et al. [28]. Exact details and sequences of the oligonucleotides will be provided upon request. All constructs were verified by sequencing.

2.2. RT-PCR

DNaseI-treated RNA was isolated using RNAwiz (Ambion) and used for reverse transcription. PCR involved 35 cycles for FHL2 or 25 cycles for HPRT under standard conditions. The following primers were used: FHL2 5'-GTCCTACAAGGATCGGCACTGG-3'; 5'-CCAGCTCCCGTGAAAATA-3'; HPRT 5'-GTTGAGAGATCATCTCCACC-3'; 5'-AGCGATGATGAACCAGGTTA-3'.

2.3. Whole mount in situ hybridization

In situ hybridizations followed the previously described protocol by Herzer et al. [28]. Briefly, mouse embryos were fixed in 4% paraformaldehyde and probed with in vitro transcribed digoxigenin (DIG)-UTP labeled probe. The hybridization was detected with alkaline phosphatase (AP) conjugated anti-DIG antibody and visualized using BM purple (Roche) as AP substrate.

2.4. Cell culture and transfections

293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

Transient transfection assays were performed according to the standard calcium-phosphate coprecipitation method [26]. The total amount of transfected DNA was kept constant (4 µg) by adding pCMX and pUC18 (Clontech), respectively. Luciferase activity was assayed as recommended by the manufacturer (Promega) in an EG&G luminometer (Berthold). Relative light units were normalized to β-galactosidase activity and protein concentration using the Bradford dye-assay (BioRad). All experiments were repeated at least three times. Student's *t*-test was used to determine significant differences ($P \leq 0.05$).

2.5. GST pulldown assay

Expression of the GST fusion proteins (Pharmacia) and the coupled *in vitro* transcription–translation reactions (Promega) in the presence of [³⁵S]methionine were performed according to the manufacturer's instructions. GST pulldown assays were performed as described [23] using buffer containing 150 mM KCl. Ten percent of the *in vitro* translated protein was loaded as input.

2.6. Preparation of nuclear extracts

Extracts from 293 cells, transfected with the respective constructs, were prepared as described [20]. Briefly, the cytoplasmic fraction was obtained by hypotonic lysis of the cells and removed quantitatively. Intact nuclei were collected by centrifugation and nuclear proteins extracted in high salt buffer. Insoluble material was pelleted and the concentration of the supernatant was determined using the Bradford dye assay (Bio-Rad).

2.7. Yeast two-hybrid analysis

Yeast two hybrid studies were performed as recommended by the manufacturer (Clontech). Briefly, HF7c cells were transformed using the LiAc method [29] with pGBKT7, pGBKT7-WT1, or pGBKT7-WT1–FIAD (carrying tryptophan prototrophy markers) in concert with either pACT2 or pACT2-FHL2 (carrying leucine prototrophy markers). Single yeast colonies were resuspended in TE and parallel-plated on selection media lacking leucine and tryptophan to assess cotransformation efficiency and on selection media lacking leucine, tryptophan, and histidine to assay the interaction of bait and prey. Yeast were cotransformed with Gal4-DBD-p53 and Gal4-AD-SV40 largeT as positive control. Plates were incubated at 30 °C and then photographed (3 and 7 days of incubation for double or triple dropout plates, respectively).

2.8. Coimmunoprecipitation assays and Western blot analysis

293 cells were co-transfected with 10 µg pCWL0 and either 10 µg pCMX-FHL2 or 10 µg pCMX-Flag-FHL2.

Four micrograms of α-Flag antibody (M5, Sigma) was immobilized on 40 µl 1:1 slurry of gamma-bind-Sepharose G™ (Pharmacia) and incubated with 200 µg precleared nuclear extracts. Precipitated complexes were washed five times in IP-buffer (20 mM Tris–HCl pH 8.0, 250 mM NaCl, 0.5% NP40, 0.1 µg/µl BSA, and 0.5 mM Pefablock) and separated on 10% SDS gels. Western blots were decorated with the α-WT1 C-19 antibody (Santa Cruz). Secondary antibody and chemoluminescence procedures were performed according to the manufacturer's instructions (Amersham).

2.9. Immunostainings

293 cells were seeded out on fibronectin-coated coverslips and transiently transfected with 0.2 µg pCWL0 and pCMX-FHL2. Cells were fixed, permeabilized with 0.2% Triton X100 and incubated with rabbit α-FHL2 [23] and mouse α-WT1 F6 antibodies (Santa Cruz). Following repeated washing steps with PBS, protein localization was visualized by secondary antibodies coupled to fluorescent dyes Alexa488 (α-FHL2, green emission) or Alexa546 (α-WT1, red emission, both Molecular Probes). Nuclei were counterstained with DAPI (Roche Diagnostics).

3. Results

3.1. FHL2 and WT1 are coexpressed in mouse embryonic gonads

To investigate the FHL2 expression pattern during mouse embryonic development, we performed RT-PCR analyses with FHL2-specific primers in different tissues. Besides the described expression of FHL2 in the heart (Refs. [23,30], and data not shown), we noticed that FHL2 is expressed in both, embryonic testes and, to a weaker extent, in embryonic ovaries (Fig. 1A). Whole-mount *in situ* hybridizations with an FHL2-specific probe showed that FHL2 expression is most prominent at E13.5 (Fig. 1B) and fades to background levels during later stages of differentiation (data not shown). Interestingly, the product of the tumor suppressor gene WT1 shows an overlapping expression pattern with FHL2 during this period in testis as well as in ovary (Fig. 1B; Ref. [31] and data not shown). These results led us to investigate a possible role of FHL2 in the regulation of factors involved in gonadal differentiation, such as WT1, MIS, and DAX1.

3.2. FHL2 interacts with WT1 *in vitro*

To analyze a potential interaction between FHL2 and WT1 *in vitro*, GST pulldown experiments were performed with bacterially expressed GST–FHL2 and several *in vitro* translated [³⁵S]methionine-labeled proteins. Fig. 2A

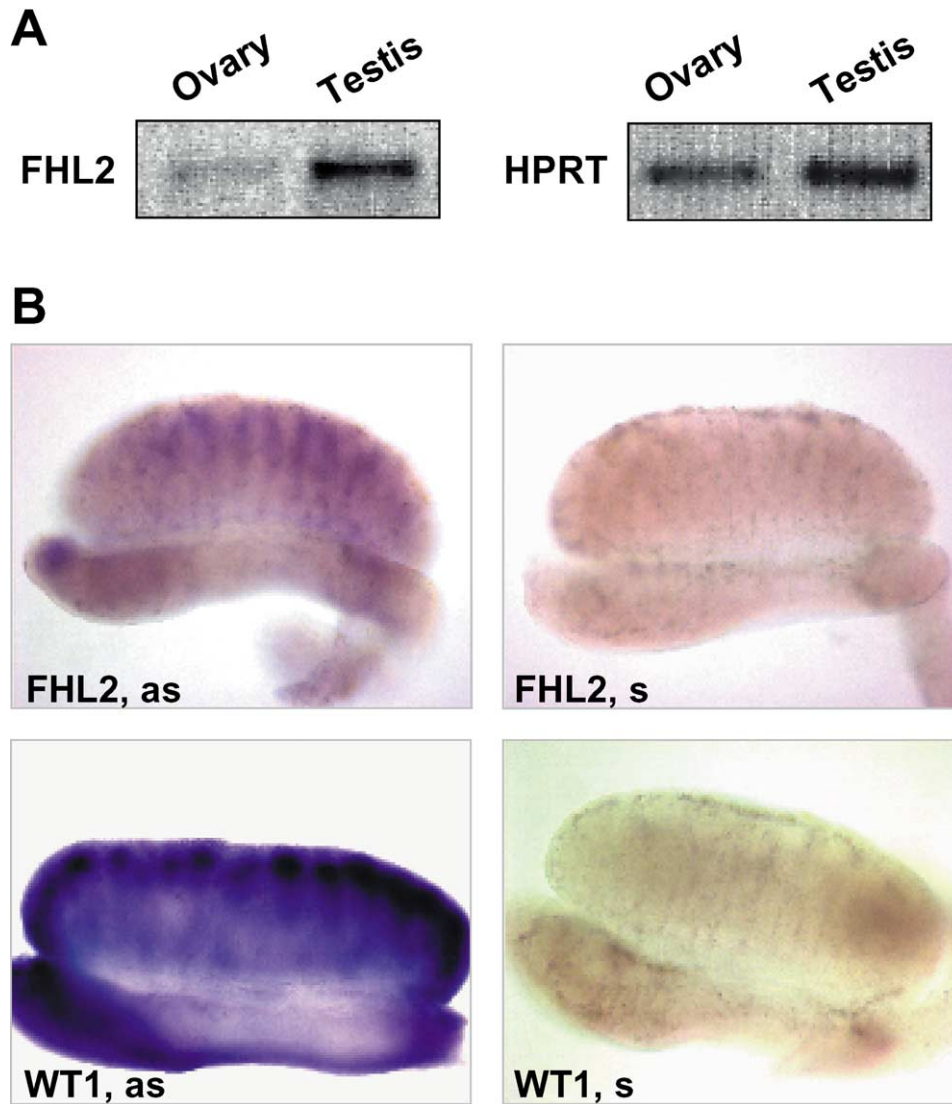


Fig. 1. FHL2 is expressed in embryonic gonads. (A) RT-PCR analyses with FHL2-specific primers demonstrate expression of FHL2 in mouse gonads at E13.5 (left panel). The RT-PCR for the housekeeping gene HPRT is used as a control for comparable amounts of cDNA (right panel). (B) Whole mount in situ hybridization shows FHL2 (upper left) and WT1 (bottom left) expression in gonads at E13.5 (as depicts antisense hybridization). Hybridization to the sense probes (s) is shown as control in the corresponding right panels.

demonstrates that WT1 specifically associates with GST-FHL2 while no interaction with the control GST protein is detected. In addition, other transcription factors involved in WT1-dependent gene regulation such as SF1, p53, or SOX9 fail to interact thus demonstrating the specificity of the interaction between GST-FHL2 and WT1 (Fig. 2A). To define the domains of FHL2 that mediate the interaction with WT1, different LIM-domain deletion mutants were generated and expressed as GST fusion proteins. Fig. 2B shows that GST-FHL2 (LIM 0–2) interacts with WT1 as strong as full-length GST-FHL2 while the corresponding C-terminal deletion mutant GST-FHL2 (LIM 3–4) lost the ability to interact with WT1. Deletion mutants containing either LIM0–1 or LIM1–2 still bind to WT1 albeit with reduced affinity.

However, none of the single LIM domains interacted with WT1 (Fig. 2B).

In reciprocal experiments, we demonstrate that the N-terminus of WT1 does interact with FHL2, while the C-terminal zinc finger domain of WT1 (aa 298–429) fails to do so. Further WT1 deletion mutants delineate the FHL2 interaction domain (FIAD) in WT1 to amino acids 182–298. Interestingly, the FIAD overlaps with the described activation domain of WT1 (amino acids 182 to 250) (Fig. 2C and Ref. [19]).

3.3. FHL2 interacts with WT1 in vivo

To investigate whether FHL2 interacts with WT1 in vivo, yeast two-hybrid assays were performed. Full-length WT1

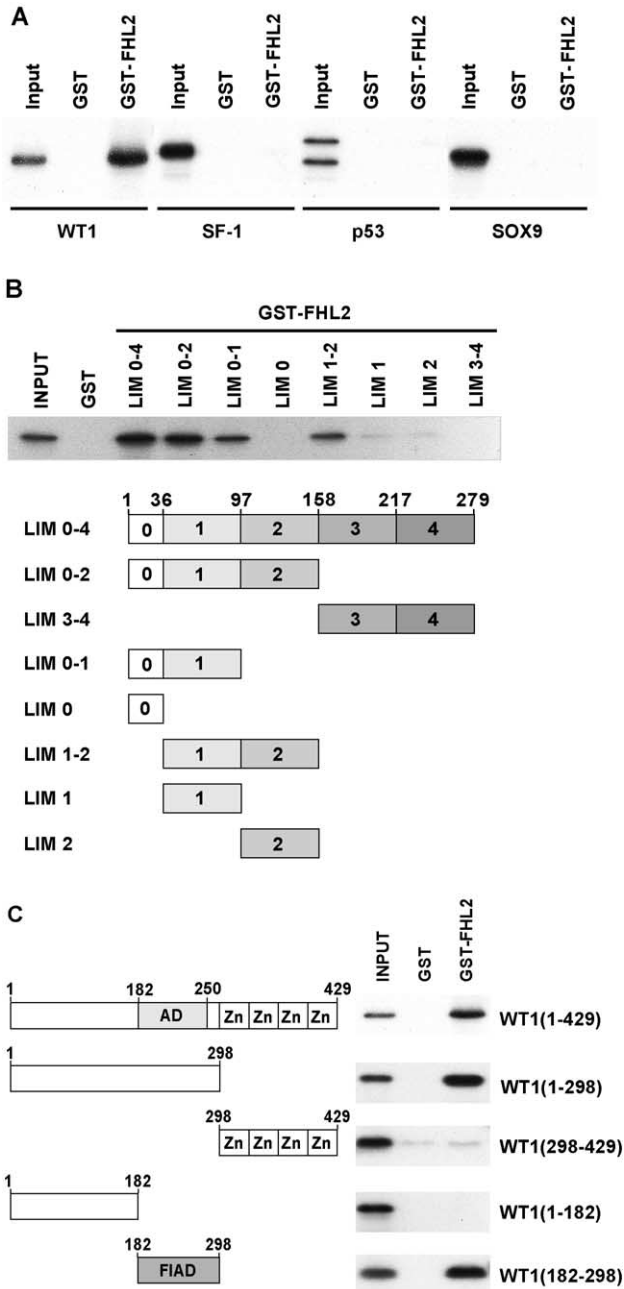


Fig. 2. FHL2 and WT1 specifically interact in vitro. GST-pulldown experiments were performed to assay for binding of [³⁵S]methionine-labeled proteins to the indicated GST-FHL2 fusion proteins. The corresponding input control is 10%. (A) FHL2 specifically interacts with WT1. No interaction is detectable between FHL2 and other factors involved in WT1-dependent gene regulation, such as SF1, p53, and SOX9. (B) The WT1 interaction domain in FHL2 is mapped to the N-terminal LIM-domains 0–2. A schematic representation of the different FHL2 deletion mutants fused to GST is shown. (C) The FHL2 interaction domain (FIAD) in WT1 is mapped to a region overlapping with the activation domain (AD) of WT1 and is located between amino acids 182 and 298. A schematic representation of the WT1 deletion mutants is depicted.

or the FHL2 interaction domain FIAD were used as bait proteins, and full-length FHL2 fused to the Gal4 activation domain (FHL2-AD) was used as prey. Fig. 3A shows that

cotransformation of either full-length WT1 or the FIAD of WT1 with FHL2 allows yeast growth on media lacking the nutritional selection marker histidine, thus confirming interaction in vivo.

The in vivo interaction between FHL2 and WT1 is further demonstrated by coimmunoprecipitation experiments (Fig. 3B). Nuclear extracts from 293 cells cotransfected with either FHL2 or Flag-tagged FHL2 and WT1 were immunoprecipitated using the α -Flag antibody M5. Western blot analyses show that the Flag-FHL2/WT1 complex is formed in vivo and that WT1 is coimmunoprecipitated, whereas no WT1 protein is detectable in immunoprecipitates from 293 cells transfected with untagged FHL2 and WT1. The unrelated signal, indicated as M5, is generated due to the detection of M5 antibody heavy chain by the secondary antibody. To analyze the subcellular distribution of FHL2 and WT1, we performed immunofluorescence analyses in 293 cells transfected with expression plasmids for WT1 and FHL2 (Fig. 3C). FHL2 is found both in the cytoplasm and in the nucleus and colocalizes with nuclear WT1. Taken together, our data show interaction of FHL2 and WT1 in vivo and demonstrate an overlapping subcellular expression pattern of both proteins.

3.4. FHL2 modulates WT1-dependent transcription

To examine the potential modulation of WT1 function by FHL2, the DAX1 promoter was chosen as a target gene because WT1 is known to upregulate DAX1 expression during gonadal development [20]. As described, transient transfection of increasing amounts of WT1 (–/–) resulted in DAX1-reporter gene activation. Importantly, coexpression of FHL2 and WT1 (–/–) mediates significant superactivation of the DAX1 promoter in a dose-dependent manner (Fig. 4A). No transactivation is observed with FHL2 alone (Fig. 4A) or when the WT1(+KTS) or WT1(–KTS/+Exon5) isoforms are coexpressed with FHL2 (data not shown).

To investigate the potential regulation of WT1 by FHL2 on a gene expressed in testis, the MIS promoter was chosen. In testis, WT1 is known to associate and synergize with SF1 in the upregulation of MIS gene expression [4,8]. Transient transfections were performed with the MIS-RE1-LUC reporter construct that contains two copies of the WT1/SF1 binding site. As described, cotransfection of either WT1 (–/–) or SF1 led to little activation, whereas coexpression of both WT1 (–/–) and SF1 synergistically activated the MIS reporter (Ref. [8] and Fig. 4B). Expression of increasing amounts of FHL2 in the presence of WT1 (–/–) and SF1 results in additional, significant superactivation of the MIS reporter. The controls show that this superactivation is dependent on both, WT1 (–/–) and SF1, and is not mediated by FHL2 alone (Fig. 4B). Similar results were obtained in other cell lines such as Cos-7 or CV1 (data not shown). Therefore, in summary, our data

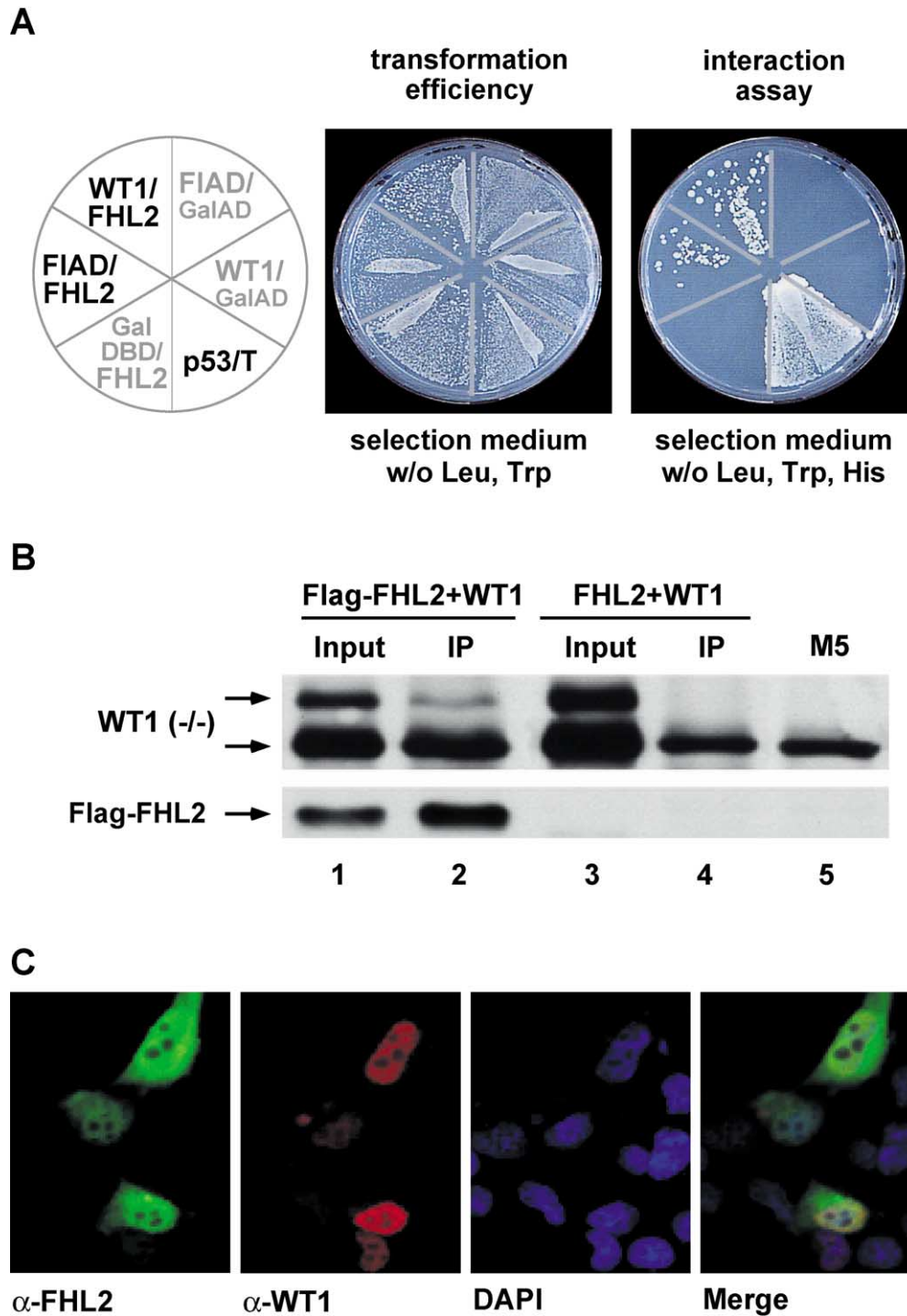


Fig. 3. FHL2 interacts with WT1 in vivo. (A) Yeast two-hybrid analyses demonstrate the interaction of either WT1 or WT1–FIAD (aa 182–298) with FHL2 in vivo. WT1 and FIAD correspond to WT1 and WT1–FIAD fused to the Gal4–DBD, FHL2 indicates a FHL2 fusion to the Gal4-activation domain. Gal-DBD and Gal-AD represent the empty expression vectors. Yeasts transformed with Gal4-DBD-p53 and Gal4-AD-SV40-largeT serve as positive growth control. Yeasts are grown in presence of 5 mM 3-aminotriazole. (B) WT1 coimmunoprecipitates with FHL2. Nuclear extracts of 293 cells transfected with WT1 and either FHL2 or Flag-FHL2 are immunoprecipitated with α -Flag-specific antibody M5. Ten percent of the extract used for immunoprecipitation (IP) is loaded as an input control in lanes 1 and 3. The immunoprecipitated complexes are separated in lanes 2 and 4. Western blots were either decorated with an α -WT1-specific antibody (upper panel) or with M5 (lower panel). WT1 appears as a double band. The lower band comigrates with the M5 antibody heavy chain that is detected by the secondary antibody (lane 5, indicated M5). Specific coimmunoprecipitation of WT1 is only achieved when Flag-tagged FHL2 is present in the lysate (lane 2, upper panel). No signal was obtained using untagged FHL2 protein (upper panel, lane 4, and lower panel, lanes 3–4). (C) FHL2 and WT1 colocalize in the nucleus. FHL2 immunoreactivity is detected in both the cytoplasm and the nucleus (green fluorescence), WT1 immunoreactivity (red fluorescence) is detectable in the nucleus only. Nuclei are counterstained with DAPI. Subcellular colocalization of FHL2 and WT1 in the nucleus is confirmed by overlay (Merge).

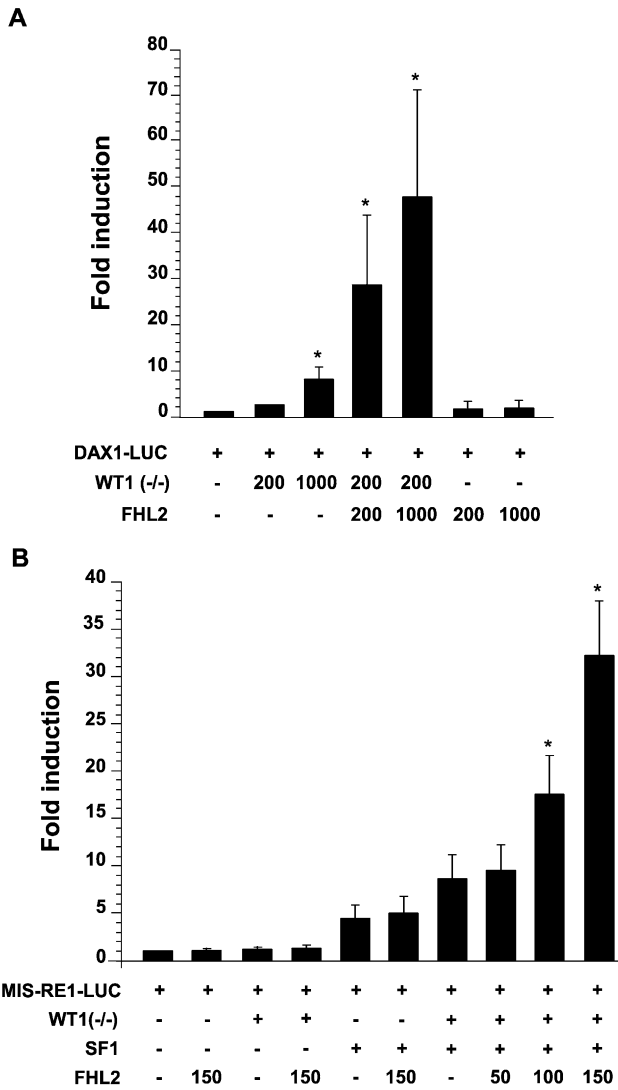


Fig. 4. FHL2 coactivates WT1-dependent gene expression. (A) FHL2 potentiates WT1 activation on the DAX1 promoter. Cotransfections were carried out using a DAX1-reporter plasmid (0.5 μ g/well) and the indicated amounts of expression plasmids for WT1 and FHL2 in 293 cells. (B) The synergistic effect of SF1 and WT1 is potentiated by FHL2. The MIS reporter plasmid (0.5 μ g/well) is cotransfected with expression plasmids for SF1 and WT1 (50 ng each) and increasing amounts of FHL2 in 293 cells. Cotransfection of 150 ng FHL2 alone or in combination with either with 50 ng WT1 or SF1 does not result in significant superactivation. Asterisks indicate significance ($P \leq 0.05$).

demonstrate that FHL2 functions as a bona fide coactivator in WT1-dependent gene regulation.

4. Discussion

In this study, we investigated the expression of the LIM-only coactivator FHL2 during mouse gonadal development and detected that FHL2 can function as a coactivator of WT1 in the differentiating gonads.

A crucial event in female sex development is the differentiation of the Müllerian ducts into oviduct, uterus, and the upper end of the vagina. In order to create a permissive environment for the differentiation of the Müllerian ducts, it is a prerequisite of utmost importance to ensure that MIS is not expressed (Fig. 5). The orphan nuclear receptor DAX1 was shown to function as a repressor for SF1, possibly by recruiting the nuclear hormone receptor co-repressor N-CoR [22] and thus to antagonize WT1/SF1 induced MIS expression. In addition, SF1 expression in ovaries dramatically declines during the period between E13.5 and E16.5 [32,33]. DAX1 is a direct target gene of WT1 and WT1 binds to and activates the DAX1 promoter in ovaries [20]. Our results now show that FHL2 significantly coactivates WT1-dependent DAX1 reporter activity (Fig. 4A). The biological importance of DAX1 in ovarian differentiation is still elusive, though. It has been shown by Yu et al. [34] that targeted deletion of DAX1 does neither affect ovarian development nor female fertility.

The synergistic effect of WT1 and SF1 in the upregulation of MIS gene expression is well documented in vitro (Ref. [8] and Figs. 4B and 5). The importance of SF1 in the control of MIS expression in testes has been demonstrated in mice lacking SF1 binding sites in the MIS promoter. MIS level in these mice was more than three times reduced. Surprisingly though, the amount of MIS was sufficient to provide a local environment necessary for Müllerian duct regression [7]. It has been suggested that the upregulation of MIS expression by SF1 is rather important for non-Müllerian duct tissue such as testicular Sertoli and Leydig cells to modulate their differentiation and function [35,36]. Expression of very high MIS levels in transgenic mice on the other hand can disrupt the differentiation of Leydig cell precursors [36]. Our data now demonstrate that FHL2 and WT1 not only exhibit an overlapping expression pattern in developing testis, but also that FHL2 superactivates the synergistic transcriptional induction of the MIS promoter by WT1/SF1 (Fig. 4B). The dispensibility of the SF1 binding site in the MIS promoter points out that FHL2 may not be involved in the initiation of the Müllerian duct regression in males [7].

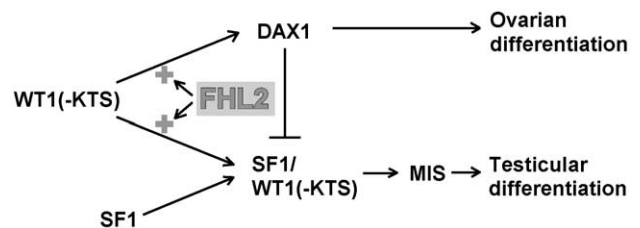


Fig. 5. Putative role of FHL2 in the fine-tuning of gene regulation during gonadal differentiation. In testicular differentiation, FHL2 directly interacts with WT1 and promotes activation of MIS expression. In ovarian differentiation, on the other hand, the WT1/FHL2 interaction results in the transcriptional activation of DAX1 and subsequently ensures repression of MIS expression by antagonizing SF1 action.

Recently, FHL2-deficient mice have been published [30,37]. So far, no phenotype in gonadal differentiation has been described, further indicating that FHL2 rather plays a modulating than a decisive role. A possible explanation for the difficulty to detect a gonadal phenotype might also be the functional redundancy of the closely related LIM-only family members FHL1, FHL3, FHL4, and ACT [38], which are all as well expressed in the developing gonads (data not shown).

FHL2 specifically interacts with WT1 *in vitro*, but does not associate with other transcription factors involved in gonadal regulation processes, such as SF1, SOX9, or p53 (Fig. 2A). We mapped the FHL2 interaction domain (FIAD) in WT1 to amino acids 182 to 298, which overlaps with the described transactivation domain of WT1 [19]. Although recent studies identified several proteins binding to different domains of WT1 [39–42], so far CBP and PAR4 are the only transcriptional cofactors described to bind to WT1. The interaction with PAR4 is dependent on the presence of Exon 5 in the WT1 transactivation domain [43], and the CBP-interaction domain in WT1 was mapped to the first two zinc fingers [11]. In contrast, our data demonstrate that FHL2 is the first coactivator that directly associates with the WT1 transactivation domain of the (–KTS/–Exon5) isoform.

The WT1 interaction domain in FHL2 is mapped to LIM domains 0–2 (Fig. 2B). No single LIM domain suffices for interaction, suggesting that the 3D structure of LIM domains 0 to 2 is required to form the proper interaction surface for WT1 binding. As the association of WT1 and FHL2 was verified by yeast two-hybrid assays and coimmunoprecipitation experiments, we demonstrate that both proteins are capable of interacting *in vivo* (Fig. 3A,B). In addition, immunofluorescence analyses show subcellular colocalization of FHL2 and WT1 and further support the physiological relevance of their interaction (Fig. 3C).

In summary, our data present a model in which FHL2 acts as a bona fide coactivator for WT1 during the process of gonadal differentiation (Fig. 5): In the emerging testicular organ, FHL2 directly supports the transcriptional activation of MIS gene expression by SF1 and WT1. FHL2 interacts with WT1 and thereby is part of the transcriptional coactivator complex enhancing WT1 function. In ovarian differentiation, on the other hand, the presence of FHL2 and its direct interaction with WT1 is responsible for the transcriptional upregulation of DAX1. Taken together, FHL2 coactivates WT1-dependent transcription of factors that are involved in both testicular and ovarian differentiation.

Acknowledgements

We thank Manfred Gessler, Thomas Dobner, and Jerry Pelletier for providing plasmids and all the members of the Schüle laboratory for the support and fruitful discussions.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (En 280/2-4) to C.E. and (SFB 364/C8) to R.S.

References

- [1] A.H. Sinclair, P. Berta, M.S. Palmer, J.R. Hawkins, B.L. Griffiths, M.J. Smith, J.W. Foster, A.M. Frischauf, R. Lovell-Badge, P.N. Goodfellow, *Nature* 346 (1990) 240–244.
- [2] R.R. Behringer, M.J. Finegold, R.L. Cate, *Cell* 79 (1994) 415–425.
- [3] B. Vigier, F. Watrin, S. Magre, D. Tran, N. Josso, *Development* 100 (1987) 43–55.
- [4] W.H. Shen, C.C. Moore, Y. Ikeda, K.L. Parker, H.A. Ingraham, *Cell* 77 (1994) 651–661.
- [5] D.S. Lala, P.J. Hornsby, *Mol. Cell. Endocrinol.* 89 (1992) 19–24.
- [6] S. Honda, K. Morohashi, M. Nomura, H. Takeya, M. Kitajima, T. Omura, *J. Biol. Chem.* 268 (1993) 7494–7502.
- [7] N.A. Arango, R. Lovell-Badge, R.R. Behringer, *Cell* 99 (1999) 409–419.
- [8] M.W. Nachtigal, Y. Hirokawa, D.L. Enyeart-VanHouten, J.N. Flanagan, G.D. Hammer, H.A. Ingraham, *Cell* 93 (1998) 445–454.
- [9] S.L. Madden, D.M. Cook, J.F. Morris, A. Gashler, V.P. Sukhatme, F.J. Rauscher, *Science* 253 (1991) 1550–1553.
- [10] F.J. Rauscher, *FASEB J.* 7 (1993) 896–903.
- [11] W. Wang, S.B. Lee, R. Palmer, L.W. Ellisen, D.A. Haber, *J. Biol. Chem.* 276 (2001) 16810–16816.
- [12] W. Bruening, N. Bardeesy, B.L. Silverman, R.A. Cohn, G.A. Machin, A.J. Aronson, D. Housman, J. Pelletier, *Nat. Genet.* 1 (1992) 144–148.
- [13] J. Pelletier, W. Bruening, C.E. Kashtan, S.M. Mauer, J.C. Manivel, J.E. Striegel, D.C. Houghton, C. Junien, R. Habib, L. Fouser, et al., *Cell* 67 (1991) 437–447.
- [14] J.A. Kreidberg, H. Sariola, J.M. Loring, M. Maeda, J. Pelletier, D. Housman, R. Jaenisch, *Cell* 74 (1993) 679–691.
- [15] D.A. Haber, R.L. Sohn, A.J. Buckler, J. Pelletier, K.M. Call, D.E. Housman, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 9618–9622.
- [16] H. Nakagama, G. Heinrich, J. Pelletier, D.E. Housman, *Mol. Cell. Biol.* 15 (1995) 1489–1498.
- [17] S.H. Larsson, J.P. Charlier, K. Miyagawa, D. Engelkamp, M. Rasoulzadegan, A. Ross, F. Cuzin, V. van Heyningen, N.D. Hastie, *Cell* 81 (1995) 391–401.
- [18] R.C. Davies, C. Calvio, E. Bratt, S.H. Larsson, A.I. Lamond, N.D. Hastie, *Genes Dev.* 12 (1998) 3217–3225.
- [19] Z.Y. Wang, Q.Q. Qiu, T.F. Deuel, *J. Biol. Chem.* 268 (1993) 9172–9175.
- [20] J. Kim, D. Prawitt, N. Bardeesy, E. Torban, C. Vicaner, P. Goodyer, B. Zabel, J. Pelletier, *Mol. Cell. Biol.* 19 (1999) 2289–2299.
- [21] M. Ito, R. Yu, J.L. Jameson, *Mol. Cell. Biol.* 17 (1997) 1476–1483.
- [22] P.A. Crawford, C. Dorn, Y. Sadovsky, J. Milbrandt, *Mol. Cell. Biol.* 18 (1998) 2949–2956.
- [23] J.M. Müller, U. Isele, E. Metzger, A. Rempel, M. Moser, A. Pscherer, T. Breyer, C. Holubarsch, R. Buettner, R. Schüle, *EMBO J.* 19 (2000) 359–369.
- [24] A. Swain, R. Lovell-Badge, *Genes Dev.* 13 (1999) 755–767.
- [25] J. Altschmied, J. Duschl, *BioTechniques* 23 (1997) 436–438.
- [26] H. Greschik, J.M. Wurtz, P. Hublitz, F. Köhler, D. Moras, R. Schüle, *Mol. Cell. Biol.* 19 (1999) 690–703.
- [27] C. Thäte, C. Englert, M. Gessler, *Oncogene* 17 (1998) 1287–1294.
- [28] U. Herzer, A. Crocoll, D. Barton, N. Howells, C. Englert, *Curr. Biol.* 9 (1999) 837–840.
- [29] D. Gietz, A. St Jean, R.A. Woods, R.H. Schiestl, *Nucleic Acids Res.* 20 (1992) 1425.
- [30] Y. Kong, J.M. Shelton, B. Rothermel, X. Li, J.A. Richardson, R. Bassel-Duby, R.S. Williams, *Circulation* 103 (2001) 2731–2738.

- [31] J.F. Armstrong, K. Pritchard-Jones, W.A. Bickmore, N.D. Hastie, J.B. Bard, *Mech. Dev.* 40 (1993) 85–97.
- [32] X. Luo, Y. Ikeda, K.L. Parker, *Cell* 77 (1994) 481–490.
- [33] Y. Ikeda, W.H. Shen, H.A. Ingraham, K.L. Parker, *Mol. Endocrinol.* 8 (1994) 654–662.
- [34] R.N. Yu, M. Ito, T.L. Saunders, S.A. Camper, J.L. Jameson, *Nat. Genet.* 20 (1998) 353–357.
- [35] M.M. Lee, C.C. Seah, P.T. Masiakos, C.M. Sottas, F.I. Preffer, P.K. Donahoe, D.T. Maclaughlin, M.P. Hardy, *Endocrinology* 140 (1999) 2819–2827.
- [36] C. Racine, R. Rey, M.G. Forest, F. Louis, A. Ferre, I. Huhtaniemi, N. Josso, N. di Clemente, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 594–599.
- [37] P.H. Chu, W.M. Bardwell, Y. Gu, J. Ross Jr., J. Chen, *Mol. Cell. Biol.* 20 (2000) 7460–7462.
- [38] G.M. Fimia, D. De Cesare, P. Sassone-Corsi, *Mol. Cell. Biol.* 20 (2000) 8613–8622.
- [39] S. Maheswaran, S. Park, A. Bernard, J.F. Morris, F.J. Rauscher III, D.E. Hill, D.A. Haber, *Proc. Natl. Acad. Sci. U. S. A.* 90 (1993) 5100–5104.
- [40] R.W. Johnstone, R.H. See, S.F. Sells, J. Wang, S. Muthukkumar, C. Englert, D.A. Haber, J.D. Licht, S.P. Sugrue, T. Roberts, V.M. Rangnekar, Y. Shi, *Mol. Cell. Biol.* 16 (1996) 6945–6956.
- [41] R.W. Johnstone, J. Wang, N. Tommerup, H. Vissing, T. Roberts, Y. Shi, *J. Biol. Chem.* 273 (1998) 10880–10887.
- [42] L.M. McKay, B. Carpenter, S.G. Roberts, *Oncogene* 18 (1999) 6546–6554.
- [43] D.J. Richard, V. Schumacher, B. Royer-Pokora, S.G. Roberts, *Genes Dev.* 15 (2001) 328–339.
- [44] J.M. Müller, E. Metzger, H. Greschik, A.-K. Bosserhoff, L. Mercep, R. Buettner, R. Schüle, *EMBO J.* 21 (2002) 736–748.