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Functional conservation of interactions between a homeodomain cofactor and a mammalian FTZ-F1 homologue

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Nuclear receptors are master regulators of metazoan gene expression with crucial roles during development and in adult physiology. Fushi tarazu factor 1 (FTZ-F1) subfamily members are ancient orphan receptors with homologues from *Drosophila* to human that regulate diverse gene expression programs important for developmental processes, reproduction and cholesterol homeostasis in an apparently ligand-independent manner. Thus, developmental and tissue-specific cofactors may be particularly important in modulating the transcriptional activities of FTZ-F1 receptors. In *Drosophila*, the homeodomain protein Fushi tarazu acts as a cofactor for FTZ-F1 (NR5A3), leading to the hypothesis that a similar type of homeodomain cofactor–nuclear receptor relationship might exist in vertebrates. In this study, we have identified and characterized the homeodomain protein Prox1 as a co-repressor for liver receptor homologue 1 (LRH1/NR5A2), a master regulator of cholesterol homeostasis in mammals. Our study suggests that interactions between LRH1 and Prox1 may fulfil roles both during development of the enterohepatic system and in adult physiology of the liver.

Keywords: nuclear receptor; homeodomain; Prox1; FTZ-F1; LRH1

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INTRODUCTION

Nuclear receptors (NRs) are master regulators of metazoan gene expression programmes governing crucial processes during development and in adult physiology. Whereas ligand-binding may primarily trigger transcriptional responses of ligand-activatable receptors, the action of tissue-specific or developmental cofactors may have equally important roles, particularly in the case of orphan receptors where ligand binding is uncertain or can be excluded (Li *et al*, 2003).

Members of the Fushi tarazu factor 1 (FTZ-F1, NR5A3) subfamily belong to the category of ancient orphan receptors with homologues from *Drosophila* to human. Mammalian homologues include steroidogenic factor 1 (SF1/NR5A1), which regulates reproductive development and steroidogenesis (Hammer & Ingraham, 1999), and liver receptor homologue 1 (LRH1/NR5A2), which regulates cholesterol homeostasis in the enterohepatic system by modulating the expression of genes encoding enzymes involved in bile acid synthesis, bile salt circulation and reverse cholesterol transport (Francis *et al*, 2003). Recent structure–function analysis indicates that the LRH1 ligand-binding domain (LBD) adopts an active conformation in the absence of ligand binding and that the coregulator-binding surface may not be optimized for common cofactors (Sablin *et al*, 2003). Whereas MBF1 (Brendel *et al*, 2002) and TIF2 (Sablin *et al*, 2003) may act as coactivators, the orphan receptor SHP (NR0B2) fulfils specific roles as an inducible co-repressor in the feedback regulation of LRH1 target genes involved in bile acid synthesis (Goodwin *et al*, 2000; Lu *et al*, 2000). Other cofactors might be particularly relevant for LRH1 activity regulation during development and under circumstances where SHP is insufficient or unavailable, for example under normal dietary conditions in liver.

Previous studies in *Drosophila* had identified the homeodomain protein Fushi tarazu (FTZ) as a developmental cofactor (Guichet *et al*, 1997; Yu *et al*, 1997), which was subsequently demonstrated to interact with LRH1 (Schwartz *et al*, 2001). As there are no FTZ homologues in vertebrates, functionally related homeodomain proteins may replace its function. Recently, the homeodomain protein Prox1 was demonstrated to interact with FTZ-F1 homologues in zebrafish and, in particular, to cooperate

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in vivo with FF1b (the SF1 homologue) during the development of the inter-renal primordium (Liu *et al*, 2003), raising the possibility that Prox1 may act as a cofactor in mammals as well.

In this study, we describe the identification of Prox1 as a co-repressor for LRH1. Characteristics of this interaction as well as overlapping expression patterns and developmental functions suggest that the relationship between LRH1 and Prox1 might be particularly important in regulating gene expression patterns during mammalian liver and pancreas development and in adult physiology of the enterohepatic system.

RESULTS

Characterization of Prox1–LRH1 interactions

NR-interacting proteins that had been identified in previous two-hybrid screenings (Johansson *et al*, 1999) were tested for interactions with LRH1 in a yeast two-hybrid growth assay. Strong interactions were observed with the homeodomain protein Prox1, an uncharacterized candidate cofactor. Interactions of Prox1 or SHP (for comparison) with LRH1 variants were quantified using liquid β-galactosidase assays (Fig 1A) and revealed that the minimal LBD (Sablin *et al*, 2003) was sufficient for interaction and also necessary, as no interaction was observed after deleting helix 12 (data not shown). Prox1 domain mapping and mutagenesis (Fig 1B) revealed that interactions with LRH1 required an N-terminal region containing two putative NR boxes, with NR1 (consensus LxxLL motif) being more important than NR2 (non-consensus IxxLL motif). The critical involvement of both NR boxes in determining the interactions of Prox1 with LRH1 was directly confirmed in mammalian reporter assays (see below and supplementary Fig 2 online).

These findings and the direct character of LRH1–Prox1 interactions were supported by *in vitro* binding studies using purified glutathione *S*-transferase (GST)–LRH1 protein and radio-labelled Prox1 fragments (Fig 2A). Whereas the N-terminal fragments spanning the NR-box 1/2 domain bound well to LRH1, no binding was observed with two C-terminal fragments spanning the homeo-prospero domain including an LxxLL motif that apparently does not have any role in high-affinity LRH1 binding. Finally, coexpression of green fluorescent protein (GFP)–Prox1 and LRH1 in mammalian cells resulted in the relocalization of LRH1 from a uniform pattern (Fig 2B, panel I) to specific Prox1-containing nuclear structures (Fig 2B, panel II). We conclude that LRH1 and Prox1 do interact in yeast, in mammalian cells and *in vitro*, and that these interactions require LRH1 amino-acid (aa) residues 317–560, that is, the entire LBD, and Prox1 aa residues 48–132 containing two NR-box motifs.

Expression pattern of Prox1 and LRH1

We next estimated relative mRNA expression levels in human tissues, and found that Prox1 and LRH1 are highly expressed in the liver and pancreas and at lower levels also in the ovary and small intestine (Fig 3A). Furthermore, a splicing variant (3.5 kb) of the Prox1 mRNA is seen in the testis, which is interesting as testis is a target tissue of SF1. To demonstrate the expression of Prox1 and LRH1 in the adult liver, immunohistochemistry revealed that both proteins are present in virtually all hepatocyte nuclei (Fig 3B).

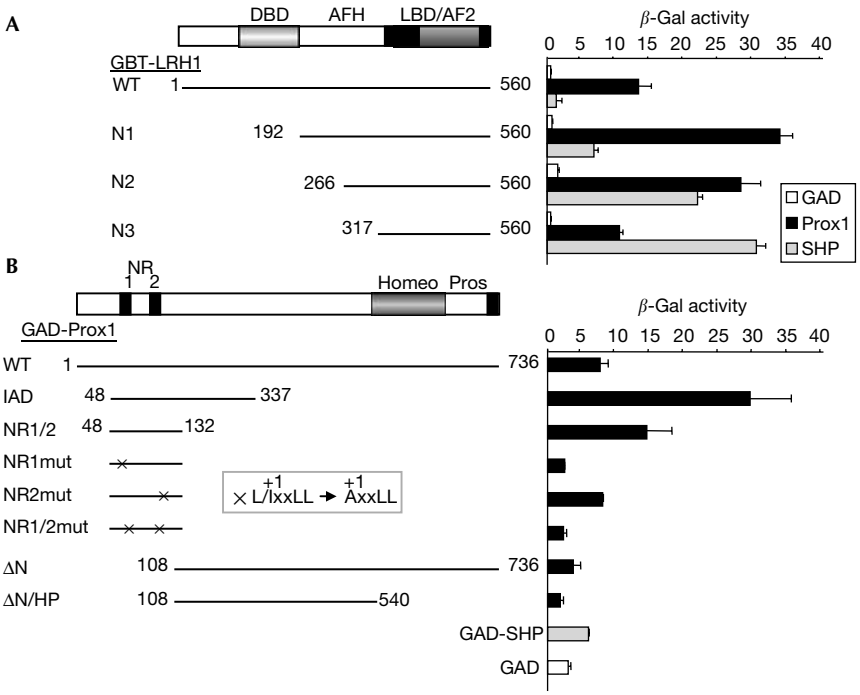

Fig 1 | Yeast two-hybrid analysis of LRH1 interactions with Prox1. (**A**) Gal4-LRH1 variants were tested for interaction with GAD-Prox1 (aa 48–337) or SHP (aa 1–260). The schematic LRH1 domain structure highlights DNA-binding domain (DBD), LBD and transcription regulation functions (AFH, AF2). (**B**) GAD-Prox1 variants were tested for interaction with Gal4-LRH1 (aa 192–560). The schematic Prox1 domain structure highlights putative NR-boxes 1 and 2 and the DNA-binding/regulatory domain (homeo/pros).

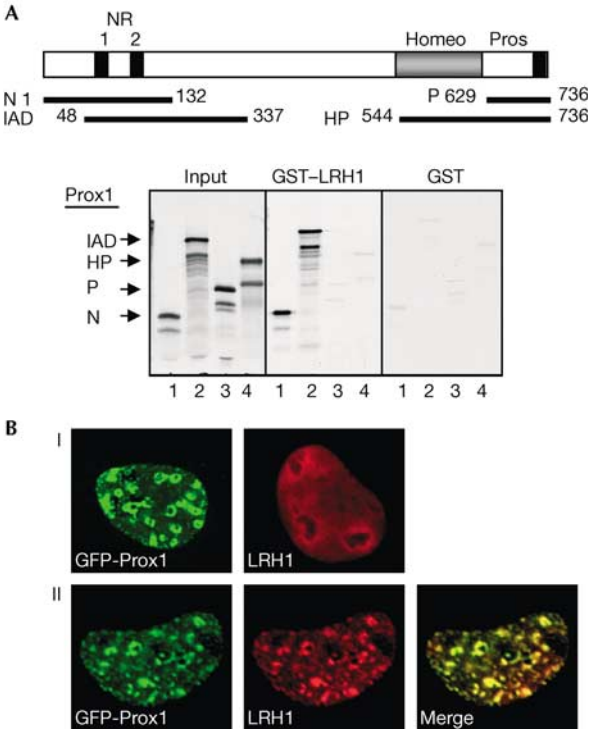

Fig 2 | Characterization of LRH1 and Prox1 interactions. (**A**) GST pull-down analysis. Binding of ^{35}S-methionine-labelled Prox1 fragments to GST–LRH1 (aa 192–560) or GST alone was analysed. Input represents 20% of the material used for the pull-down. (**B**) Intracellular localization analysis. GFP-tagged Prox1 and VP16-tagged LRH1 (0.5 μg plasmid) were expressed individually (panel I) or coexpressed (panel II) in Cos7 cells and visualized directly or indirectly using anti-VP16 antibody. Representative fluorescence images for Prox1 (green), for LRH1 (red) and merged images (yellow) following superimposition are shown.

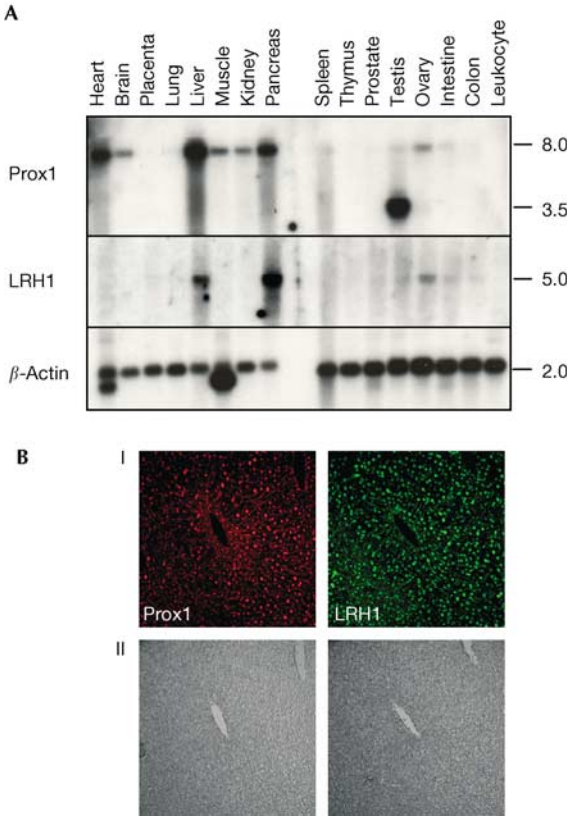

Fig 3 | Tissue distribution and cellular coexpression of LRH1 and Prox1 in the liver. (**A**) Human multiple tissue northern blots containing 2 μg of poly(A)$^+$ mRNA from each tissue were probed successively with ^{32}P-labelled LRH1, Prox1 and β-actin cDNAs. RNA molecular weights are in kilobases. (**B**) Adult mice liver samples were subjected to immunohistochemistry as described in Methods. Representative fluorescence images for Prox1 (red) and for LRH1 (green) are shown in panel I. Corresponding tissue samples without staining are shown in panel II.

These results were confirmed by *in situ* hybridization showing strong signals for both LRH1 and Prox1 mRNA in hepatocytes (supplementary Fig 1 online). In summary, our data suggest that Prox1 is expressed in all LRH1 target tissues and is probably a physiologically relevant cofactor of LRH1, particularly in the adult liver of mammals.

Inhibition of LRH1 activity by Prox1

The effect of Prox1 on LRH1 transcriptional activity was analysed in a number of different experimental settings (Fig 4) using the *shp* promoter, an established LRH1 target (Lu *et al*, 2000). First, we found that LRH1 activation of the *shp-luc* reporter in Cos7 cells was repressed by coexpression of Prox1 (Fig 4A). Repression by Prox1 was as effective as repression by SHP (Fig 4A) at equal expression levels as judged from immunoblots (data not shown). Importantly, repression was not simply due to squelching as an equally expressed NR-box 1/2 mutant Prox1 did not repress (supplementary Fig 2 online), further confirming the importance of the N-terminal NR boxes 1 and 2 in determining Prox1–LRH1 interactions (Fig 1B). Second, transfections of the HuH7 human liver cell line revealed that Prox1 and the p160 coactivator TIF2 effectively antagonized each other on the *shp* promoter in a dose-dependent manner (Fig 4B). Third, LRH1 overexpression increased endogenous SHP mRNA levels in HuH7 cells, whereas Prox1 coexpression antagonized this increase (Fig 4C). Fourth, decreasing endogenous Prox1 expression by RNA interference revealed that Prox1-specific but not control short interfering RNA (siRNA) increased LRH1-dependent reporter activity (Fig 4D). These results collectively suggest a co-repressor function of Prox1.

Intrinsic transcriptional features of Prox1

Experiments were carried out to investigate whether or not Prox1 would directly repress transcription and, if so, which cofactors target the putative repressor function and may act in concert with Prox1? First, analysis of Prox1 variants, in comparison to the two established repressors SHP and thyroid hormone receptor (TR)α, in a repressor assay clearly revealed that Prox1 can act as a strong transcriptional repressor (Fig 5), Domain mapping revealed the existence of an N-terminal potent repressor domain (aa 108–337), whereas the C-terminal homeo-prospero domain (aa 544–736) was not required for Prox1 repression and had only a weak repressor potential on its own.

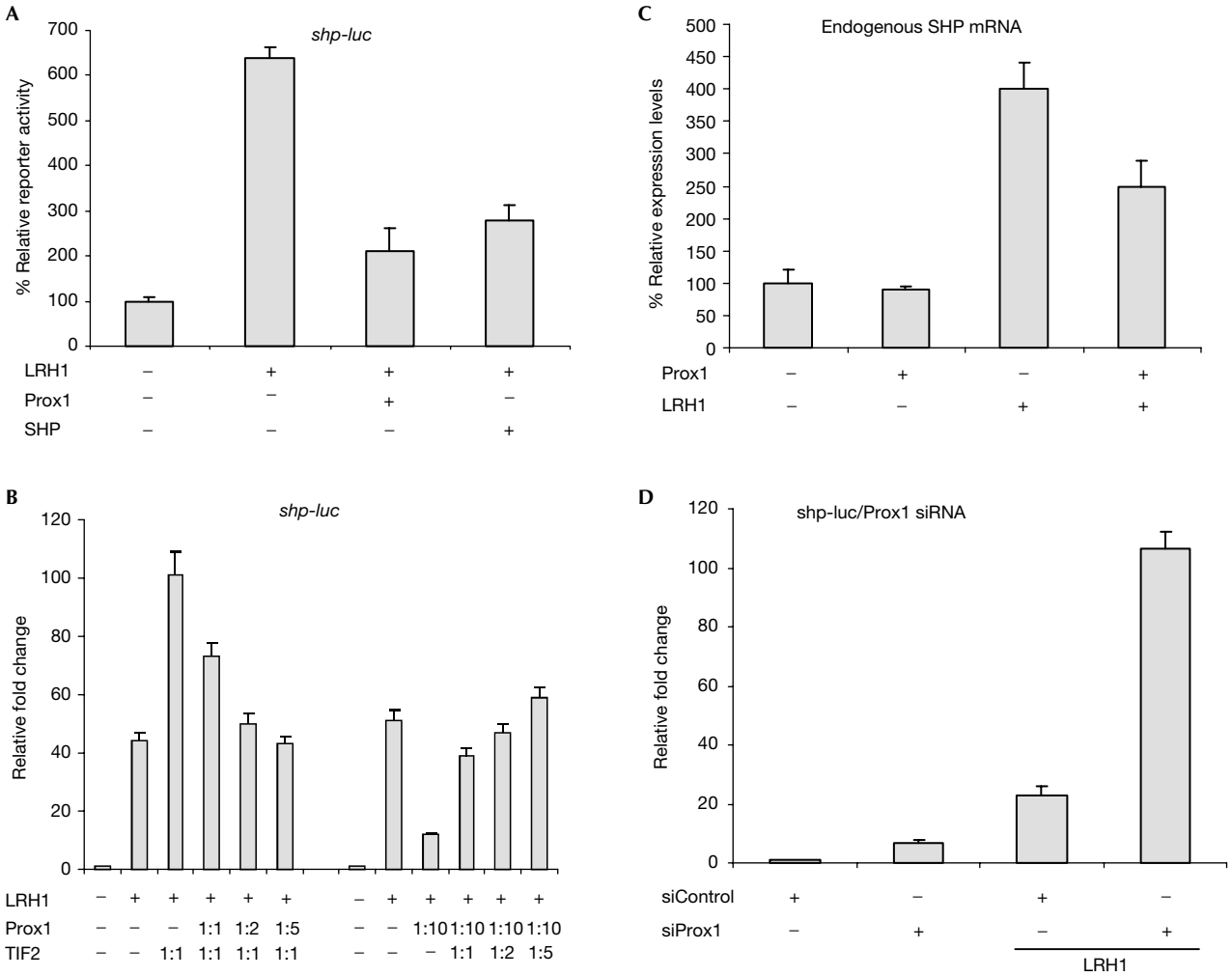

Fig 4 | Effects of Prox1 on LRH1 activity. (**A**) Effect of Prox1 on LRH1-dependent reporter gene activity. Cos7 cells received 100 ng *shp-luc* reporter, 100 ng pSG5-LRH1, 200 ng pFlag-Prox1 or 200 ng pFlag-SHP plasmid as indicated, and luciferase activities were related to the activity of the reporter in the absence of exogenous LRH1, Prox1 and SHP, which was set to 100%. (**B**) Effect of Prox1 on TIF2 coactivation of LRH1-dependent reporter gene activity. HuH7 cells received 500 ng of the *shp-luc* reporter, 200 ng of pSG5-LRH1, 200/400/1,000 ng (1:1/1:2/1:5) of pFlag-Prox1 and 200/400/1,000 ng (1:1/1:2/1:5) of pSG5-TIF2 as indicated. Luciferase activities were related to the activity of the reporter alone, which was set to 1. (**C**) Effect of Prox1 on the expression of endogenous SHP mRNA. HuH7 cells received 200 ng pSG5-LRH1 and/or 1,800 ng pFlag-Prox1 as indicated. Expression levels of endogenous SHP cDNA in the presence of exogenous LRH1 and/or Prox1 were related to the levels in their absence (control), which was set to 100%. (**D**) Effect of reducing endogenous Prox1 levels by RNA interference on reporter activity. HuH7 cells received 500 ng *shp-luc*, 100 ng pSG5-LRH1, 10 μl Prox1 siRNA duplex oligos (20 μM) or 10 μl control siRNA (20 μM) as indicated. Luciferase activities of the co-transfections were related to the activity of the reporter alone, which was set to 1. All individual experiments (**A–D**) were performed with equal amounts of DNA by filling up with the respective empty vector.

Assuming histone deacetylases as potential cofactors involved in Prox1 repression, we investigated whether various class I and II HDACs (Fig 6A and data not shown) would change their localization pattern upon coexpression with GFP–Prox1 or vice versa. HDAC3 was clearly identified as a candidate Prox1 partner, whereas HDAC2 did not colocalize (Fig 6A). The direct character of HDAC3 interactions was further supported by GST pull-down assays (Fig 6B). HDAC3 was specifically retained by a Prox1 fragment containing the repressor domain but not by the non-repressing homeo-prospero domain or by GST alone. Finally, endogenous Prox1–HDAC3 complexes were detected by co-immunoprecipitation from HuH7 cell extracts (Fig 6C). We conclude that Prox1 has intrinsic transcriptional repression potential that is largely dependent on an N-terminal repression domain and that may involve recruitment of HDAC3.

DISCUSSION

A number of independent studies indicate that Prox1 and LRH1 are coexpressed throughout development of endoderm-derived tissues including liver, pancreas and intestine in mice and humans (Oliver *et al*, 1993; Galarneau *et al*, 1996; Tomarev *et al*, 1996; Rausa *et al*, 1999; Burke & Oliver, 2002). Functional inactivation

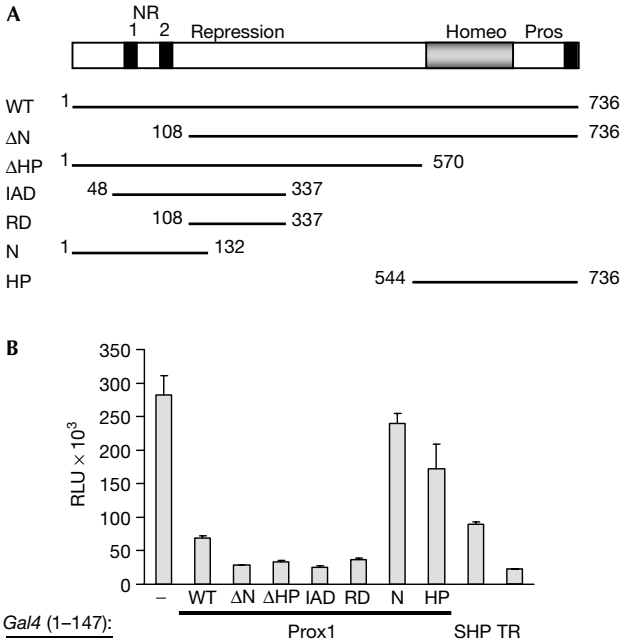

Fig 5 | Analysis of Prox1 repression. (**A**) Schematic Prox1 domain structure and repressor constructs. (**B**) Repressor assay. Gal4-tagged expression constructs for Prox1, SHP and TRα (each 400 ng plasmid) were analysed for repression of a *luc* reporter (500 ng plasmid) containing a complex promoter with activator (HNF1-VP16)- and repressor (Gal4 fusion protein)-binding sites (Båvner *et al*, 2002).

of the mouse *Prox1* gene caused embryonic lethality due to multiple developmental defects, including abnormal cell proliferation and migration during liver morphogenesis (Sosa-Pineda *et al*, 2000). Expression of LRH1 is evident in the same tissues of adult mammals, for example, in liver hepatocytes and bile ducts and exocrine pancreas (Galarneau *et al*, 1996; Annicotte *et al*, 2003). Although we were able to demonstrate coexpression of Prox1 with LRH1 in mouse liver, the specific roles of Prox1 in adult physiology of liver, pancreas, testis and ovary and particularly its relationship to relevant LRH1 cofactors such as SHP or DAX-1 in these tissues remain to be elucidated. One hypothesis could be that, for example in liver, Prox1 may modulate LRH1 target gene expression during development and under normal physiological conditions, whereas SHP may primarily serve as a metabolic sensor under conditions of increased metabolic activity such as high-cholesterol diet. The negative effect of Prox1 on LRH1 (this study) and SF1 (Liu *et al*, 2003) seen in cell culture systems may be physiologically significant, as it is currently unclear whether LRH1 acts as an activator of target gene transcription *in vivo*. A recent study highlights this issue by demonstrating that heterozygous LRH1 knockout mice express unexpectedly fivefold to sevenfold higher levels of CYP7A and CYP8B genes than wild-type mice (Del Castillo-Olivares *et al*, 2004). It is conceivable that co-repressors such as Prox1 may actually be particularly important to suppress 'constitutively' active NRs including LRH1, whereas a majority of NRs would not require Prox1-like co-repressors in the absence of activating ligands.

Despite extensive work aimed at understanding Prox1 function, little is currently known about its direct molecular targets, that is, regulated genes and regulatory proteins. Thus, LRH1 represents one of the first target factors of Prox1 action. Further, the characterization of Prox1 as a potent repressor of transcription and the possible connection to HDAC3 is particularly interesting, as this deacetylase is a component of a conserved co-repressor complex involved in repression by NRs (Heinzel *et al*, 1997). Whereas future work has to dissect the mechanisms of Prox1 co-repressor action, we found that LRH1 activity is increased by trichostatin A (A.B. and E.T., unpublished data), consistent with a role of HDACs in modulating LRH1 activity. Many homeodomain proteins act both as transcriptional activators and repressors in a context-dependent manner, so alternative mechanisms of repression or activation have to be considered for Prox1 as well.

The ability of Prox1 to modulate mammalian gene expression patterns by acting as a cofactor for LRH1 and perhaps other NRs is probably a characteristic of all vertebrates, as Prox1 was recently demonstrated to cooperate with FF1b (SF1/NR5A1) during the development of the inter-renal primordium in zebrafish (Liu *et al*, 2003). Intriguingly, the principle of a homeodomain protein acting as a transcriptional coregulator for FTZ-F1 members is evolutionarily conserved as this relationship resembles *Drosophila* FTZ-F1/FTZ, which is a developmental homeodomain protein unique to flies and one of the earliest characterized LxxLL coregulators (Guichet *et al*, 1997; Yu *et al*, 1997; Schwartz *et al*, 2001). Thereby, the identification of mammalian Prox1 as an LRH1 coregulator fulfils the original proposal that '…interactions of this sort might allow homeodomain proteins to modulate the activity or target range of nuclear hormone receptors' and '…that other NR–homeodomain protein interactions may be important and common in developing organisms' (Guichet *et al*, 1997).

METHODS

Plasmids/oligonucleotides. Plasmid constructs were generated using standard cloning procedures and verified by restriction enzyme analysis and DNA sequencing. Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene). Details are available on request.

Protein–protein interaction assays. Experimental conditions of yeast two-hybrid assays, GST pull-down assays, intracellular localization analysis and co-immunoprecipitations are essentially as described previously (Johansson *et al*, 1999; Båvner *et al*, 2002; Holter *et al*, 2002) and are provided as supplementary information online.

Analysis of mRNA and protein expression. Human multiple tissue northern blots (MTN blot #7760-1 and 7759-1, Clontech) were hybridized for 1 h with probes against human Prox1, human LRH1 and human β-actin according to the manufacturer's instructions. *In situ* hybridization and immunohistochemistry procedures are given as supplementary information.

Cell cultures and transient transfections. *Cell culture*: Human hepatoma HuH7 and monkey kidney Cos7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. *Reporter assays:* Cells were plated in six-well plates and transiently transfected using Lipofectamine 2000 (Life Technologies) and incubated for 24 h. Relative luciferase units (RLU) represent the mean ± s.d. of duplicate transfections and were reproduced in independent experiments.

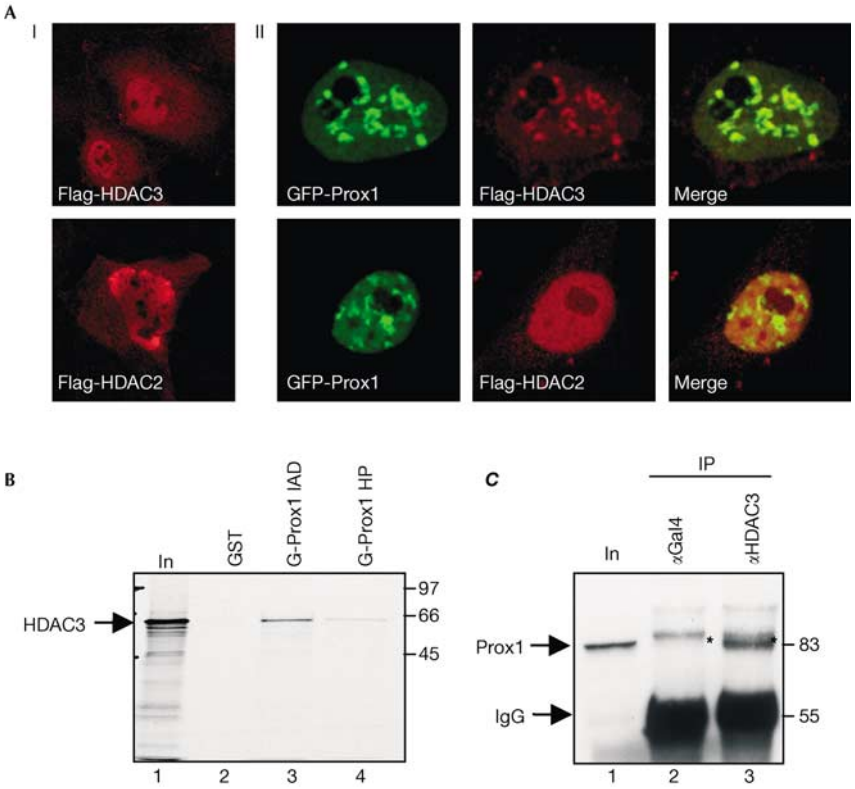

Fig 6 | Characterization of Prox1 interactions with HDAC3. (**A**) Intracellular localization analysis. GFP-tagged Prox1 and Flag-tagged HDACs (0.5 μg plasmid) were expressed individually (panel I) or coexpressed (panel II) in Cos7 cells and visualized directly or indirectly using anti-Flag antibody. Representative fluorescence images for HDACs (red) and Prox1 (green) are shown. Merged images (yellow) following superimposition depict colocalization in the case of HDAC3. (**B**) GST pull-down assay. Binding of ^{35}S-methionine-labelled HDAC3 to GST–Prox1 IAD (aa 48–337), GST–Prox1 HP (aa 544–736) or GST alone was analysed. Input (In) corresponds to 20% of the material subjected to the pull-down. (**C**) Co-immunoprecipitation of endogenous Prox1 and HDAC3. Immune complexes from HuH7 whole-cell extracts (300 μg total protein) were isolated using HDAC3 antibody or using Gal4 antibody (control). Precipitates were analysed using a rabbit polyclonal Prox1-specific antibody. The input (In) corresponds to 20% of the material subjected to immunoprecipitation. (*) Nonspecific band; (IgG) position of the immunoglobulin heavy chain (approximately 55 kDa).

RNA interference: Transfection of siRNA duplexes was performed in HuH7 cells using Lipofectamine 2000. siRNA sequences are given as supplementary information online.

mRNA extraction and quantification by real-time PCR. Total RNA from cell cultures was isolated using the RNeasy mini-kit (Qiagen). A 0.5 μg portion of RNA was treated with DNase I (Invitrogen) and subsequently transcribed into cDNA by Superscript II (Invitrogen). mRNA expression was quantified by Taqman (SHP) or SYBRgreen (18S; internal standard) qPCR on an ABI 7700 machine (Applied Biosystems).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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