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► **To cite this version:**

Tomas Jakobsson, Waffa Osman, Jan-Åke Gustafsson, Johanna Zilliacus, Anette Wärnmark. Molecular basis for repression of liver X receptor-mediated gene transcription by the receptor-interacting protein 140. *Biochemical Journal*, 2007, 405 (1), pp.31-39. 10.1042/BJ20070004 . hal-00478730

**HAL Id: hal-00478730**

**<https://hal.science/hal-00478730>**

Submitted on 30 Apr 2010

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# Molecular basis for repression of Liver X Receptor mediated gene transcription by the Receptor-interacting protein 140

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Short title: Repression of LXR activity by RIP140

Keywords: LXR, RIP140, nuclear receptor, transcription, coregulatory protein

Abbreviation used: aa, amino acid; AD, activation domain; ATRX, X-linked alpha thalassaemia mental retardation; BSC40, monkey kidney cells; Cidea, cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector A; CPT1, Carnitine palmitoyltransferase-1; CtBP, corepressor C-terminal binding protein; GFP, green fluorescent protein; GLUT4, glucose transporter; H12, helix 12; HDAC, histone deacetylase protein; h, human; Huh7, human hepatoma cells; LBD, ligand binding domain; LXR, liver X receptors; LXRE, LXR responsive element; N-CoR, nuclear receptor corepressor; NLS, nuclear localization signal; NR, nuclear receptor; PCR, polymerase chain reaction; PEPCK, phosphoenolpyruvate carboxykinase; PGC1, PPAR gamma-coactivator-1; RIP140, receptor-interacting protein 140, RT-PCR, real time PCR; RXR; retinoid X receptor; SMRT, silencing mediator of retinoid and thyroid hormone action protein; TAF-172, TATA box-binding protein (TBP)-associated factors 172; TRAP220, thyroid hormone receptor-associated protein 220; TRITC, tetramethyl rhodamine isothiocyanate; Ucp1, uncoupling protein1.

Similarities in physiological roles of Liver X receptors (LXR) and corepressor Receptor-interacting protein 140 (RIP140) in regulating energy homeostasis and lipid and glucose metabolism suggest that the effects of LXR could at least partly be mediated by recruitment of the corepressor RIP140. In the present study, we have elucidated the molecular basis for regulation of LXR transcriptional activity by RIP140. LXR is evenly localized in the nucleus and neither the N-terminal domain nor the ligand-binding domain is necessary for nuclear localization. Both LXR subtypes, LXR $\alpha$  and  $\beta$ , interact with RIP140 and colocalize in diffuse large nuclear domains. Interaction and colocalization are dependent on the ligand-binding domain of the receptor. The C-terminal domain of RIP140 is sufficient for full repressive effect. None of the C-terminal NR-boxes is required for the corepressor activity whereas the NR-box like motif as well as additional elements in the C-terminal region are required for full repressive function. The C-terminal NR-box like motif is necessary for interaction with LXR $\beta$  whereas additional elements are needed for strong interaction with LXR $\alpha$ . In conclusion, our results suggest that corepression of LXR activity by RIP140 involves an atypical binding mode of RIP140 and a repression element in the RIP140 C-terminus.

## INTRODUCTION

The liver X receptor (LXR) is a ligand inducible transcription factor and member of the nuclear receptor superfamily [1]. There are two known paralogues, LXR $\alpha$  (NR1H3) and LXR $\beta$  (NR1H2). The two subtypes have a high degree of sequence similarity but display different expression patterns. Expression of LXR $\alpha$  is restricted to liver, kidney, adipose tissue, intestine and macrophages while LXR $\beta$  is ubiquitously expressed [2]. Oxidized cholesterol derivatives, so called oxysterols, have been shown to bind to and activate the LXRs [3, 4]. LXRs bind to target DNA (LXR response element) as a permissive heterodimer with another member of the nuclear receptor superfamily, the retinoid X receptor (RXR). LXRs have been identified as key players in the regulation of cholesterol homeostasis, lipid and glucose metabolism as well as immune and inflammatory responses [reviewed in [5, 6]]. LXRs affect whole body energy balance. LXR $\alpha/\beta$ -deficient mice are leaner than wild-type littermates and are also resistant to obesity when fed a high fat and cholesterol diet [7-9]. LXR knockout mice have an increased energy expenditure and increased expression in skeletal muscle and adipose tissue of the mitochondrial protein Ucp1 that uncouples respiration [7, 9]. In accordance, Ucp1 expression is down regulated by LXR agonist in adipose tissue [10]. LXRs also regulate glucose metabolism. Synthetic LXR agonists improve glucose tolerance in rodent diabetic models [11, 12]. LXRs repress genes involved in glucose production, such as PEPCCK, and activate genes involved in glucose utilization, such as glucokinase, in hepatocytes [10-13]. In adipocytes, LXRs induce GLUT4 expression that contributes to increased glucose uptake in the cells [12, 14]. Failure of proper regulation of energy balance and lipid and glucose metabolism may lead to a number of diseases, including atherosclerosis, obesity and diabetes. These diseases are considered to be the main causes of mortality in the Western society today. LXRs are therefore interesting potential targets for drug treatment of several diseases.

Nuclear receptor mediated gene transcription involves recruitment to nuclear receptors of a large number of coregulatory proteins that form dynamic multi-protein complexes [reviewed in [15]]. Coregulators function as bridging and/or stabilizing factors, inhibitors of specific protein-protein interactions or modifiers of other proteins with enzymatic activity (i.e. acetylation, methylation, phosphorylation, ubiquitination). These events initiate (coactivators) or inhibit (corepressors) chromatin remodeling and/or the assembly of the general transcription machinery. Receptor-interacting protein RIP140 is a corepressor protein that is highly expressed in metabolic tissues such as liver, muscle and adipose tissue and has an important role in regulation of lipid and glucose metabolism [reviewed in [16]]. The physiological function of RIP140 is partly similar to that of

LXRs. Mice devoid of RIP140 are lean and are resistant to high-fat diet-induced obesity [17]. Adipocytes lacking RIP140 have an increased energy expenditure and elevated expression of proteins involved in energy expenditure such as *Ucp1*, *Cidea* and *Cpt1b* [17, 18]. Furthermore, RIP140-knockout mice on high-fat diet have an improved glucose tolerance. RIP140-depleted adipocytes have an increased glucose uptake and increased expression of genes involved in glucose uptake and glycolysis such as GLUT4 and hexokinase 2 [19].

RIP140 interacts with many ligand-activated nuclear receptors, including LXR [20, 21]. RIP140 contains nine NR-boxes (LxxLL-motifs) and one NR-box like motif (LxxML) [22]. NR-boxes are found in almost all coactivators identified and bind in a ligand-dependent manner to the ligand-binding domain of nuclear receptors [23]. Furthermore, the NR-box is necessary and sufficient for interaction between the receptor and the coregulator. The corepressors N-CoR and SMRT contain a related motif, the CoRNR box (L/IxxI/VI) that mediates the binding to unliganded nuclear receptors [24-26]. RIP140 has been suggested to repress gene transcription by competing with coactivators for binding to the receptor [22]. However, RIP140 also has an intrinsic repression activity that is at least partly mediated by recruitment of histone deacetylase proteins (HDACs) and the corepressor C-terminal binding protein (CtBP) [27-29]. The intrinsic repression activity has been mapped and includes several domains of RIP140 [30-32].

The similarities in physiological roles of LXRs and RIP140 in regulating energy homeostasis and lipid and glucose metabolism suggest that the effects of LXR could at least partly be mediated by recruitment of the corepressor RIP140. The aim of the present study was to delineate specific determinants for the repressive effect of RIP140 on LXR mediated gene transcription. We first demonstrate that both LXR subtypes, LXR $\alpha$  and  $\beta$ , interact with RIP140 and that RIP140 colocalizes with the LXRs in large nuclear domains. Furthermore, both interaction and colocalization is dependent on the ligand-binding domain of the receptors. We also show that the main repression activity of RIP140 resides in its C-terminal part and that all NR-boxes within this domain of RIP140 do not need to be intact for the repressive effect of RIP140. Moreover, our data prove the importance of an NR-box like motif and integrity of the C-terminal domain of RIP140 for obtaining full repression by RIP140 on LXR mediated gene transcription. Interestingly, we also find LXR subtype differences in binding to RIP140. The NR-box like motif in RIP140 is necessary and sufficient for the interaction with LXR $\beta$  whereas more than one intact NR-box is needed in order for RIP140 to interact strongly with LXR $\alpha$ .

## EXPERIMENTAL

### Plasmid constructs

For localization studies, human (h) full-length LXR $\alpha$  (aa 1-447), hLXR $\alpha$   $\Delta$ LBD (aa 1-214), hLXR $\alpha$   $\Delta$ N (aa 96-447), hLXR $\alpha$   $\Delta$ H12 (aa 1-436), full-length hLXR $\beta$  (aa 1-461), hLXR $\beta$   $\Delta$ LBD (aa 1-230), hLXR $\beta$   $\Delta$ N (aa 85-461) and hLXR $\beta$   $\Delta$ H12 (aa 1-450) were generated by polymerase chain reaction (PCR) and subcloned into the *Xba*I and *Xho*I site of the pcDNA3-FLAG plasmid [33]. Plasmid pEGFP-RIP140, expressing green fluorescent protein (GFP)-fused hRIP140, has been described previously [34]. Expression plasmid for transient transfection studies, pSG5-HA-hRIP140, expressing HA-tagged hRIP140, has been described previously [22]. Deletion constructs in the pSG5-HA plasmid, hRIP140/1-281, hRIP140/1-472, hRIP140/431-745, hRIP140/431-1158, and NR-box mutation constructs have been described previously [32]. Deletion constructs in the pSG5-HA plasmid, hRIP140/863-1158, hRIP140/965-1158, hRIP140/747-1060 and hRIP140/747-935, were generated by subcloning PCR fragments into the *Bgl*II site of the pSG5-HA plasmid. pSG5-HA-hRIP140 FL mut NR10 were generated using Quik-Change XL Site-Directed Mutagenesis Kit (Stratagene) according to the protocol provided by the manufacturer. The reporter plasmids 3xLXRE luciferase reporter, kindly provided by KaroBio AB, Sweden, and RSV  $\beta$ -gal reporter were used. For yeast two-hybrid experiments full-length hLXR $\alpha$  (aa 1-447), hLXR $\alpha$  ligand-binding domain (aa 205-447), hLXR $\alpha$  N-terminal domain (aa 1-291), hLXR $\alpha$  DNA-binding domain (aa 96-214), hLXR $\beta$  full-length (aa 1-461), hLXR $\beta$  ligand-binding domain (aa 219-461), hLXR $\beta$  N-terminal domain (aa 1-258) and hLXR $\beta$  DNA-binding domain (aa 85-230) were generated by PCR and subcloned into the *Sfi*I and *Bam*HI site of the pGBKT7 plasmid (Clontech) to create the GAL4 DBD construct. GAL4 AD fusion protein hRIP140/863-1158, hRIP140/965-1158, hRIP140/747-1060, hRIP140/747-935, hRIP140/747-1158 and hRIP140/747-1158 mut NR10 were generated by PCR and subcloned into the *Sfi*I and *Bam*HI site of the pACT2 plasmid (Clontech). GAL4 AD fusion protein hRIP140/560-1158 was isolated in a yeast-two hybrid screen from a pACT2 cDNA library (Clontech).

### Confocal microscopy

COS-7 cells were plated on cover slips in six-well plates and, the following day, transfected with expression plasmids using the Fugene 6 transfection reagent (Roche). One hundred ng of expression plasmid for GFP-fused proteins and 200 ng of the expression plasmid for FLAG-tagged proteins were used. Next day fresh medium was added containing the ligands 1  $\mu$ M T0901317 and incubated overnight. After 24 h, incubated cells were fixed with 3% paraformaldehyde in 5% sucrose-

phosphate-buffered saline (PBS) rinsed with PBS and permeabilized with PBS containing 0.1% Tween 20 (PBS-T). Cells transfected with FLAG-tagged proteins were blocked with 5% goat serum (Jackson ImmunoResearch) in PBS-T for 1 h. Thereafter the cells were incubated with primary antibody diluted in PBS-T for 1.5 h. Anti-FLAG M5 monoclonal antibody (Sigma) was used at a dilution of 1:200. Cells were then washed in PBS-T and incubated with secondary antibody conjugated with tetramethyl rhodamine isothiocyanate (TRITC; Jackson ImmunoResearch) diluted at 1:200 in PBS-T for 1 h. Cells were washed and fixed on slides with ImmunoMount (Shandon). Subcellular images were determined with a TCSSP Multiband Confocal imaging system (Leica). Fluorescence images for more than 100 transfected cells were studied for each experiment and the images shown are representative for the majority of cells studied.

### Reporter gene assays

Human hepatoma cells (Huh7) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% (v/v) fetal-bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. The day before transfection, cells were seeded onto 24 well-plates ( $4 \times 10^4$  cells/well), and on the following day, transfected with 2.5-50 ng pSG5-HA-RIP140 expression vector, 100 ng 3xLXRE luciferase reporter and 10 ng RSV β-gal reporter using Lipofectamine 2000 transfection reagent (Invitrogen). To keep vector amount constant in each transfection, appropriate amount of empty pSG5 vector was added. Five hours after transfection medium was changed to medium containing 250 nM T0901317 or DMSO as vehicle and cells were cultured for 24 h. Luciferase and β-galactosidase activities were measured using luciferin and ATP reagents (BioThema) and a Galacto-Star kit (Tropix), respectively, in microplate luminometer (Thermo Electron corp.). All experiments were repeated at least three times with triplicates in each, and luciferase activity was normalized to β-galactosidase activity.

### Yeast two-hybrid assays

For yeast two-hybrid assays different combinations of pGBKT7-GAL4 DBD and pACT2-GAL4 AD fusion constructs (100 ng each) were cotransformed into *Saccharomyces cerevisiae*, strain Y187 (Clontech) and plated on medium lacking leucine and tryptophan for selection. Liquid β-galactosidase assay was performed in the presence of 250 nM T0901317 or DMSO as vehicle and β-galactosidase units were calculated according to the protocol provided by the manufacturer (BD Biosciences). All experiments were repeated at least three times with triplicates in each.

## Western Blotting

Whole cell extracts were prepared from Huh7 cells transfected with HA-tagged RIP140 plasmids. The cell pellet was washed in PBS and resuspended in extraction buffer (10 mM Tris-HCl [pH 7.4], 300 mM NaCl, 1 mM dithiothreitol (DTT), 0.1% Igepal CA-630, Complete Mini protein inhibitor cocktail [Roche]), incubated for 15 min on ice and homogenized. After centrifugation (3.000 rpm, 10 min) the supernatant was analyzed by Western blotting with anti-HA.11 monoclonal antibody (MMS-101P, Nordic Biosite).

## RT-PCR

For quantification of LXR mRNA levels in Huh7 cells total RNA was isolated using E.Z.N.A. Total RNA kit ((Omega Bio-tek) according to the manufacturer's instructions. First strand cDNA synthesis of RNA (1 µg) was performed using Superscript II reverse transcriptase (Invitrogen) with random hexamers. mRNA expression was quantified using 10 ng cDNA/reaction by SYBR green (18S as internal standard) quantitative PCR applying the comparative Ct analysis using the Applied Biosystems 7500 real-time PCR. Following primers (5'-3') were used: LXR $\alpha$  F: CCCCATGACCGACTGATGTT, R: GAGGCTCACCAGTTTCATTAGCAT; LXR $\beta$  F: GGAGGACCAGATCGCCCT, R: AGCAGCATGATCTCGATAGTGG.



## RESULTS

### LXR is localized in the nucleus

To analyze the intracellular localization of LXR $\alpha$  and  $\beta$ , we transfected COS-7 cells with plasmids expressing FLAG-tagged LXR $\alpha$  and  $\beta$ , respectively. LXR $\alpha$  and LXR $\beta$  were distributed evenly in the nucleus both in the absence and presence of the synthetic LXR agonist ligand T0901317 (LXR $\alpha$ : Figure 1B, panels a-b. The results for LXR $\beta$  are identical to those for LXR $\alpha$  and are therefore presented in the Supplementary Materials, Figure 1.) The same localization was also found using fluorescent HcRed-tagged LXR $\alpha$  and  $\beta$  (data not shown). To study the influence of different LXR domains on receptor localization we used FLAG-tagged LXR constructs where the N-terminal domain ( $\Delta$ N), ligand-binding domain ( $\Delta$ LBD) or helix 12 ( $\Delta$ H12) of LXR  $\alpha$  or  $\beta$  was deleted (Figure 1A). Transfection of COS-7 cells with the deletion constructs revealed that LXR $\alpha$  and  $\beta$   $\Delta$ N proteins had a foci like distribution in the nucleus, whilst LXR $\alpha$  and  $\beta$   $\Delta$ LBD and  $\Delta$ H12 proteins were evenly distributed in similarity to full length LXR $\alpha$  and  $\beta$  (LXR $\alpha$ : Figure 1B, panels c, e and g. LXR $\beta$ : Supplementary Materials, Figure 1). The intracellular localization of the deleted LXR $\alpha$  and  $\beta$  proteins did not change in the presence of the LXR ligand (LXR $\alpha$ : Figure 1B, panels d, f, and h. LXR $\beta$ : Supplementary Materials, Figure 1). Collectively, these data suggest that wild type LXR is localized in the nucleus and that neither the N-terminal domain nor the ligand-binding domain is indispensable for nuclear localization of LXR $\alpha$  or  $\beta$ , indicating the importance of the DNA-binding domain and the hinge region for nuclear targeting. Furthermore, our data show that removal of the N-terminal domain of LXR changes the intranuclear localization of the receptor, suggesting that this domain is important for formation of the correct receptor structure and/or association with protein complexes that contribute to normal intranuclear localization.

### LXR $\alpha$ and LXR $\beta$ interact with RIP140

To identify proteins interacting with LXRs in the presence of the synthetic LXR agonist ligand T0901317 we performed yeast two hybrid screens. Several of the isolated clones from different human cDNA libraries using either LXR $\alpha$  or LXR $\beta$  as bait encoded RIP140. These results confirm the interaction between LXRs and RIP140 and indicate a role for RIP140 in LXR mediated gene regulation. We further verified the interaction between LXR and RIP140 in a GST-pull-down analysis with GST-fused LXR $\alpha$  and *in vitro* translated RIP140. RIP140 interacted with LXR $\alpha$  both in the absence and presence of LXR ligand T0901317 (data not shown).

To elucidate which domains of LXR $\alpha$  and LXR $\beta$  that interact with RIP140, *Saccharomyces cerevisiae* yeast strain Y187 containing an integrated GAL4-responsive lacZ reporter gene was cotransformed with pGBKT7-GAL4 DBD-LXR fusion constructs (Figure 2) and the pACT2-GAL4 AD-RIP140 C-terminus (aa 560-1158). Transcriptional activity, as measurement of interaction, was then assayed using a liquid  $\beta$ -galactosidase assay. Strong interaction was seen between the C-terminus of RIP140 (aa 560-1158) and the full length LXR $\alpha$  and  $\beta$  proteins, respectively, in the presence of the synthetic LXR ligand T0901317 (Figure 2). The interaction of RIP140 (aa 560-1158) with full length LXR $\alpha$  was only slightly enhanced by ligand, whereas the interaction with LXR $\beta$  was strongly ligand dependent. The ligand-binding domains of LXR $\alpha$  and LXR $\beta$  interacted in a ligand-dependent manner with RIP140 C-terminus while no binding of the DNA-binding domain of either LXR was seen. The N-terminal domain of LXR $\alpha$  interacted weakly with RIP140, both in the presence and absence of ligand, while the N-terminal domain of LXR $\beta$  did not bind. In summary, the C-terminal part of RIP140 readily binds both LXR subtypes and the ligand-binding domain is necessary for strong interaction.

### **LXR ligand-binding domain is necessary for colocalization with RIP140**

To investigate whether LXR $\alpha$  and  $\beta$  colocalize with RIP140, GFP-tagged RIP140 was expressed alone or together with FLAG-tagged LXR $\alpha$  and  $\beta$ , respectively, in COS-7 cells. Figure 3A shows that GFP-RIP140 is localized in small, discrete foci in the nucleus, as previously shown [32]. The localization of RIP140 was not changed in the presence of LXR ligand T0901317 (Figure 3A). However, coexpression of RIP140 with LXR $\alpha$  in the absence of ligand resulted in redistribution of RIP140 from the small foci to an even distribution (Figure 3B, panels a-c). Moreover, in the presence of LXR ligand, both LXR $\alpha$  and RIP140 were redistributed and colocalized in diffuse large nuclear domains (Figure 3B, panels d-f). The same localization pattern was seen coexpressing LXR $\beta$  with RIP140 (Supplementary Materials, Figure 2) In summary, the localization of the coexpressed proteins was distinct from the individually expressed proteins. These results suggest that RIP140 and LXR colocalize in the nucleus already in the absence of ligand but that the ligand changes the subnuclear localization of the RIP140/LXR complex.

To determine which domains of LXR are important for colocalization with RIP140, FLAG-tagged LXR deletion constructs were coexpressed with GFP-RIP140. Coexpression of RIP140 with LXR $\alpha$  $\Delta$ N and LXR $\beta$  $\Delta$ N, both in the absence and presence of ligand, distributed RIP140 to the LXR $\Delta$ N typical nuclear foci (LXR $\alpha$ : Figure 3C, panels a-f. LXR $\beta$ : Supplementary Materials, Figure 2), suggesting that the N-terminal domain of LXR is dispensable for colocalization with

RIP140. In contrast, coexpression of LXR $\alpha$  $\Delta$ LBD or LXR $\beta$  $\Delta$ LBD did not change the localization of RIP140, neither in the absence nor presence of LXR ligand, but rather RIP140 stayed in the small RIP140 characteristic foci (LXR $\alpha$ : Figure 3D panels a-f. LXR $\beta$ : Supplementary Materials, Figure 2), showing that the ligand-binding domain of LXR is necessary for redistribution of RIP140. The conserved helix 12 in the very C-terminus of nuclear receptor ligand-binding domains has been shown to be important for coactivator binding but not for binding of the corepressor proteins SMRT and NCoR [reviewed in [35]]. Interestingly, RIP140 colocalizes with LXR $\alpha$   $\Delta$ H12 and LXR $\beta$   $\Delta$ H12 in diffuse large nuclear domains in presence of ligand, in the same way as with full length LXR (LXR $\alpha$ : Figure 3E, panels a-f. LXR $\beta$ : Supplementary Materials, Figure 2), suggesting that helix 12 in LXR is not required for colocalization with RIP140.

In summary, these data show that RIP140 and LXR are redistributed by ligand and colocalize in diffuse large nuclear domains. The ligand-binding domain is necessary for colocalization, whereas the N-terminal domain is dispensable.

### **The C-terminal part of RIP140 represses LXR mediated transcriptional activity**

RIP140 has been shown to repress gene activation of an LXR responsive element (LXRE)-regulated reporter gene in monkey kidney cells (BSC40) overexpressing LXR $\alpha$  and RXR $\alpha$  [20]. To determine whether RIP140 can repress gene activation mediated by either LXR subtype, we overexpressed LXR $\alpha$  and LXR $\beta$ , respectively, together with RXR $\alpha$  in COS-7 cells. Increasing amount of a RIP140 expression plasmid showed a dose dependent repressive effect of both LXR $\alpha$  and LXR $\beta$  mediated gene activation (data not shown). To examine if RIP140 could repress transcriptional activation mediated by endogenous LXR, human hepatoma (Huh7) cells were used. Quantitative RT-PCR showed that the Huh7 cells express  $3.2 \pm 0.9$  fold higher LXR $\alpha$  mRNA levels compared to LXR $\beta$  (value is the mean  $\pm$  S.D. of three independent experiments, n=9). Addition of the LXR ligand T0901317 strongly upregulated the LXRE-controlled reporter gene activity in the cells (Figure 4B). When cells were transfected with increasing amount of a RIP140 expressing vector and grown in the presence of ligand, a strong dose dependent repression of the reporter gene activity was observed (Figure 4B). In addition, non-ligand induced reporter gene activity was repressed, which could be explained by our interaction studies that showed binding between the LXRs and RIP140 even in the absence of ligand. These results demonstrate that RIP140 can repress transcriptional activity mediated by endogenous LXR.

To obtain further molecular insight into the repressive effect of RIP140, different deletion constructs of RIP140 (Figure 4A) were expressed in Huh7 cells and their influence on endogenous LXR mediated gene activation was studied. Figure 4C shows that the repressive activity of RIP140 mainly resides in the C-terminal part of RIP140 and that the N terminus and the middle part of RIP140 also have some repressive potential. However, the data suggest that the C-terminal part, aa 747-1158, is sufficient for the full repressive effect of RIP140.

### **C-terminal NR-box like motif (NR10) is important for full repressive activity of RIP140**

The C-terminus of RIP140 (aa 747-1158) contains two NR-boxes (LxxLL motifs) and one NR-box like motif (LxxML). To determine if the NR-boxes are necessary for the repressive activity of RIP140 on LXR mediated gene activation, we transfected Huh7 cells with vectors expressing RIP140 aa 747-1158, intact or mutated in either one NR-box or several NR-boxes in different combinations (Figure 5A). Mutation of the NR-box like motif NR10 individually (mut NR10) or in combination (mut NR 8,10, NR 9,10 and mut NR8,9,10) significantly reduced the repressive effect of the C-terminus, whereas mutations in other NR-boxes individually (mut NR8 and mut NR9) or in combination (mut NR 8,9) had no effect (Figure 5C). To verify the importance of the NR-box like motif NR10 in the context of full length RIP140, mutation in NR10 was introduced in full length RIP140 and the repressive effect was studied in Huh7 cells. The results show that mutation of the NR-box like motif NR10 significantly reduces the repressive activity of full length RIP140 (Figure 5D). To insure that the difference is not due to the level of protein expressed, Huh7 cells were transfected with full length HA-tagged RIP140 or HA-tagged RIP140 mut NR10 (Figure 5B). The expression level of the mutant protein was similar to the wild type protein supporting that the difference in activity of the two proteins shown in Figure 5D, reflect that the NR10 motif is important for the repressive function. Thus the data clearly show the importance of the NR-box like motif for the repressive activity of RIP140 in LXR mediated transcriptional activation.

### **The integrity of the C-terminal part of RIP140 is needed for full repressive activity**

To determine whether the NR-box like motif NR10 is the only determinant for repression in RIP140 C-terminus, deletion constructs of RIP140 C-terminus were tested for their repressive potential on LXRE-controlled reporter gene activity in Huh7 cells (Figure 6A). All deletion constructs containing the NR-box like motif NR10 showed repression capability (Figure 6B, see constructs 747-1158, 863-1158 and 965-1158). In contrast, deletion of the region containing the NR-box like

motif NR10 abolished the repression activity (Figure 6B, see constructs 747-1060 and 747-935), confirming the role of the motif for repression. Moreover, deletion of aa 747-862 also reduced the repressive activity (Figure 6B, see construct 863-1158), demonstrating that the NR-box motif is not sufficient for full repression. In summary, our data suggest that the presence of the NR-box like motif NR10 and the integrity of the C-terminal region are needed for full repressive activity.

### **RIP140 interacts differently with the LXR subtypes**

To clarify the relative importance of the two LXR subtypes for RIP140's repressive activity on LXR mediated gene transcription, a yeast two hybrid experiment was performed where the determinants for RIP140's interaction with LXR  $\alpha$  and  $\beta$ , respectively, were identified. The C-terminal domain of RIP140 (aa 747-1158) interacts with LXR $\beta$  in a ligand enhanced manner (Figure 7B), while LXR $\alpha$  interacted ligand independently (Figure 7A). The deletion constructs containing the NR-box like motif NR10 interacted with LXR $\beta$  (Figure 7B, see constructs 863-1158 and 965-1158). Interestingly, the deletion constructs lacking the motif (747-1060 and 747-935) or containing a mutation of the motif (mut NR10) did not bind to LXR $\beta$  (Figure 7B). In contrast, LXR $\alpha$  interacted with construct 747-1060, which lacks the NR-box like motif NR10, and with the mut NR10 construct. The interactions were, however, weak compared to the 747-1158 construct (Fig. 7B). Constructs containing just one NR box motif, 965-1158 or 747-935, had a total loss of binding towards LXR $\alpha$ . Taken together these results indicate that LXR $\alpha$  and  $\beta$  interact differently with RIP140 C-terminal domain. The NR-box like motif NR10 is necessary for interaction with LXR $\beta$  whereas, in the case of LXR $\alpha$ , our data suggest that more than one NR-box is needed to obtain any binding; for full binding capacity the NR-box like motif must be present.

## DISCUSSION

In this study we have characterized the molecular basis for repression of LXR regulated transcription by RIP140 that could contribute to the physiological role of LXRs in control of energy homeostasis and lipid and glucose metabolism.

We have shown that both LXR $\alpha$  and LXR $\beta$  readily bind RIP140 and that the interaction is ligand dependent with LXR $\beta$  and almost ligand independent with LXR $\alpha$ . A recent study described a weaker affinity of the corepressors NCoR and SMRT to unliganded LXR $\alpha$  than to LXR $\beta$  [36], which together with our results could imply that LXR $\alpha$  already in the absence of ligand has adopted a conformation that preferably binds NR-box containing coregulators.

An important regulatory mechanism for protein function is specific intracellular localization. Some nuclear receptors, such as the glucocorticoid receptor, are translocated into the nucleus upon ligand binding, whereas others are present within the nucleus both in the absence and presence of ligand. The subnuclear localization of nuclear receptors can also be regulated by ligand or by formation of protein complexes. We observed a nuclear localization of LXR $\alpha$  and LXR $\beta$  both in the absence and presence of the agonist ligand T0901317. Mapping studies show that neither the N-terminal domain nor the ligand-binding domain is necessary for nuclear localization, suggesting that the DNA-binding domain and hinge region are important for this to occur. Our data support a recent study that identified a nuclear localization signal (NLS) within LXR hinge region [37]. The study also identified an NLS in the N-terminal domain of LXRs [37]. Interestingly, our data show that while the N-terminally deleted LXRs were still localized in the nucleus, the subnuclear localization had changed from an even pattern to nuclear foci. Although the significance of the altered localization is unknown it could reflect changes in composition of receptor protein complexes affecting nuclear distribution.

We have previously demonstrated that a redistribution of nuclear RIP140 and the glucocorticoid receptor correlates with corepression of glucocorticoid receptor activity by RIP140 [32]. In this study we observed a ligand-induced redistribution of LXR and RIP140 to diffuse large nuclear domains. Similar findings have also been reported for the androgen receptor [38]. Although the functional significance of the nuclear localization pattern is unclear it might reflect formation of new multiprotein complexes.

Our yeast two-hybrid data and colocalization studies show that the LXR ligand-binding domain is necessary for interaction with RIP140. RIP140 has nine NR-boxes and one NR-box like motif. NR-box motif LxxLL mediates the interaction of numerous coactivators, such as p160 family of coactivators, TRAP220 and PGC-1, with nuclear receptor ligand-binding domains. Crystal structures have shown that agonist induced conformational change in the nuclear receptor ligand-binding domain leads to formation of a recognition surface for the NR-box consisting of helices 3-5 and helix 12 [reviewed in [39]]. Thus the conserved helix 12 in the very C-terminus of NR ligand-binding domains is important for coactivator binding. The corepressors N-CoR and SMRT contain a related motif, the CoRNR box (L/IxxI/VI) that mediates the binding to unliganded nuclear receptors [24-26]. The CoRNR box binding site overlaps partially with the NR box binding site on nuclear receptor ligand-binding domains. However, helix 12 is not needed for CoRNR box binding [reviewed in [40]]. Our data show that the colocalization of RIP140 in nuclear domains with LXRs is independent of the presence of helix 12. Interestingly, we have identified a CoRNR box motif in the C-terminus of RIP140 (aa 1042-1046, IKWVI) that, in addition to the NR-boxes, might contribute to receptor binding.

We and others have identified several RIP140 domains with intrinsic repression activity [30-32]. To fully understand the mechanism of RIP140 mediated repression, the activity of RIP140 on nuclear receptor regulated genes must also be studied. Here we show that the C-terminus of RIP140 (aa 747-1158) is sufficient for repression of LXR mediated transcription in Huh7 cells. Previous studies have also pointed to the role of this region for repression of GR and AR regulated genes [32, 38]. The C-terminus contains two NR-boxes (NR8 and NR9) and one NR-box like motif (NR10). Our data show that mutation of the NR-box like motif (LYYML) dramatically reduces the repressive potential. Since mutation of the NR-box like motif also abolished binding to LXR $\beta$  and strongly reduced binding to LXR $\alpha$ , the main role of this motif in corepression is to interact with the receptor. The NR-box like motif has also been shown to be important for ligand induced binding of RIP140 to retinoic acid receptor [28]. In contrast, the main determinant for corepression of GR activity is NR8 and mutation of the NR-box like motif NR10 does not have any effect [32]. Our repression study using deletion constructs of the C-terminal part of RIP140 confirm the importance of the NR-box like motif for repression of LXR mediated transcriptional activation, but also show that in order to achieve full repressive activity the integrity of the whole C-terminal RIP140 is needed. When elucidating the role of the two LXR subtypes in RIP140 mediated corepression, we demonstrate the importance of RIP140 NR-box like motif for interaction with LXR $\beta$  while for LXR $\alpha$  more than one NR-box seems to be important for binding. The clear differences in NR-box preferences of LXR $\alpha$  and  $\beta$  for binding to RIP140 suggest that the two LXR subtypes have different

affinity and/or interaction dynamics. The requirement for more than one NR-box for LXR $\alpha$  binding suggests that individual NR-boxes only have weak affinity for LXR $\alpha$ . For sufficient affinity several NR-boxes are needed for binding and rebinding. Our data suggest that the different biological functions of the two LXR subtypes are not only due to subtype specific expression patterns and availability of coregulators, but also to different binding dynamics of a coregulator such as RIP140 to the receptor subtypes.

To further address the importance of NR-box like motifs, the LxxML motif was scanned using the ScanProsite tool [41] against Swiss-Prot/Trembl databases [42]. Proteins found were then further scanned for LxxLL motifs. A selection of identified proteins are shown in Figure 8. The LxxML motif was found in the orphan nuclear receptor DAX-1 [43] that contains two NR-box like motifs and one NR-box. Human DAX-1 interacts with the nuclear receptors ER $\alpha$  and ER $\beta$  through these motifs and functions as an NR-box containing corepressor in the presence of ligand [44]. Moreover, the cofactor PSU1 contains several atypical NR-boxes with the conserved sequence Lxx $\Phi$ L ( $\Phi$ =hydrophobic), including four LxxML motif, and interacts with several nuclear receptors in a ligand dependent manner [45]. Furthermore, this motif scan revealed that the chromatin remodeling protein ATRX that is a member of the Snf2/Swi2 family has one LxxML motif and two LxxLL motifs that are highly conserved [46]. The human TATA-binding protein-associated factor, TAF-172 contains two LxxML motifs and 11 LxxLL motifs [47]. Both the ATRX and the TAF-172 protein are tightly coupled to the transcriptional machinery and it could be speculated that they bind nuclear receptors. In summary, the alignments/database scanning show that NR-box like motif is highly conserved in RIP140 species, implying an important role for this motif in RIP140 function. Furthermore, this motif is found in proteins with different functions, which could suggest a role for the NR-box like motifs also in other biological contexts.

Our results on the role of the C-terminal NR-boxes and NR-box like motif in RIP140 clearly demonstrate that the determinants for RIP140 interaction and thus corepression differ between nuclear receptors. RIP140 seems to interact with LXR $\beta$  in an atypical manner that does not require the determinants for coactivator binding such as the LxxLL motif and helix 12. On the other hand, our further mapping of the RIP140 C-terminal domain showed that the NR-box like motif (NR10) is not sufficient to mediate corepression. A construct containing NR10 but with a deletion of aa 747-862 had reduced corepressive activity. Thus the integrity of the C-terminal domain (aa 747-1158) is also necessary to mediate full corepression. The function of aa 747-862 in mediating corepression is



not yet understood but might involve the formation of so far unidentified repressive protein complexes.

RIP140 is an atypical corepressor protein that can interact with ligand-activated nuclear receptors and mediate the ligand-induced repression of regulated genes. Importantly, ligand activated LXR represses genes involved in energy, lipid and carbohydrate metabolism in liver and adipose tissue [10]. In this study we have identified RIP140 as a corepressor for LXRs and characterized the molecular basis for repression. Further studies will identify which LXR regulated gene networks involve the corepression activity of RIP140. The important role of both LXRs and RIP140 in regulation of pathways related to development of human metabolic disease emphasizes the significance of understanding of RIP140 mediated repression of LXR regulated genes for a future development of therapeutics.

## ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Science Council.

Declarations of interest: Jan-Åke Gustafsson is cofounder, shareholder, deputy board member, research grant receiver and consultant of KaroBio AB.

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## FIGURE LEGENDS

### Figure 1. Intracellular localization of LXR.

(A) A schematic picture of the LXR domains used. (B) COS-7 cells were transfected with expression plasmids for FLAG-tagged full length LXR $\alpha$  and LXR $\alpha$  domains and treated with (+) or without (-) 1 $\mu$ M T0901317 overnight before visualization of the expressed protein with FLAG-specific antibody using confocal microscopy. The images are representative for 90-100% of the cells studied.

### Figure 2. Interaction of RIP140 with LXR domains.

(A, B) *Saccharomyces cerevisiae* yeast strain Y187 containing an integrated GAL4-responsive lacZ reporter gene was cotransformed with plasmids expressing GAL4 DBD fused to full length LXR $\alpha$  or LXR $\beta$  and LXR $\alpha$  or LXR $\beta$  domains and GAL4 AD-RIP140 aa 560-1158 in the presence or absence of 250 nM T0901317.  $\beta$ -galactosidase activity was assayed as a measurement of interaction. The mean and standard deviation (n=9) are collected from three independent experiments with triplicates in each.

### Figure 3. Intracellular colocalization of LXR and RIP140.

(A) COS-7 cells were transfected with plasmids expressing GFP-RIP140 and treated with (+) or without (-) 1 $\mu$ M T0901317 overnight before visualization of the protein using confocal microscopy. (B-E) COS-7 cells were cotransfected with plasmids expressing GFP-RIP140 and FLAG-LXR $\alpha$  or FLAG-LXR $\alpha$  domains. The cells were treated with (+) or without (-) 1 $\mu$ M T0901317 overnight before visualization of the expressed FLAG-LXR protein with FLAG-specific antibody using confocal microscopy. Panels a and d show the localization of FLAG-LXR protein in red and panels b and e show the localization of GFP-RIP140 in green in the same cells. Panels c and f show the merged image, where colocalization of proteins shows up as yellow. The images shown are representative from 90-100% of the cells studied.

### Figure 4. The C-terminal part of RIP140 represses LXR mediated transcriptional activity.

(A) A schematic picture of the RIP140 domains used. (B) Huh7 cells containing endogenous LXR were transfected with 3xLXRE luciferase reporter plasmid and RSV  $\beta$ -gal reporter plasmid, and increasing amounts of a plasmid expressing RIP140. (C) Huh7 cells were transfected with 3xLXRE luciferase reporter plasmid and RSV  $\beta$ -gal reporter plasmid in the presence (30 ng) or absence of



plasmids expressing RIP140 or RIP140 domains. The cells were maintained in the presence of 250 nM T0901317 or DMSO as vehicle control. The values were related to the activity obtained with ligand treatment in the absence of RIP140 protein. The mean and standard deviation (n=3) of a representative experiment is shown.

**Figure 5. C-terminal NR-box like motif (NR10) is important for full repressive activity of RIP140.**

(A) A schematic picture of the RIP140 mutations used. (B) The expression levels of transfected HA-tagged full length and NR10 mutant RIP140 in Huh7 cells were compared. The values on the left are molecular sizes in kilo-daltons. (C) Huh7 cells containing endogenous LXR were transfected with 3xLXRE luciferase reporter plasmid and RSV  $\beta$ -gal reporter plasmid in the presence (10 ng) or absence of plasmids expressing the C-terminal domain of RIP140 (aa 747-1158) or proteins containing mutations of the indicated NR boxes in the context of RIP140/747-1158. (D) Huh7 cells were transfected with 3xLXRE luciferase reporter plasmid and RSV  $\beta$ -gal reporter plasmid in the presence (10 ng) or absence of plasmids expressing full length RIP140, full length RIP140 containing a mutation of NR10, the C-terminal domain of RIP140 (aa 747-1158) or the C-terminal domain containing a mutation of NR10. The cells were maintained in the presence of 250 nM T0901317 or DMSO as vehicle control. The values were related to the activity obtained with ligand treatment in the absence of RIP140 protein. The mean and standard deviation (n=3) of a representative experiment is shown.

**Figure 6. The integrity of the C-terminal part is needed for full repressive activity.**

(A) A schematic picture of the RIP140 domains used. (B) Huh7 cells containing endogenous LXR were transfected with 3xLXRE luciferase reporter plasmid and RSV  $\beta$ -gal reporter plasmid in the presence (10 ng) or absence of plasmids expressing RIP140 domains. The cells were maintained in the presence of 250 nM T0901317 or DMSO as vehicle control. The values were related to the activity obtained with ligand treatment in the absence of RIP140 protein. The mean and standard deviation (n=3) of a representative experiment is shown.

**Figure 7. Interaction of LXR $\alpha$  and LXR $\beta$  with different RIP140 constructs.**

(A, B) *Saccharomyces cerevisiae* yeast strain Y187 containing an integrated GAL4-responsive lacZ reporter gene was cotransformed with plasmids expressing GAL4 DBD-LXR $\alpha$  (A) or LXR $\beta$  (B) and GAL4 AD-RIP140 constructs in the presence or absence of 250 nM T0901317.  $\beta$ -galactosidase

activity was assayed as measurement of interaction. The mean and standard deviation (n=9) are collected from three independent experiments with triplicates in each.

**Figure 8. Database scanning for LxxML motifs.**

Proteins identified using ScanProsite tool against Swiss-Prot/Trembl databases with LxxML motif as search pattern. Proteins with LxxML motifs were further scanned for LxxLL motifs.

Figure 1

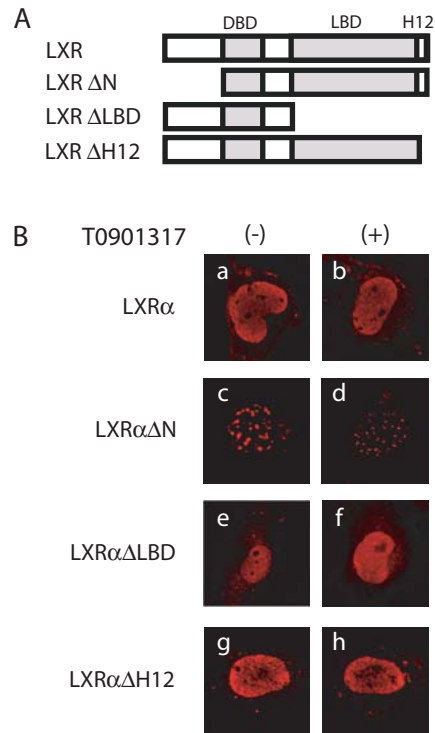


Figure 2

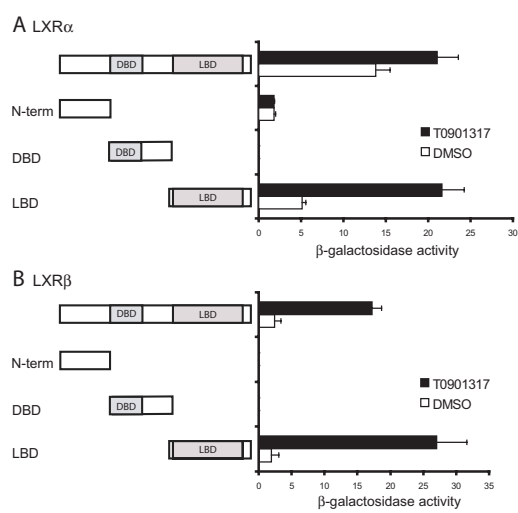


Figure 3

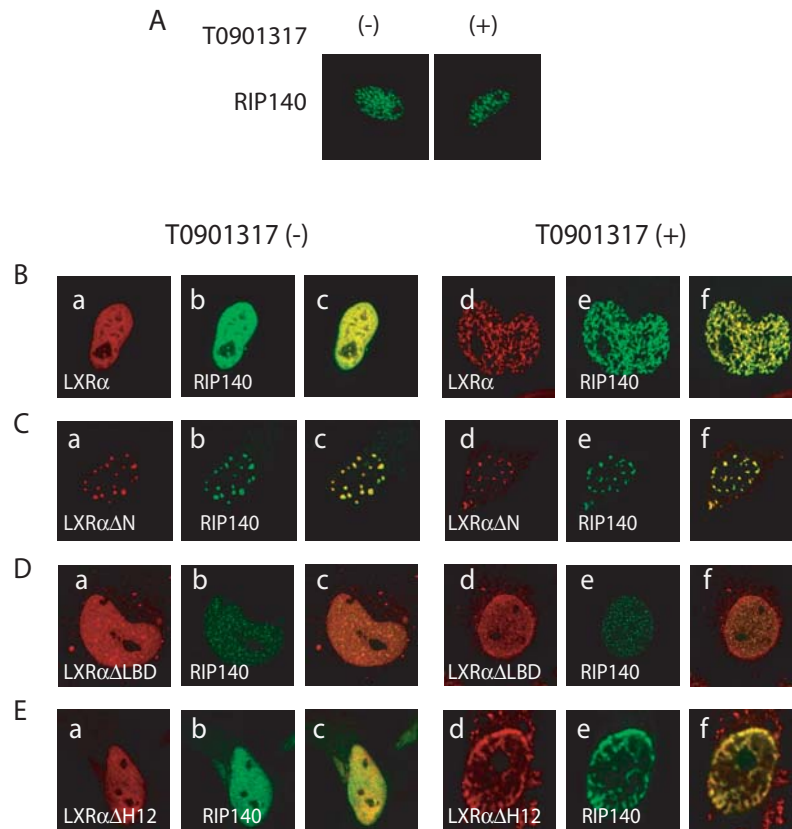
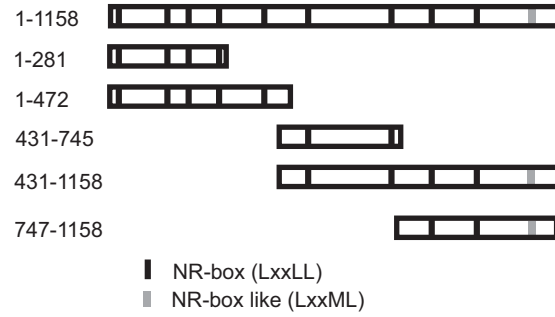
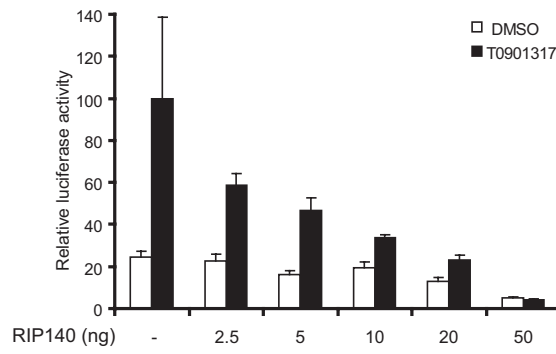


Figure 4

A



B



C

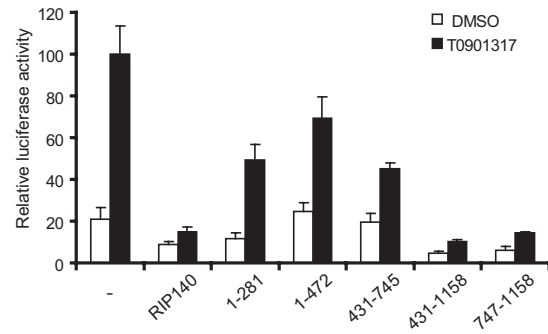


Figure 5

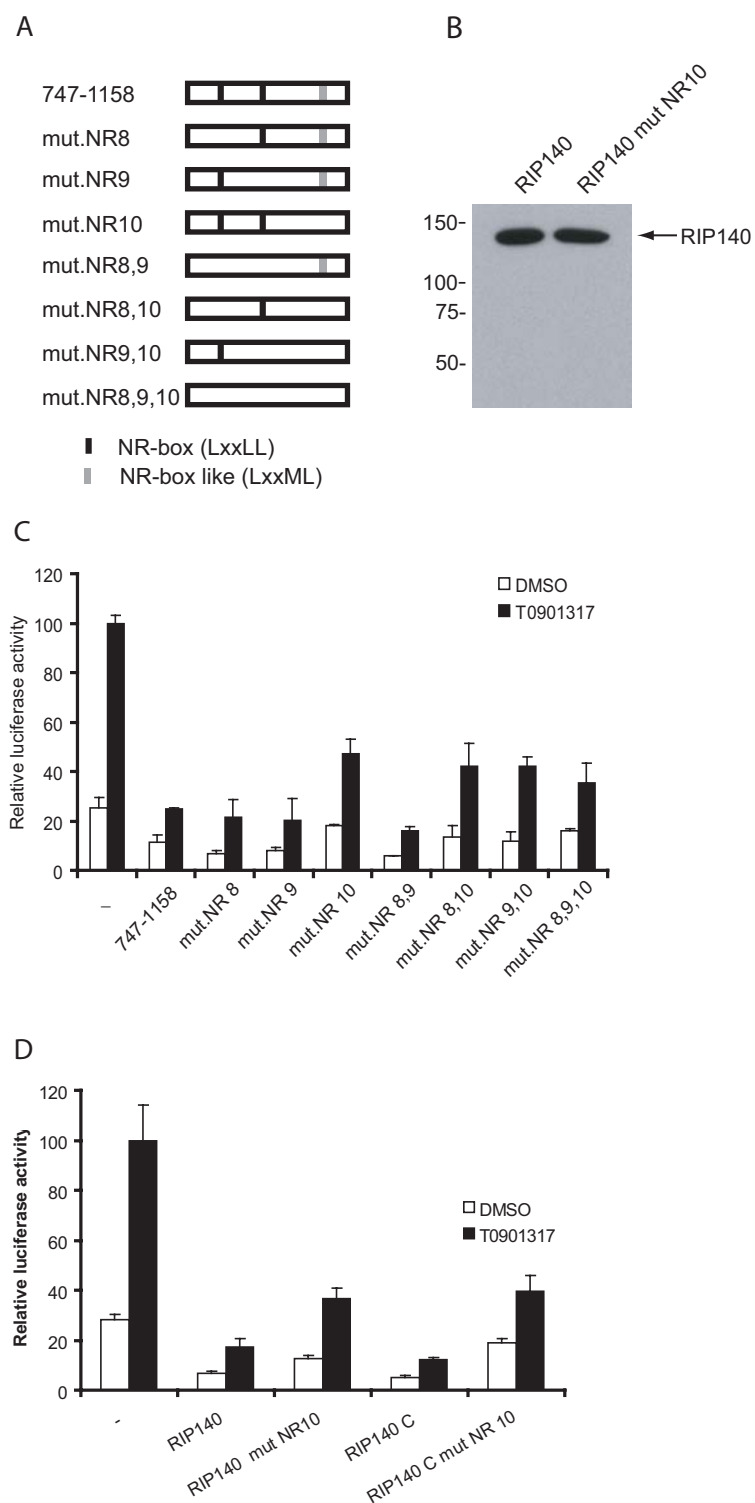


Figure 6

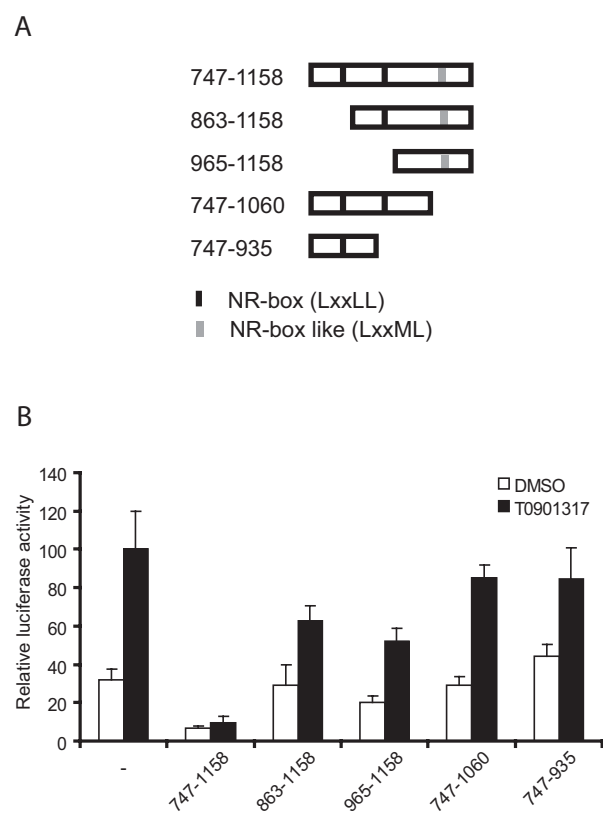




Figure 7

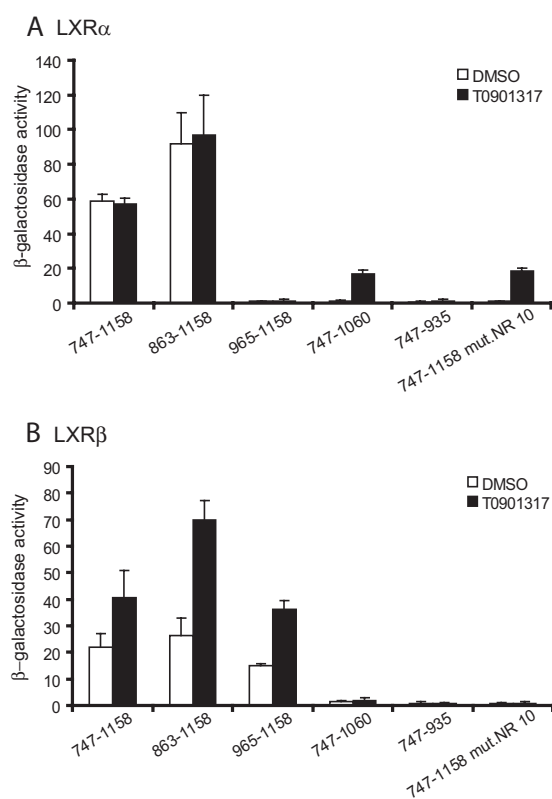


Figure 8

Protein	LxxML	LxxLL
Diacylglycerol kinase gamma	1	1
Rho guanine nucleotide exchange factor 12	1	2
Rho guanine nucleotide exchange factor 11	1	5
Homeodomain-interacting protein kinase 3	2	-
Homeobox protein TGIF2LX	1	-
Transcriptional regulator ATRX	1	2
TATA-binding protein-associated factor 172	2	11
DAX-1	2	1
p63-MAPK	1	3
5'-AMP-activated protein kinase catalytic subunit	1	1
Protein AHC1 (ADA HAT complex component 1 ( yeast)	1	-
ATP-binding cassette sub-family A member 1	2	6
ATP-binding cassette sub-family A member 12	2	4
ATP-binding cassette sub-family C member 8	3	7