Supporting Information

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SI Text

SI Materials. ***Hormones, inhibitors.*** JAK2 inhibitor AG490 was obtained from Calbiochem. Recombinant human Prl and the pure antagonist Del1–9-G129R-hPrl (1) were produced in *E. coli* and purified as described previously (2).

Plasmids. The pcDNA3(+)-human $PrlR_{WT}$ expression vector and the LHRE-luciferase (firefly) reporter plasmid were described previously (3, 4). Mutations I146L and Y587F were introduced by oligonucleotide-directed mutagenesis using the QuikChange II Mutagenesis kit from Stratagene. The coding sequences of vectors were verified on both strands to confirm the presence of the mutations of interest and the absence of unexpected mutations.

Primers. Mutagenesis, PCR, and sequencing primers were from Eurogentec (Oligold quality). Primer sequences are available upon request.

SI Methods. ***Mutation screening.*** The A-to-C substitution in exon 6 (leading to I146L mutation) abolishes XapI/ApoI restriction site. Hence, while digestion of exon 6 PCR product amplified from $PrlR_{WT}$ exhibits 2 bands (181 and 142 bp), the mutated allele remains undigested, which gives rise to an additional band of 323 bp in heterozygous patients (Fig. S1). This method was used to confirm the mutation of interest.

Culture and generation of stable HEK 293 and MCF-7 cell clones. HEK293 and MCF-7 cells were routinely cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen). Cells were transfected (Fugene 6, Roche) as indicated using plasmids encoding the PrlR of interest (2 μg/10 cm petri dish), and for HEK293 only, with the LHRE-luciferase reporter plasmid (0.4 μg/dish). Stable clones and populations (i.e., mixed clones) were selected by, and cultured in, growth medium containing 500 μg/ml active G-418 (geneticin).

Transient transfections of HEK293 cells. HEK293 cells plated in 96-well plates (50,000 cells/well) were in 0.5% FCS medium and cotransfected (Fugene 6, Roche) using plasmids encoding the PrlR of interest (20 ng/well), the LHRE-luciferase reporter gene (4 ng/well) and the *Renilla* luciferase gene (4 ng/well) as an internal (Prl-unresponsive) control of transfection efficiency, as earlier described (5). For luciferase assays (see below), cells were stimulated for 16–20 h by human Prl on the day after transfection.

Culture and generation of stable Ba/F3 cell populations. This murine pro-B cell line is dependent on IL-3 for growth. Cells (10^6) were electroporated (Gene Pulser, BIO-RAD; 200V and 950 μF) using 10–20 μg of pcDNA3(+)-$PrlR_{WT}$ or pcDNA3(+)-$PrlR_{I146L}$, then populations stably expressing either receptor were selected by several passages in G418-containing medium. Ba/F3-$PrlR_{WT}$ and Ba/F3-$PrlR_{I146L}$ cells were routinely maintained in RPMI medium 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 500 μg/ml G-418, and 4 nM human Prl as growth factor (instead of IL-3).

Binding assays. Radiolabeling of human Prl was performed using carrier-free Na[^{125}I] (GE Healthcare) by the IODOGEN method as described (6). Binding affinities of PrlRs were determined using cell homogenates prepared from stable HEK293 clones and Ba/F3 populations as reported (7). For determining the number of cell surface receptors, binding assays were performed using living cells, as described (8). Displacement curves were analyzed by using nonlinear curves fitting (model: homologous competitive binding curves, one class of binding sites, PRISM software).

LHRE-luciferase reporter assay. Luciferase assays were performed as earlier described using either firefly luciferase (stable clones) or dual luciferase (transient transfections) kits (Promega), and a luminometer (Lumat LB 9501, Berthold) (7). The experiments were routinely performed at least three times in triplicate.

Signaling studies. Cells were starved overnight in FCS-free medium (HEK293, MCF-7) or 4–6 h in medium containing 1% FCS (Ba/F3). Then, cells were stimulated using various concentrations of human Prl and/or PrlR antagonist or AG490 (time and concentrations are indicated in the legends). Cell lysates were prepared as previously described (9). For immunoprecipitation studies, 0.5–1 mg of total lysates were incubated with anti-human PrlR (clone 1A2B1, Zymed, 5 μg/ml), or polyclonal anti-STAT5 (C-17, Santa Cruz Biotechnology, 1 μg/ml). Immunoprecipitated samples were analyzed using 7.5% SDS/PAGE, then transferred onto nitrocellulose membranes (Bio-Rad) as described (9). Immunoblotting involved either anti-phosphotyrosine antibody (clone 4G10, Upstate, 1:10,000 dilution) or anti-phosphorylated STAT5 (clone AX1, Advantex BioReagents, 1:5,000 dilution). Antigen-antibody complexes were revealed using either horseradish peroxidase conjugated with anti-mouse (1:4,000 dilution) or with anti-rabbit (1:10,000 dilution) antibody (GE Healthcare), as previously described (9). Membranes were stripped and re-blotted with the anti-PrlR mAb (clone 1A2B1, Zymed, 1:1,000 dilution) or anti-Stat5 Ab (C-17, Santa Cruz, 1:1,000 dilution) to ensure equal loading.

Immunohistochemical analysis. Three μm sections of paraffin-embedded, formalin-fixed tissues were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by incubating slides in 0.3% H_2O_2 for 10 min at room temperature, and nonspecific binding of Ig was minimized by preincubation in normal horse serum for 30 min at room temperature. To reveal phosphorylated STAT5, we strictly followed the procedures previously described for its immunodetection in human breast tissue (10); sections were heated in a microwave-cooker with antigen-retrieval solution AXAR1 (Advantex BioReagents). To reveal total STAT5, sections were heated in a microwave-cooker with citrate buffer. The primary antibodies were diluted in 1% BSA PBS and incubated with the samples overnight (4 °C): AX1 0.6 μg/ml, anti-STAT5 (C-17) 2 μg/ml, anti-PrlR (1A2B1) 5 μg/ml, anti-fatty acid synthase (H-300, Santa Cruz) 2 μg/ml. Antigen-antibody complexes were detected using ABC Elite system (Vector Laboratories) using 3,3′-diaminobenzidine as chromogen.

SI Results. ***Additional SNPs.*** Besides sequencing exons, which identified missense SNPs in exons 5 and 6 (see main text), intronic regions bordering exons were also sequenced, which identified various SNPs reported in NCBI SNP database. Nonexhaustively, these included the following SNPs (with their frequencies in MFA population): rs16872636 (3/74), rs6885088 (1/74), rs12152833 (2/74), and rs2047741 (9/74, one homozygous).

Binding affinity of $PrlR_{I146L}$. The binding affinity of $PrlR_{WT}$ and $PrlR_{I146L}$ for human Prl was determined using HEK293 stable clones. It was very close for both receptors, with affinity constants (K_d) of 0.64 nM ($PrlR_{WT}$) and 0.38 nM ($PrlR_{I146L}$) (Fig. S3). The affinity for the pure PrlR antagonist Del1–9-G129R-

hPrl was also similar for both receptors (not shown), i.e., 10-fold lower than for WT human Prl (1).

1. Bernichtein S, *et al.* (2003) Development of pure prolactin receptor antagonists. *J Biol Chem* 278:35988–35999.
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3. Lochnan HA, Buteau H, Richards S, Edery M, Kelly PA (1995) Functional activity of the human prolactin receptor and its ligands. *Mol Cell Endocrinol* 114:91–99.
4. Goffin V, *et al.* (1996) Antagonistic properties of human prolactin analogs that show paradoxical agonistic activity in the Nb2 bioassay. *J Biol Chem* 271:16573–16579.
5. Dos Santos C, *et al.* (2004) A common polymorphism of the growth hormone receptor is associated with increased responsiveness to growth hormone. *Nat Genet* 36:720–724.
6. Goffin V, Norman M, Martial JA (1992) Alanine-scanning mutagenesis of human prolactin: Importance of the 58–74 region for bioactivity. *Mol Endocrinol* 6:1381–1392.
7. Kinet S, Bernichtein S, Kelly PA, Martial JA, Goffin V (1999) Biological properties of human prolactin analogs depend not only on global hormone affinity, but also on the relative affinities of both receptor binding sites. *J Biol Chem* 274:26033–26043.
8. Berlanga JJ, *et al.* (1997) Prolactin activates tyrosyl phosphorylation of insulin receptor substrate-1 and phosphatidylinositol-3-OH kinase. *J Biol Chem* 272:2050–2052.
9. Llovera M, *et al.* (2000) Human prolactin (hPRL) antagonists inhibit hPRL-activated signaling pathways involved in breast cancer cell proliferation. *Oncogene* 19:4695–4705.
10. Nevalainen MT, *et al.* (2004) Signal transducer and activator of transcription-5 activation and breast cancer prognosis. *J Clin Oncol* 22:2053–2060.
11. Elkins PA, *et al.* (2000) Ternary complex between placental lactogen and the extracellular domain of the prolactin receptor. *Nat Struct* Biol 7:808–815.

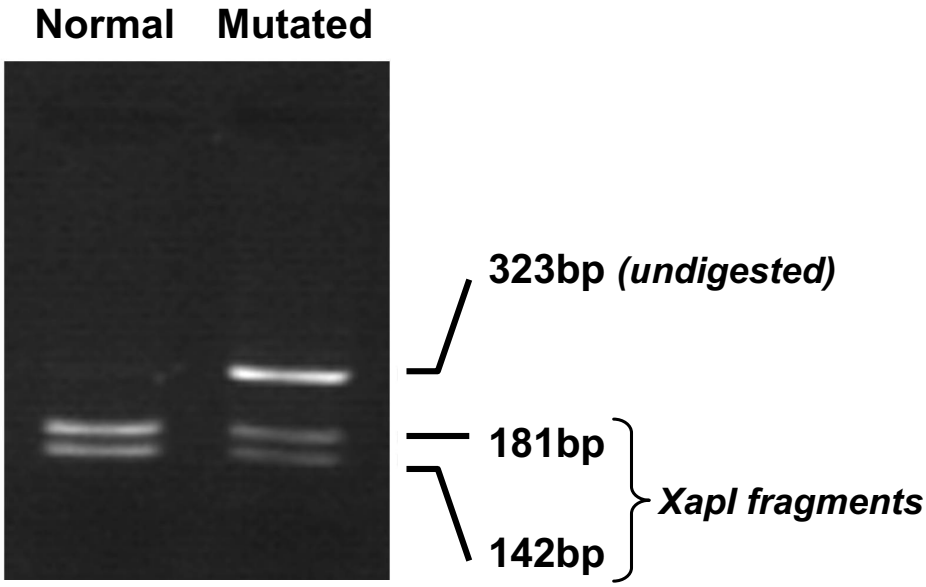

Fig. S1. PCR products amplified from WT or mutated PrlR exon 6 digested using XapI/ApoI and analyzed by agarose electrophoresis.

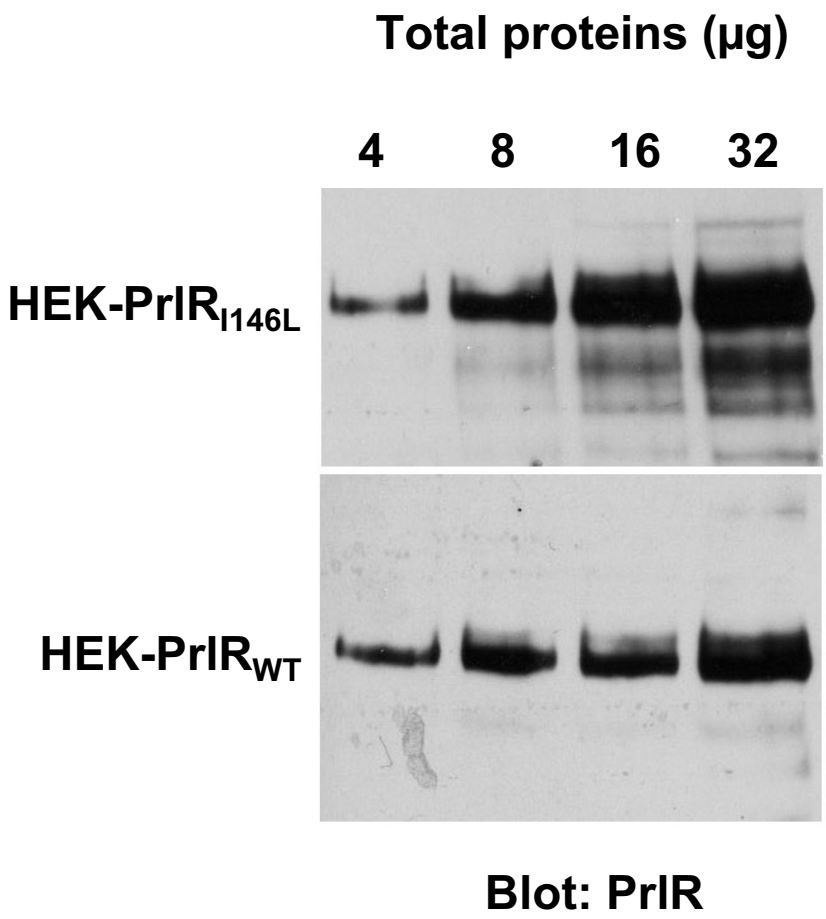

Fig. S2. Comparison of the expression levels of PrlR$_{WT}$ and PrlR$_{I146L}$ in HEK293 stable clones (parental HEK cells do not express endogenous PrlR). Serial dilutions of cell lysates were prepared from each clone and analyzed by PrlR immunoblotting. Similar dilutions gave rise to similar signal intensity for clones expressing either receptor.

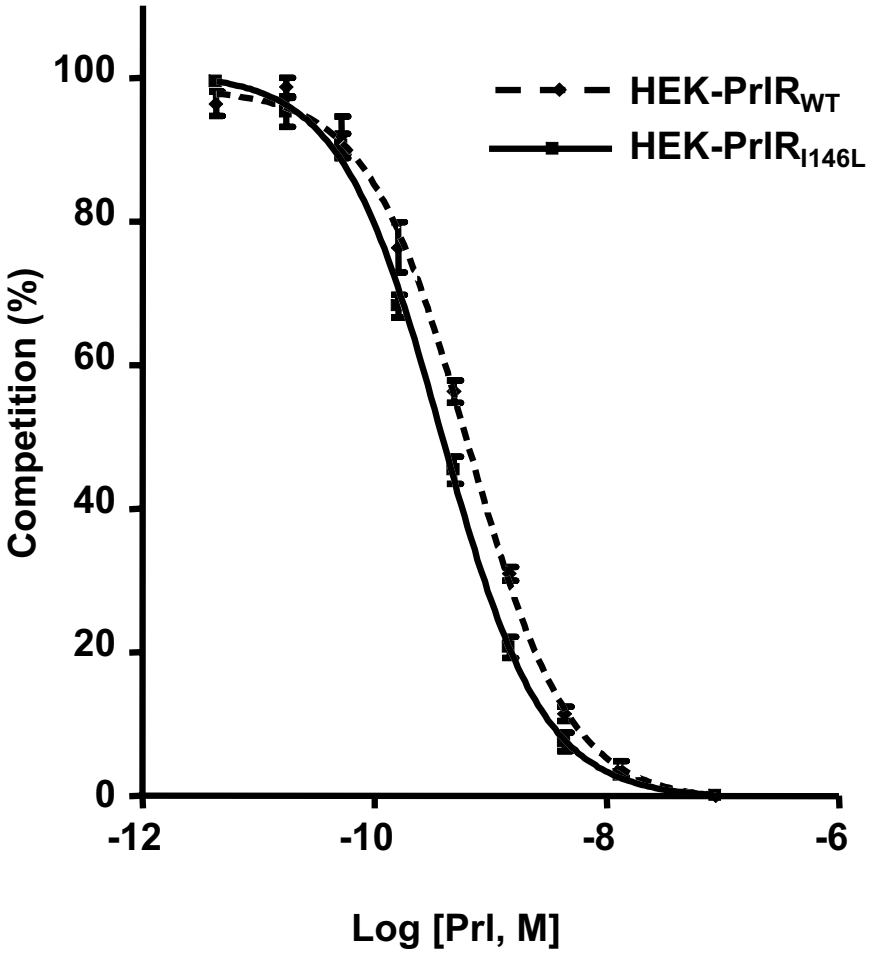

Fig. S3. Displacement curves of ^{125}I-Prl by unlabelled human Prl using HEK293-PrlR$_{WT}$ and HEK293-PrlR$_{I146L}$ cell homogenates. This figure shows that I-to-L substitution does not affect binding affinity of the PrlR for human Prl. Data are shown as means ± SD (four independent experiments performed in duplicate).

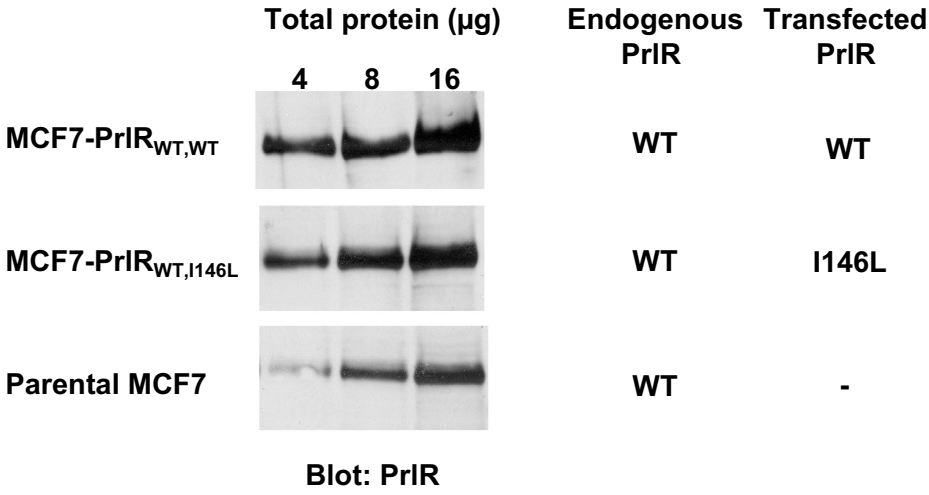

Fig. S4. Comparison of the expression levels of PrlR$_{WT}$ and/or PrlR$_{I146L}$ MCF-7 stable clones. Serial dilutions of cell lysates were prepared from each clone and analyzed by PrlR immunoblotting. Similar dilutions gave rise to similar signal intensity for clones expressing either receptor. Transfected MCF-7 clones (MCF7-PrlR$_{WT,WT}$ versus MCF7-PrlR$_{WT,I146L}$) expressed approximately twice more PrlR than the parental cell line (*Bottom*).

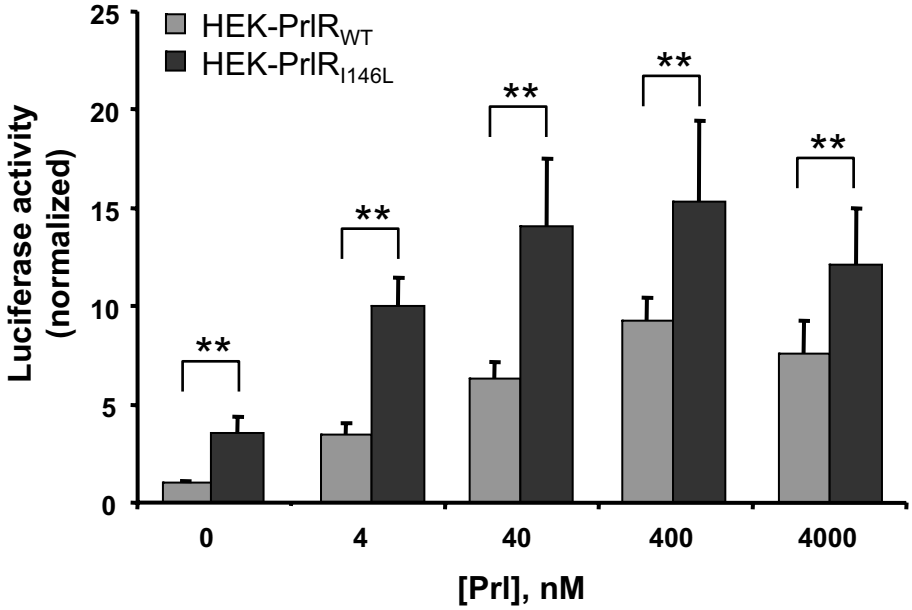

Fig. S5. Prolactin dose-response of LHRE-luciferase gene induction. Both HEK293-PrlR$_{WT}$ and HEK293-PrlR$_{I146L}$ clones also stably incorporated the Prl-responsive LHRE-luciferase gene (see *SI Text*). Luciferase activity in HEK293-PrlR$_{WT}$ (gray bars) or HEK293-PrlR$_{I146L}$ (black bars) was measured after 24 h treatment with increasing concentrations of Prl. Data are normalized to basal luciferase activity of HEK293-PrlR$_{WT}$ (means ± SD, six independent experiments performed in triplicates). **, $P < 0.01$.

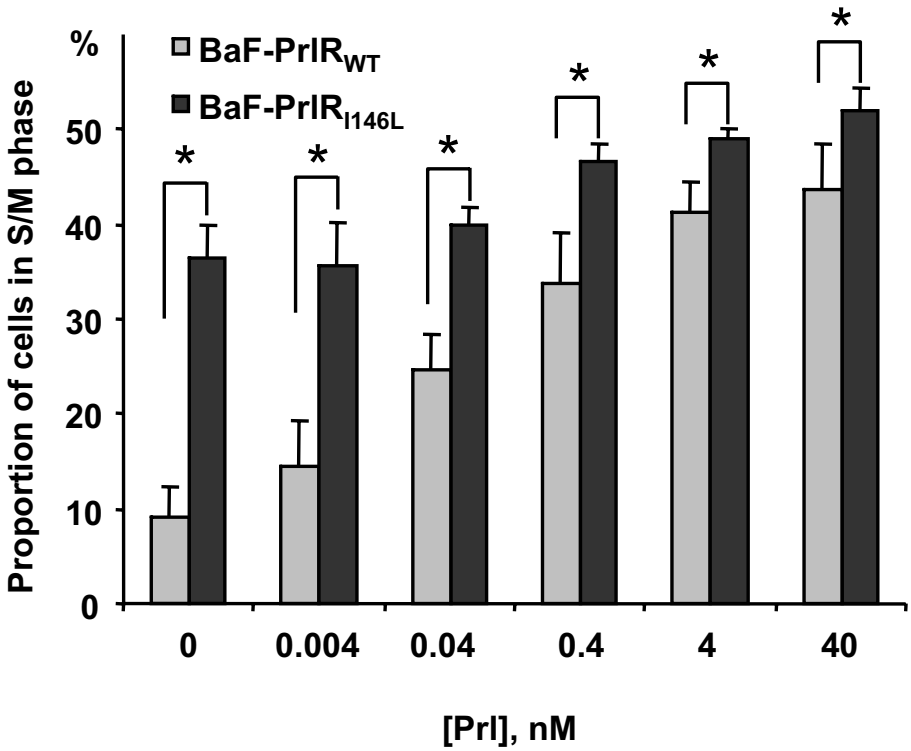

Fig. S6. Prolactin dose-response of S/M phase induction in Ba/F cells. Ba/F-PrlR$_{WT}$ (gray bars) and Ba/F-PrlR$_{I146L}$ (black bars) cells were starved by Prl depletion for 6 h, then stimulated with increasing concentrations of Prl for 24 h. Cell cycle distribution was monitored by FACS analysis (see Fig. 3*F*), and the proportion of cells in S/M phase was quantified for both populations. Data are mean ± SD, from four independent experiments. *, $P < 0.05$.

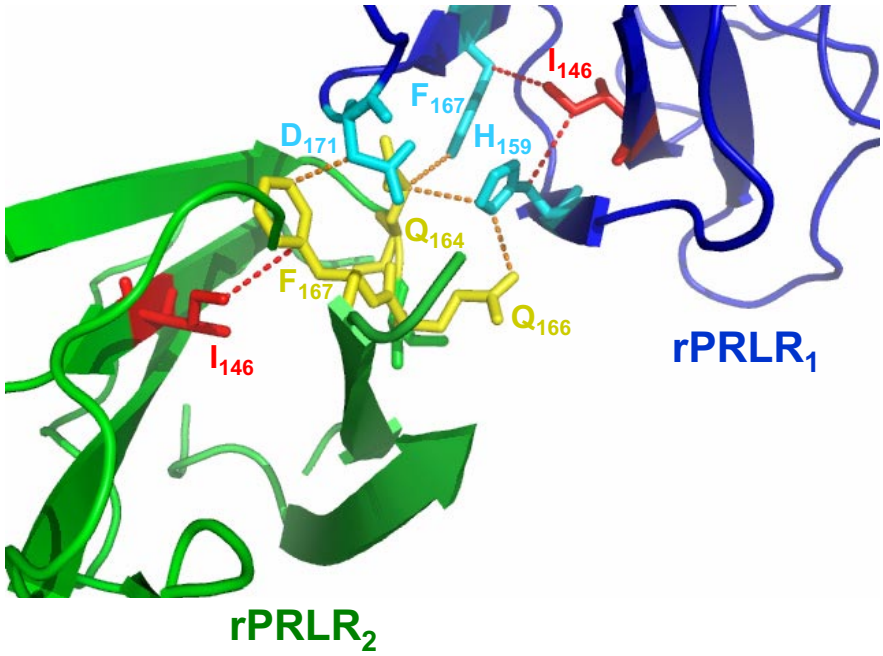

Fig. S7. Potential role of Ile_{146} at the receptor-receptor interface within the ligand-PrlR complex. This figure shows some of the interactions between the two receptor chains involved in the hormone-PrlR ternary complex, PDB ID code: 1f6f (11). The interactions symbolized by orange dotted lines involve residues H_{159}, F_{167} and D_{171} from one receptor ($PrlR_1$) and Q_{164}, Q_{166} and F_{167} from the other ($PrlR_2$). Ile_{146} is directly interacting with some of these residues (red dotted lines). We propose that I-to-L mutation could lead to local structural rearrangements favoring the unbound receptor to adopt a conformation mimicking that of the receptor bound to an agonist. This figure was generated by using The PyMOL Molecular Graphics System (DeLano Scientific).