

Both LRP5 and LRP6 Receptors Are Required to Respond to Physiological Wnt Ligands in Mammary Epithelial Cells and Fibroblasts^{*[5]}

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Background: Mammary stem cells require a specific Wnt signaling receptor, LRP5, despite co-expression of the (better studied) LRP6 receptor.

Results: Both LRP5 and LRP6 are required for mammary epithelial cell responsiveness to Wnt1/9b/10b (but not Wnt3a).

Conclusion: LRP5 co-expression with LRP6 can confer active Wnt signaling where either receptor alone cannot.

Significance: This explains aspects of stem cell regulation and tumorigenesis.

A canonical Wnt signal maintains adult mammary ductal stem cell activity, and this signal requires the Wnt signaling receptor, LRP5. However, previous data from our laboratory have shown that LRP5 and LRP6 are co-expressed in mammary basal cells and that LRP6 is active, leading us to question why LRP6 is insufficient to mediate canonical signaling in the absence of LRP5. Here, we show that at endogenous levels of LRP5 and LRP6 both receptors are required to signal in response to some Wnt ligands both *in vitro* (in mouse embryonic fibroblasts and mammary epithelial cells) and *in vivo* (in mammary outgrowths). This subgroup of canonical ligands includes Wnt1, Wnt9b, and Wnt10b; the latter two are expressed in mammary gland. In contrast, the ligand commonly used experimentally, Wnt3a, prefers LRP6 and requires just one receptor regardless of cellular context. When either LRP5 or LRP6 is overexpressed, signaling remains ligand-dependent, but the requirement for both receptors is abrogated (regardless of ligand type). We have documented an LRP5-6 heteromer using immiscible filtration assisted by surface tension (IFAST) immunoprecipitation. Together, our data imply that under physiological conditions some Wnt ligands require both receptors to be present to generate a canonical signal. We have designed a model to explain our results based on the resistance of LRP5-6 heteromers to a selective inhibitor of E1/2-binding Wnt-LRP6 interaction. These data have implications for stem cell biology and for the analysis of the oncogenicity of LRP receptors that are often overexpressed in breast tumors.

diverse as cell migration, cell polarity, and adhesion (1, 2). The β -catenin/TCF³ (so-called “canonical”) pathway is essential to the self-renewal, proliferation, and differentiation of stem and progenitor cells in a variety of contexts and is highly oncogenic when dysregulated (3, 4). Genetic and biochemical data suggest that this pathway is activated when Wnt ligands interact with members of two distinct families of cell surface receptors, the Frizzled (Fzd) receptors and the low density lipoprotein receptor-related proteins LRP5 and LRP6 (5, 6). This initiates a signaling cascade (associated with phosphorylation of LRP species) (7–9), resulting in translocation of β -catenin to the nucleus where it interacts with the LEF-1/TCF family of transcription factors to modulate transcription of Wnt target genes (10, 11). In vertebrates, there are several Wnt ligands and Fzd receptors (19 Wnt ligands and 10 Fzd homologues) but only two LRP species with defined roles in Wnt signaling (1). Although Wnt-Fzd interactions orchestrate the activation of both β -catenin/TCF-dependent and -independent pathways, the LRP family of Wnt receptors specifically mediates activation of the β -catenin/TCF arm of the pathway (12–16).

LRP5 and LRP6 are type I, single span transmembrane receptors with a large extracellular domain shown to bind several Wnt ligand species tested *in vitro* (14, 15, 17, 18). In addition to Wnt proteins, the extracellular regions of LRP5 and LRP6 also bind other agonists and antagonists of the Wnt pathway, including members of the Dkk family, Sclerostin, and Wise (17, 19–22). Presumably, the output of LRP receptor activation represents the sum total of these interactions. LRP5 and LRP6 exhibit a high degree of sequence homology, sharing 73 and 64% sequence identity in their extracellular and intracellular domains, respectively (15). This, coupled with extensive similarities in structural and biochemical properties, has led to the assumption of functional redundancy between the two recep-

The Wnt signaling pathway plays critical roles in embryonic development and tissue homeostasis and regulates processes as

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³ The abbreviations used are: TCF, T cell factor; LRP, lipoprotein receptor-related protein; IFAST, immiscible filtration assisted by surface tension; Fzd, Frizzled; MEC, mammary epithelial cell; MEF, mouse embryonic fibroblast; MCS, multiple cloning site; IRES, internal ribosome entry site; qRT-PCR, quantitative RT-PCR; IP, immunoprecipitation; rmWnt, recombinant mouse Wnt; mWnt, mouse Wnt; mLRP, mouse LRP.

tors. However, *in vivo* studies show that the two receptors mediate unique functions. Although homozygous deletion of LRP6 in transgenic mice leads to perinatal lethality, LRP5 knock-out mice are viable and fertile (18, 23, 24). Early lethality of LRP6 knock-out mice has hindered systematic, comparative studies of the relative contributions of the two receptors to Wnt signaling *in vivo*. In this study, we evaluated the relative signaling potential of LRP5 and LRP6 when expressed at endogenous levels in contrast to many other molecular structure/function studies that have relied on ectopic overexpression of the two receptors (13, 25, 26). This revealed a novel, ligand-dependent restriction of their activities.

We have shown previously that *lrp5*^{-/-} mammary glands exhibit depleted mammary stem cell activity and are protected from Wnt1-mediated tumorigenicity despite expression of LRP6 (24). The absence of LRP5, however, has no effect on Wnt3a-mediated transactivation of the canonical Wnt pathway in *lrp5*^{-/-} mammary epithelial cells (MECs) (27). These data suggest that LRP5 and LRP6 serve different functions in the mouse mammary gland, although the mechanistic details remain unknown. One intriguing hypothesis to explain the distinct roles of LRP5 and LRP6 is that different Wnt ligands activate the canonical Wnt pathway by preferentially signaling through either LRP5 or LRP6.

To test this hypothesis, we used mouse embryonic fibroblasts (MEFs) harvested from wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} embryos and treated them with different Wnt ligands. We observed that whereas Wnt3a requires LRP6 to activate the Wnt pathway another group of Wnt ligands, including Wnt1, -9b, and -10b, requires both LRP5 and LRP6 for optimal Wnt pathway activation in MEFs. Using a modified immunoprecipitation assay (IFAST), we obtained evidence that LRP5 and LRP6 exist in mixed heteromeric signaling complexes. Furthermore, we show that when these receptors are overexpressed either LRP5 or LRP6 can mediate Wnt1 class signals. The dual requirement for LRP5 and LRP6 was also observed *in vivo* for mammary gland outgrowth. Our studies illustrate an important regulatory mechanism operating *in vivo* with implications for specific ligand-receptor complexes functioning as mediators of various Wnt-dependent physiologies.

EXPERIMENTAL PROCEDURES

Cell Culture—MEFs were harvested from 13.5-day pregnant C57Bl/6 *lrp5*^{+/-} or *lrp6*^{+/-} mice (that were crossed to heterozygous males). Briefly, embryos were diced and trypsinized (0.05% trypsin, EDTA) for 10 min at 37 °C followed by resuspension in growth medium. The genotype of each individual embryo was identified (+/+, +/-, or -/-). MEFs and HEK293T cells were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (Harlan, Indianapolis, IN) and 100 units/ml penicillin/streptomycin (Invitrogen). MEFs were propagated in low oxygen chambers (1% O₂ content) to extend the lifespan of the primary cells (28). HC11 cells (gift of Nancy Hynes, Friedrich Miescher Institute, Basel, Switzerland) were cultured in RPMI 1640 medium containing 10% FBS (Harlan), 5 μg/ml insulin (Sigma-Aldrich), and 10 ng/ml recombinant human EGF (R&D Systems, Minneapolis, MN). MECs harvested from wild type or

lrp5^{-/-} mammary glands were maintained as described previously (27).

Plasmids and Reagents—cDNA constructs encoding mouse Wnt3a and Wnt10b were generously provided by Bart Williams (Van Andel Research Institute, Grand Rapids, MI) and Ormond MacDougald (University of Michigan Medical School, Ann Arbor, MI) (29, 30), respectively. Wnt1 expression plasmid (31) was subcloned into the retroviral vector Pcmmp-MCS (multiple cloning site)-IRES-eGFP. An expression plasmid for mouse LRP5 (pCMV-SPORT6-LRP5) was purchased from Open Biosystems (Huntsville, AL), and an expression plasmid for mouse LRP6 was generated by subcloning the NotI-cut cDNA from pYX-Asc-LRP6 (Invitrogen) into pCDNA3 mammalian expression vector. The LRP5-myc and LRP6-myc tagged constructs were generously provided by Gail V. W. Johnson (University of Rochester, Rochester, NY) and Anthony M. C. Brown (Weill Cornell Medical College, New York, NY), respectively (12, 26). Lentiviral vectors designed to express either Wnt1 or Wnt3a were assembled using the backbone of a self-inactivating lentiviral vector (SIN) with an EF1α promoter (a kind gift from Dr. Robert G. Hawley, American Red Cross). To create a bicistronic vector, an IRES DNA fragment was inserted at the MCS followed by a human placental alkaline phosphatase (hPAP) cDNA construct (a gift from Dr. Deborah A. Brown, State University of New York, Albany, NY) (SIN-EF1α-MCS-IRES-hPAP). Recombinant mouse Wnt3a (100 ng/ml unless otherwise indicated), Wnt5a (40 ng/ml), and Wnt9b (400 ng/ml) were purchased from R&D Systems.

Generation of Lentiviral Particles—The two series of lentiviral vectors used for this study, SIN-EF1α expression vectors that encode Wnt1 or Wnt3a (or the control MCS plasmid) and vesicular stomatitis virus G and viral polymerase/core protein constructs, were transfected into HEK293T cells using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions, and lentiviral particles were harvested from the cell supernatant 48–72 h later. Commercially available expression vectors (pLKO.1) from Open Biosystems were used to express shRNA constructs targeting *lrp5* (catalog number RMM4534-NM_008513) or *lrp6* (catalog number RMM4534-NM_008514) and packaged using Lipofectamine LTX (including a scrambled control). Cells were infected with the viral supernatant with 8 μg/ml Polybrene (Sigma) and maintained in medium with puromycin (1 μg/ml for MEFs and 6 μg/ml for HC11 cells as determined from puromycin kill curves).

Transient Transfections/Viral Transductions—All transient transfections in MEFs and HC11 cells were performed using Lipofectamine LTX reagent (Invitrogen) by following the manufacturer's protocol designed for MEFs. Briefly, 0.02 × 10⁴ cells were plated in 24-well plates, and a total amount of 0.5 μg of plasmid was added to each well. Transfections were scaled up to 6-well plates/60-mm dishes when performed for quantitative PCR or Western blot analysis.

Methods from Welm *et al.* (32) and Britt *et al.* (33) were adapted for viral transductions of MECs in suspension. 0.5 × 10⁶ wild type or *lrp5*^{-/-} MECs were plated in 24-well low attachment plates and infected with viral supernatant for 16 h (32, 33). The cells were collected, spun down at 450 × g for 5 min, and used for *in vivo* transplantation assays or plated in

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6-well plates for evaluation of transduction efficiency and for qRT-PCR analysis. To assay for transduction efficiency of Wnt1- and Wnt3a-expressing viruses, cells were stained for human placental alkaline phosphatase activity; transduction efficiency was similar (approximately 60%).

Fat Pad Assays of Outgrowth Potential in Vivo—Mammary glands of 3-week-old C57Bl/6 virgin mice were cleared of endogenous epithelium. MECs transduced with different lentiviral constructs were resuspended in DMEM containing 5 μ g/ml Matrigel and loading dye (5% glycerol, 0.5% trypan blue, 25 mM HEPES, pH 7.2). 1- μ l volumes containing 50,000 cells were injected into cleared fat pads, and outgrowths were harvested 8 weeks post-transplantation as described previously (27).

Quantitative Real Time PCR Analysis—RNA isolation, cDNA generation, and amplification by real time PCR were performed as described previously (27). Relative transcript levels were calculated using the comparative Ct method and normalized to housekeeping genes, *ywhaz* and *hprt1*. The 5' to 3' sequences of the primer pairs are detailed in Ref. 34.

Western Blot Analysis—Cells were solubilized in lysis buffer (25 mM HEPES, pH 7.4, 300 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 50 mM glycerophosphate, 0.5% Triton X-100) with freshly added protease and phosphatase inhibitors (Thermo Fisher Scientific). Western blotting analysis was performed as described previously (27). Membranes were incubated (overnight at 4 °C) with the following primary antibodies at the dilutions indicated: anti-LRP5, D5G4, 1:1000 (Cell Signaling Technology catalog number 5440); anti-LRP6 antibodies, either 1C10, 1:1000 (Abcam catalog number ab75358) or C47E12, 1:1000 (Cell Signaling Technology catalog number 3395); anti-phospho-LRP (Ser-1490), 1:1000 (Cell Signaling Technology catalog number 2568); anti- β -Actin, 1:5000 (Sigma); and anti-myc, 71D10, 1:1000 (Cell Signaling Technology catalog number 2278). To quantify the bands obtained via Western blotting, ImageJ (version 1.43u) software-based analysis was applied.

To determine the relative signal from mouse LRP5 and LRP6 proteins after Western blotting, HEK293T cells were transfected with LRP-myc constructs, and a dilution curve of total protein was loaded for SDS-PAGE. To quantify relative myc-tagged protein amounts, the Western blotting procedure and exposure times were strictly standardized. Replicated pixel numbers for each dilution of each antigen (myc, LRP5, and LRP6) were combined to assess linearity of response, and the relative signal for the specific (LRP5 and LRP6) antigens was determined with respect to the myc tag on each protein.

Dual-Luciferase Assay—To measure canonical Wnt signaling activity using the superTOP-FLASH reporter assay, MEFs or HC11 cells were transfected with the various expression vectors as indicated in the experiments together with superTOP-FLASH reporter plasmid (0.1 μ g) and *Renilla* luciferase gene (0.01 μ g) using Lipofectamine LTX (Invitrogen). The total transfected DNA amount was kept constant at 0.5 μ g by transfection with the Pcmmp-MCS-IRES-eGFP expression plasmid, and the reporter assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) as described previously (31). To normalize for transfection efficiency, firefly luciferase activity was divided by *Renilla* luciferase

activity. The value was expressed as relative luminescence units, and the average relative luminescence units were calculated for triplicate samples.

IFAST Immunoprecipitation—Wild type MEFs were treated for 2 h with Wnt3a (20 ng/ml), Wnt9b (400 ng/ml), Wnt5a (40 ng/ml), or nothing. Cells were lysed in immunoprecipitation (IP) lysis buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 1 \times protease inhibitor mixture, 1 \times phosphatase inhibitor mixture) as described previously (35). Approximately 300 μ g of lysate (1.5 μ g/ml) was incubated with either 1.2 μ g of anti-LRP5 (Cell Signaling, catalog no. 5731), 130 ng of anti-Axin1 (Cell Signaling, catalog no. 3323), 450 ng of anti-Phospho-LRP (Cell Signaling, catalog no. 2568), or 1.2 μ g rabbit IgG antibody (Sigma, catalog no. I5006), for 1 h while rotating at 4 °C. 5 μ l of prewashed protein G Dynabeads (Invitrogen catalog number 100-03D) were added to each tube, and lysates were rotated overnight with antibody and beads at 4 °C. IP complexes were purified using the IFAST method (illustrated in Fig. 7A) (36). Immune complexes were eluted in the output well in PBS containing 0.01% Tween 20 (17 μ l). Prior to the IP reaction, whole cell lysate was withdrawn for analysis. Gels were loaded with 10 μ g of whole cell lysate, 10 μ g of unbound protein (defined as the proteins remaining in the lysate after pull-through, which exceeded 95% of whole cell lysate, measured by protein assay) and 2 μ l (out of 17 μ l total) of the bound protein fraction. LRP5, LRP6, phospho-LRP, and Axin1 proteins were detected by incubating blots with the respective antibodies listed previously. To determine the specificity of the interactions, blots were incubated with anti-vinculin (Chemicon catalog number CBL233) at a 1:5000 dilution and/or anti-EGF receptor antibody (Cell Signaling Technology catalog number 2232) at a 1:1000 dilution. To calculate the efficiency of binding and pull-through of immune complexes, band intensities from the unbound and bound fractions were quantified and corrected for background using the UVP ChemiDoc-It Imaging System with Visionworks software. The total protein amount for each fraction was calculated as the product of intensities and total volumes. The percent extraction efficiency for each protein was approximated by dividing the signal equivalent to the bound protein by the signal from total (unbound plus bound) protein fractions.

RESULTS

Composition and Amount of LRP Species Are Similar in MEFs and MECs—We have shown previously that LRP5 is key to Wnt1-dependent tumor development in the murine mammary tumor virus-Wnt1 transgenic mouse model despite expression of LRP6, and that Wnt3a-dependent transactivation of the canonical Wnt pathway is not significantly affected in *lrp5*^{-/-} MECs (24, 27). To understand the molecular basis for this apparent paradox, we turned to MEFs as a potential culture model. (*lrp6*^{-/-} MECs are not readily available given the perinatal lethality of *lrp6*^{-/-} mice (23)). We derived six types of MEFs from embryos harvested from interbred *lrp*^{+/-} heterozygotes (wild type, *lrp*^{+/-}, and *lrp*^{-/-} from C57Bl/6 strains). The results we show here are derived from comparisons of wild type and knock-out MEFs (several assays also included heterozygous strains and show predictable, dose-de-

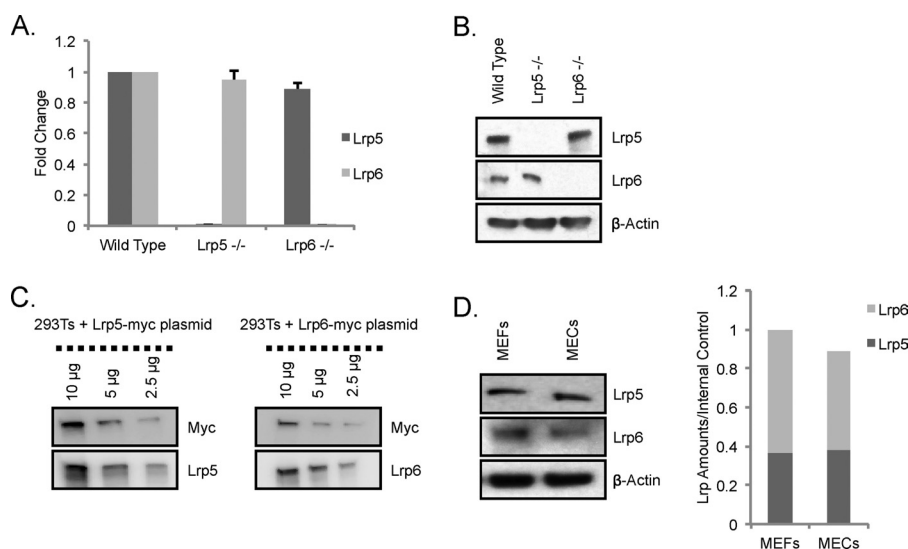


FIGURE 1. LRP5 and LRP6 expression in MEFs and MECs. *A*, *lrp5* and *lrp6* mRNA expression in wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs. To test for compensatory expression of alternate LRP species in knock-out cells, *lrp5* and *lrp6* mRNA was quantified using qRT-PCR analysis of RNA harvested from wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs. Values shown represent -fold changes compared with wild type MEFs (set to 1) (after normalization to housekeeping genes). *B*, LRP5 and LRP6 protein expression in wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs. Western blots of protein lysates prepared from wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs after probing with anti-LRP5 or -LRP6 (and β -actin) antibodies are shown. *C*, comparison of the relative signal per molecule for anti-LRP5 and anti-LRP6 antibodies. HEK293T cells were transfected with LRP5-myc and LRP6-myc plasmids, and doubling dilutions of total protein (10, 5, and 2.5 μ g) were probed with anti-myc antibody and with anti-LRP5 or -LRP6 antibody to determine the relative signal with respect to the myc tag (image analysis is described under "Experimental Procedures"). *D*, determination of relative LRP5 and LRP6 protein expression in MEFs and MECs. Protein extracts prepared from MEFs and MECs were analyzed by SDS-PAGE, and the relative amounts of LRP5 and LRP6 expressed by the two cell types were determined. Error bars show standard deviations.

pendent phenotypes; data not shown). MEF cultures were maintained in low oxygen (allowing survival over many generations) (28), and key results were repeated with separate batches of MEFs. The LRP5/LRP6 genotype of the MEF cultures did not grossly affect their morphology or growth characteristics (data not shown).

To examine the characteristics of this model, we evaluated the amounts of LRP5 and LRP6 in MEFs by various means. qRT-PCR and Western blot analyses showed that both LRP5 and LRP6 are present in cultured MEFs (Fig. 1, *A* and *B*). As expected, LRP5 and LRP6 transcripts were undetectable in RNA isolated from *lrp5*^{-/-} and *lrp6*^{-/-} MEFs, respectively (Fig. 1*A*). Western blot analysis using *lrp5*^{-/-} and *lrp6*^{-/-} MEFs confirmed the specificity of the LRP5 and LRP6 antibodies (Fig. 1*B*). Importantly, there was no compensatory up-regulation of *lrp6* mRNA or protein in the *lrp5*^{-/-} MEFs (or vice versa).

Very few studies have evaluated the function of endogenous levels of LRP5 and LRP6 as we do here. We compared the expression levels of these two receptors in MECs (the cell type of interest *in vivo*) and in MEFs (the principal assay model). To determine the relative amounts of LRP5 and LRP6, we assessed the relative detection efficiencies of LRP5 and LRP6 proteins by their cognate antibodies. Constructs encoding LRP5 and LRP6 receptors with a carboxyl-terminal myc tag were transfected into HEK293T cells. Using the internal myc tag, the relative number of LRP5 or LRP6 protein molecules could be compared (Fig. 1*C*). Specific detection with anti-LRP5 and anti-LRP6 antibodies revealed that the LRP5 antibody is ~60% more efficient at detecting its target protein than the anti-LRP6 antibody. Using this information, we calculated the ratio of endogenous LRP5 to LRP6 protein in MEFs to be 1.5. We also compared the relative expression of LRP5 and LRP6 in MECs and MEFs and

found that the ratio of LRP5:LRP6 is similar in these two cell types (Fig. 1*D*).

LRP6 Is Principal Transducer of Wnt3a-mediated Canonical Wnt Signaling in MEFs—Wnt3a is by far the most commonly used Wnt ligand for *in vitro* studies of Wnt signaling due to its solubility and availability. To test Wnt3a-mediated responses, MEFs were transfected with a plasmid encoding Wnt3a, and canonical Wnt signaling activity was measured using the β -catenin/TCF-dependent reporter assay (TOP-FLASH assay) 36 h post-transfection (31) (see supplemental Fig. S1*A*, time course of Wnt reporter activity). These responses are specific, and no activation was observed with the control construct, FOP-FLASH (data not shown).

As we showed previously for *lrp5*^{-/-} MECs, *lrp5*^{-/-} MEFs do not show dramatic differences in Wnt3a-induced β -catenin/TCF transactivation compared with wild type MEFs (TOP-FLASH was reduced by approximately 30%; Fig. 2*A*). In contrast, absence of LRP6 resulted in reduction of Wnt signaling activity by 80% (Fig. 2*A*). To confirm this result, we also assayed expression of an endogenous Wnt reporter, *Axin2* (a consistent and robust Wnt reporter across many cell types (27, 37)), using qRT-PCR. In agreement with the results from the TOP-FLASH assay, Wnt3a-dependent induction of *Axin2* mRNA was attenuated (~70%) in *lrp6*^{-/-} MEFs and was independent of LRP5 status (Fig. 2*B*).

Binding of a Wnt ligand to its cognate receptor(s) activates a signal transduction cascade that results in phosphorylation of the LRP receptors (7–9). Using an antibody shown previously to detect phosphorylation of LRP6 at Ser-1490 in response to Wnt treatment (38), we detected a band of the expected size (210 kDa) in *lrp6*^{-/-} MEFs. This suggests that the antibody is not specific to LRP6 but also detects phospho-LRP5 phosphorylated at the corresponding residue (Ser-1493). Exposure to

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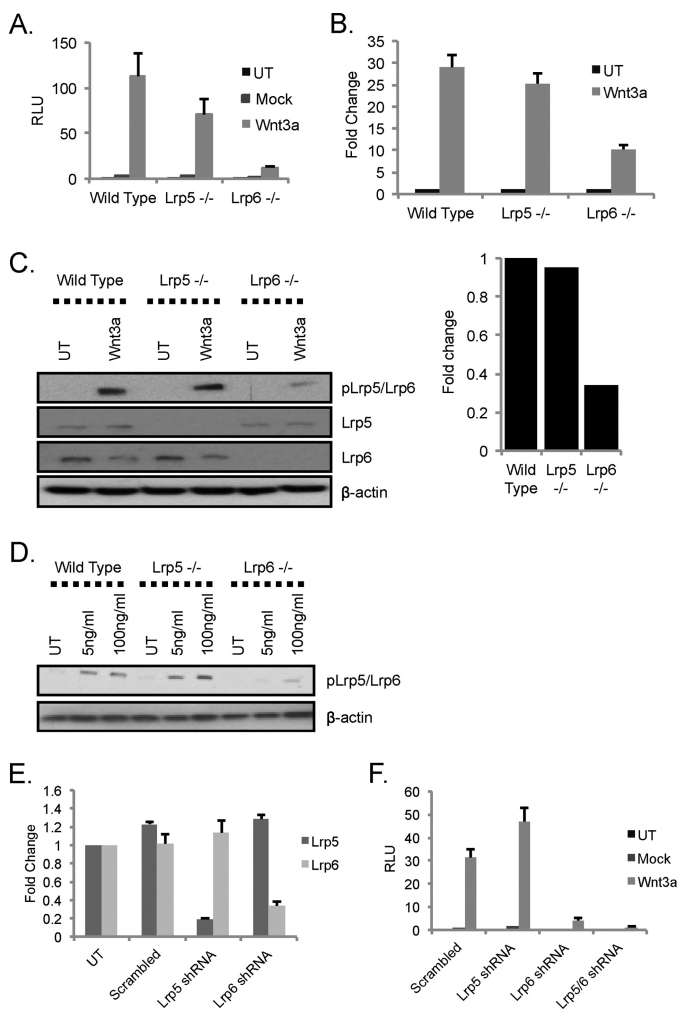


FIGURE 2. LRP6 is principal transducer of Wnt3a-mediated canonical Wnt activity in MEFs. *A*, assay of Wnt3a-induced TOP-FLASH Wnt reporter activity. Wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs were transfected with the Wnt reporter superTOP-FLASH along with a mock plasmid or a plasmid encoding mWnt3a and a transfection control (*Renilla* luciferase). 36 h post-transfection, expression of the reporter plasmid was assayed (normalized to *Renilla* luciferase). Results are shown for triplicate wells, and the results are representative of three different sets of wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs. *B*, assay of Wnt3a-induced activation of the endogenous Wnt reporter gene, Axin2. Wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs were transfected with the mWnt3a expression construct, and 36 h later, RNA was analyzed by qRT-PCR analysis for Axin2 mRNA expression. Values show -fold change over untreated samples (set to 1) following normalization to housekeeping genes *ywhaz* and *hprt1*. *C*, proximal readout of Wnt3a-mediated LRP activation in MEFs. Wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs were treated with rmWnt3A (100 ng/ml) for 16 h. Protein lysates were analyzed by immunoblotting with the indicated antibodies. Quantification of band intensities was performed using ImageJ analysis and expressed as the -fold difference in Wnt3a-mediated phospho-LRP5/6 induction levels in *lrp5*^{-/-} and *lrp6*^{-/-} MEFs relative to wild type MEFs. *D*, effect of LRP repertoire on receptor activation at a low dose of rmWnt3A. Wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs were treated with rmWnt3A (100 or 5 ng/ml) and processed as described in *C*. *E*, efficiency of RNAi-mediated knockdown of LRP5 and LRP6. Wild type MEFs were transfected with lentiviral shRNA constructs targeting *lrp5*, *lrp6*, or a scrambled control. RNA was isolated 36 h later, and relative knockdown and specificity were evaluated by qRT-PCR analysis. *F*, Wnt3a signaling in MEFs following shRNA-mediated knockdown of *lrp5* or *lrp6*. The MEFs described in *D* were assayed for Wnt responsiveness 36 h after co-transfection with superTOP-FLASH, mock plasmid, or mWnt3a and *Renilla* luciferase (processed as for *A*). UT, untransduced; RLU, relative luminescence units. Error bars show standard deviations.

rmWnt3a (for 16 h) resulted in robust phosphorylation of LRP5/LRP6 in wild type and *lrp5*^{-/-} MEFs but a 70% reduction in *lrp6*^{-/-} MEFs (Fig. 2C). To test whether this selectivity

is related to ligand concentration, we assayed lower concentrations of rmWnt3a. At 5 ng/ml, a concentration 20× less than that commonly used, reporter expression was induced 50× (compared with 400× for 100 ng/ml; supplemental Fig. S1B). Even at 5 ng/ml, LRP5 was not required for Wnt3a responses, and LRP6 was the predominant signaling receptor (Fig. 2D).

To rule out the possibility that our results from knock-out MEFs are due to chronic adaptation to the absence of LRP5 or LRP6 expression, we used an RNAi-mediated approach to test acute effects of LRP knockdown. MEFs were transduced with lentiviral shRNA constructs specifically targeting either *lrp5* or *lrp6* mRNAs (showing approximately 80 and 70% knockdown respectively; Fig. 2E), and Wnt3a-dependent TOP-FLASH activation was assayed (Fig. 2F). This approach confirmed that LRP5 has little role in transmitting signals from Wnt3a, whereas LRP6 knockdown reduced trans-activation by at least 90%. The double knockdown of LRP5 and LRP6 confirmed that any TOP-FLASH signal above background requires the presence of LRP (LRP5/LRP6 double knock-out MEFs are not available because this genotype results in gastrulation defects (15)). Taken together, these multiple assays support the identification of LRP6 as the principal transducer of Wnt3a-dependent canonical signaling. This result is supported by the recent results of MacDonald *et al.* (39), who attributed this enhanced signaling to the presence of a specific gap sequence between the phosphorylatable PPPSPXP motifs of the carboxyl-terminal domain of LRP6.

LRP5 and LRP6 Are Both Required for Efficient Wnt1-mediated Canonical Wnt Signaling in MEFs—Wnt3a is not often used *in vivo* to study gain of function in mice, and indeed our data for the unique functionality of LRP5 were derived from murine mammary tumor virus-Wnt1 transgenic mice. Therefore, we turned next to the study of Wnt1. The same experimental series performed for Fig. 2 were used to evaluate the roles of LRP5 and LRP6 in Wnt1-induced signaling (Fig. 3). A dramatic loss of canonical Wnt activity in response to Wnt1 was observed in the absence of either LRP5 or LRP6 (approximately 80%; Fig. 3A). Recently, structure/function analyses have proposed different binding sites for Wnt ligands on LRP6 receptors. Specifically, these studies have provided evidence that Wnt3a belongs to a class of ligands that binds the E3-E4 domain, whereas Wnt1, Wnt9b, and Wnt10b belong to a different class that binds the E1-E2 domain of LRP6 (40, 41). Using this information and the expression of Wnt ligands in mouse mammary gland to guide our choices, we tested whether Wnt9b and Wnt10b also require both LRP receptors and found that they too show a dual requirement for the LRP receptors (Fig. 3A).

The requirement for both LRP receptors is also evident from measurement of endogenous Axin2 mRNA expression levels (reduced equivalently by 50% in both *lrp5*^{-/-} and *lrp6*^{-/-} MEFs; Fig. 3B) and by assay of activated phospho-LRP (reduced by at least 70% in each knock-out cell strain; Fig. 3C). We compared results from knock-out cell strains with those derived from cells subjected to shRNA-mediated knockdown of *lrp5* and *lrp6* mRNAs (Fig. 3D). Loss of either LRP species resulted in the loss of 90% of Wnt signaling activity as measured by the TOP-FLASH reporter. We conclude that, for the Wnt1 class of

LRP5 and LRP6 Receptors Are Required for Some Wnt Ligands

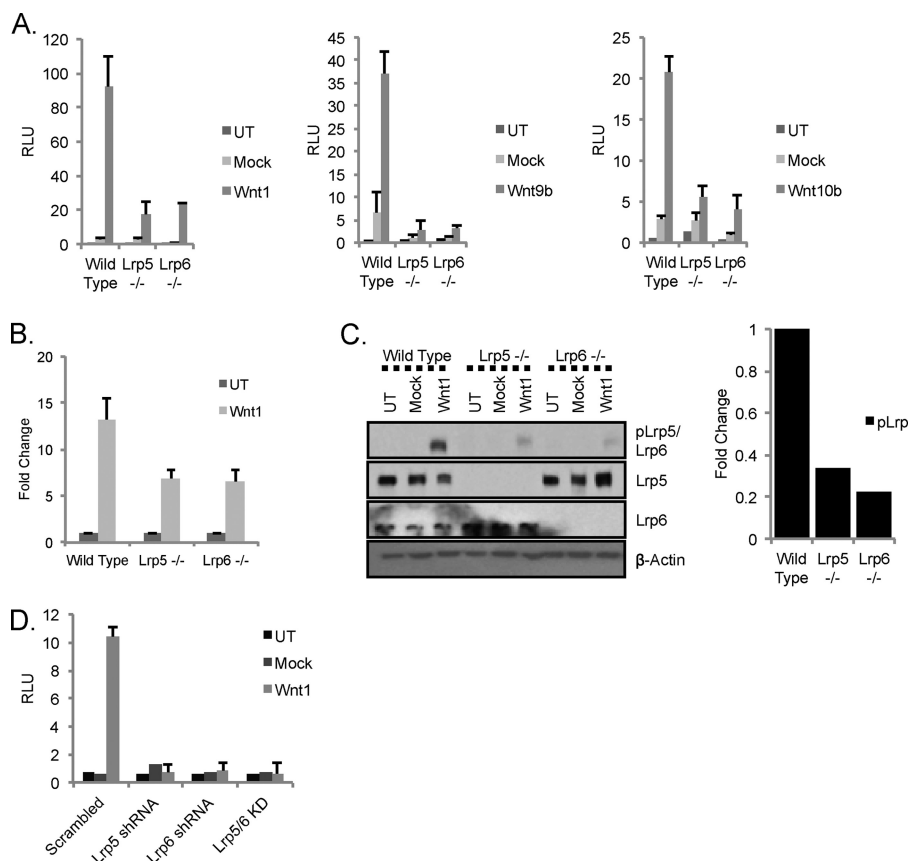


FIGURE 3. LRP5 and LRP6 are both required for efficient signaling in response to Wnt1. *A*, absence of either LRP5 or LRP6 results in loss of canonical Wnt activity in response to Wnt1. Wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs were transfected with the Wnt reporter superTOP-FLASH (and *Renilla* luciferase) along with plasmids encoding mWnt1 or mWnt10b (left- and right-hand side, respectively) (or without the ligand expression plasmids; mock) or with ectopic addition of mWnt9b (400 ng/ml; middle panel). Cells were processed as described for Fig. 2*A*. *B*, Wnt1-dependent transcriptional activation of Axin2. Wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs were plated, transfected with mWnt1 plasmid, and processed for analysis of Axin2 mRNA levels (as per Fig. 2*B*). *C*, Wnt1-mediated proximal canonical Wnt signaling. Wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs were transduced with lentiviral constructs expressing Wnt1 or the vector backbone (*Mock*) or untransduced (*UT*). 36 h later, proteins were analyzed by Western blotting as described for Fig. 2*C*. Relative phospho-LRP activation was quantified (right-hand side). *D*, Wnt1 signaling in MEFs following shRNA-mediated knockdown of *lrp5* or *lrp6*. Wild type MEFs were transduced with lentiviral shRNA constructs targeting *lrp5*, *lrp6*, or both mRNAs. 24 h later, cells were transfected with TOP-FLASH, *Renilla* luciferase, and mWnt1 expression construct and processed as for Fig. 2*D*. RLU, relative luminescence units; KD, knockdown. Error bars show standard deviations.

ligands (defined here to include Wnt9b and -10b and to exclude Wnt3a), there is a functional interaction between LRP5 and LRP6 receptors, resulting in the requirement for both receptors to be present to generate an active Wnt signaling complex.

Exogenous Expression of LRP5 or LRP6 Eliminates Requirements for Specific Wnt-LRP Complexes—We tested whether the requirement for both receptor species was also true when LRP species are overexpressed for the following two reasons. First, most previous studies have used overexpression to test for ligand-receptor signaling activity, and none have demonstrated the dual requirement for LRP receptors observed in our study. Second, LRP overexpression has been linked to the pathogenesis of breast tumors (42, 43), and this condition is therefore likely to be important physiologically.

Wild type MEFs were transfected with expression constructs for LRP5 and LRP6, producing 9× and 2.5× overexpression, respectively (Fig. 4*A*). To test whether cells overexpressing LRP receptors to the levels reported in Fig. 4*A* are still ligand-dependent for Wnt activation, these cells were transfected with the TOP-FLASH Wnt reporter (Fig. 4*B*). Signaling was undetectable (over background) without the introduction of Wnt ligands. To evaluate the properties of cells expressing higher

levels of just one LRP species, *lrp5*^{-/-} and *lrp6*^{-/-} MEFs were transfected with LRP6 and LRP5, respectively (and with LRP5 and LRP6 for comparison). LRP6 expression in *lrp6*^{-/-} MEFs produced the (approximately proportional) rescue of Wnt3a-dependent responses predicted (Fig. 4*C*). Interestingly, overexpression of LRP5 was also able to rescue Wnt3a-dependent responses (despite being largely ineffective at transducing Wnt3a signals at endogenous levels/ratios). Most surprising was the result of testing for Wnt1 responses when LRP5 was overexpressed in *lrp6*^{-/-} MEFs (high levels of LRP5 present). In this case, LRP5 alone was sufficient, and the dual requirement was eliminated. In fact, LRP5 and LRP6 were equally effective at rescuing responses to both Wnt ligand types (Fig. 4*D*).

Not All Cell Types Show Ligand-specific Receptor Requirements—To further develop the idea that endogenous levels of LRP5 and LRP6 modulate ligand-receptor specific signaling, we identified cells with endogenous LRP5 and LRP6 expression levels that are different from MECs and MEFs. Thus, the HC11 mouse mammary epithelial cell line, a clonal derivative of the COMMA-1D cell line, that has been used previously as an *in vitro* model for Wnt signaling in the mammary gland (44, 45)

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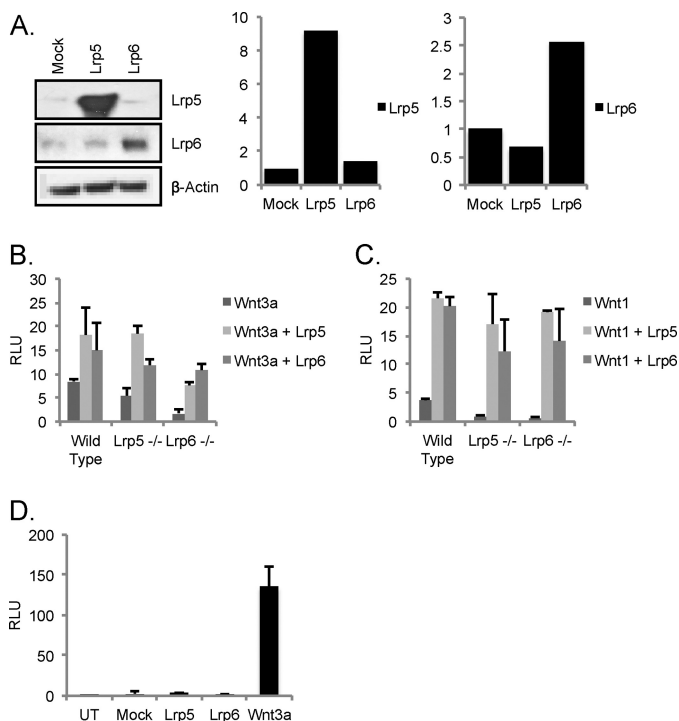


FIGURE 4. Ectopic expression of either LRP5 or LRP6 eliminates requirement for both LRP5 and LRP6. *A*, overexpression of LRP5 and LRP6 in wild type MEFs. Wild type MEFs were transfected with constructs encoding full-length mLRP5, mLRP6, or a control construct (GFP plasmid; Mock). 36 h later, protein extracts were probed with anti-LRP antibodies as indicated, and the relative amount of expression was quantified. *B–D*, assay of ligand dependence of signaling in MEFs overexpressing LRP species. To test whether MEFs overexpressing LRP receptors to the levels reported in *A* were still ligand-dependent for Wnt transactivation, cells were transfected with the TOP-FLASH Wnt reporter (B), and luciferase activity was measured 36 h later. Signaling was undetectable (over background) without the introduction of Wnt ligands (*D*). To test the signaling properties of single receptor species in knock-out backgrounds, wild type, *lrp5*^{-/-}, and *lrp6*^{-/-} MEFs were transfected with the Wnt reporter superTOP-FLASH (and the transfection control *Renilla* luciferase) together with mWnt3a (*B*) or mWnt1 (*C*) and either LRP5 or LRP6 receptor. 36 h post-transfection, cells were lysed, and Wnt signaling activity was measured. *UT*, untransduced; *RLU*, relative luminescence units. Error bars show standard deviations.

was examined. Evaluation of mRNA levels of LRP5 and LRP6 showed that HC11 cells express both LRP5 and LRP6 but have 2 \times higher *lrp5* mRNA and 4 \times higher LRP5 protein (Fig. 5, *A* and *B*). The overall amount of LRP5/6 protein is 2-fold higher in MEFs, and the ratio of LRP5:LRP6 protein is \sim 0.25 for MEFs compared with \sim 3.0 for HC11 cells. Knocking down LRP5 or LRP6 alone (Fig. 5C) had broadly similar effects on Wnt3a and Wnt1 responses. Knockdown of LRP5 was more effective than knockdown of LRP6, but neither alone produced a loss of function that was comparable with the knockdown of both species (Fig. 5, *D* and *E*). Given the relative receptor species concentration, this result was predictable. There is therefore little evidence from HC11 cells to suggest that loss of function of one receptor impacts the function of the remaining receptor species.

Dual Requirement for Both LRP5 and LRP6 for Wnt1-dependent Responses Is Also Observed in Vivo—We considered the possibility that the dual requirement revealed by MEFs could be associated with a regulatory mechanism unique to this model system. To test whether the dual dependence applies to other cell types and to determine the importance of this for specific

Wnt responses, we evaluated a Wnt-dependent preneoplastic physiology *in vivo*.

Wild type and *lrp5*^{-/-} MECs were transduced with lentiviral constructs encoding Wnt1 or Wnt3a (in suspension; see “Experimental Procedures”), transferred to cleared mammary fat pads of wild type mice, and allowed to develop *in vivo* for 8 weeks. Some of these cells were transferred to primary culture to evaluate their infection efficiency and to test their Wnt signaling response (by qRT-PCR analysis of Axin2 mRNA expression). Consistent with our previous findings from mWnt3a-treated MECs (27), the absence of LRP5 did not affect the robust induction of Axin2 in response to Wnt3a. However, the response to Wnt1 was ablated in the absence of LRP5 (Fig. 6A).

The outgrowth of MECs *in vivo* in cleared fat pads in 3-week-old recipient mice relies on ductal stem cell activity, which in turn depends on LRP5-dependent Wnt signaling activity (24, 27, 46, 47). Thus, *lrp5*^{-/-} MECs (50,000) did not show mammary gland repopulating activity when compared with MECs from wild type mice (Fig. 6, *B* and *C*). MECs expressing Wnt1 from the lentiviral expression vector developed into hyperplastic ductal outgrowths that are also typical of glands from murine mammary tumor virus-Wnt1 transgenic mice (Fig. 6D and Ref. 32). This colonization and outgrowth activity were dependent on expression of LRP5 (Fig. 6E). This is consistent with our previous results from crossing murine mammary tumor virus-Wnt1 mice into the *lrp5*^{-/-} background in which subsequent tumor development also depends upon LRP5 expression (24). In contrast, we found that Wnt3a expression induced hyperplasia *in vivo* regardless of expression of LRP5 (Fig. 6, *F* and *G*), matching the results *in vitro* from the analysis of MEFs.

Do LRP5 and LRP6 Physically Interact?—Given the functional co-dependence of LRP5 and LRP6 we have shown here, we hypothesized that these two proteins physically interact. We anticipated that evaluating this hypothesis would not be technically simple given that no prior studies have shown interactions of either of these receptors at endogenous levels (prior analyses have focused on overexpressed receptor interactions). Therefore, we used the “IFAST” adaptation of the immunoprecipitation technique because this technique eliminates typical dilutive wash steps present in traditional purification methods. We suspected it could be better at capturing low affinity and short lived complex components. This technique also dramatically reduces the purification time (36).⁴ A scheme is shown in Fig. 7A. Pull-through of magnetic beads coated with nonspecific IgG illustrates the specificity of this technique (Fig. 7B). Using lysates from MEFs treated under various conditions, LRP5 immunoprecipitates were probed for the presence of other Wnt signaling components, including LRP6, phospho-LRP, and Axin1 (Fig. 7C). LRP5 and LRP6 were pulled through together irrespective of Wnt treatment, whereas specificity controls (vinculin and EGF receptor) were not. Phospho-LRP was highly induced by Wnt3a, less induced by Wnt9b, and pulled through in both LRP5-associated fractions.

⁴ S. Goel, E. N. Chin, S. A. Fakhraldeen, S. M. Berry, D. J. Beebe, and C. M. Alexander, unpublished data.

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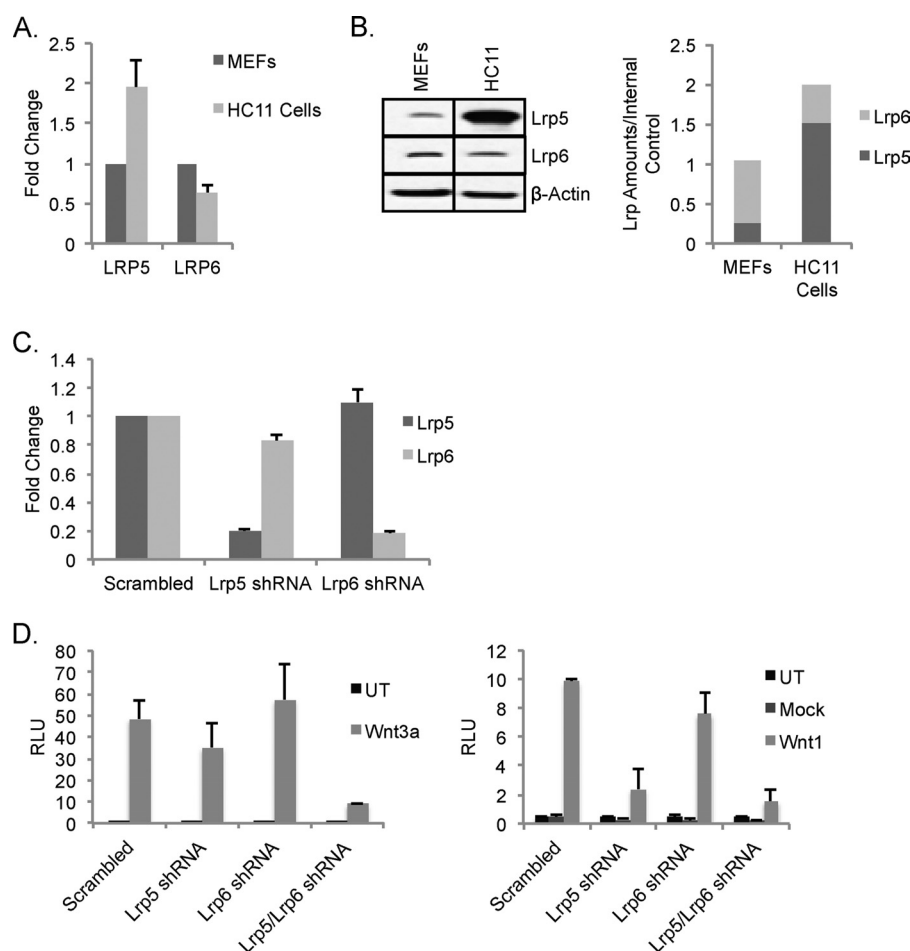


FIGURE 5. Mammary epithelial cells with endogenously high expression levels of LRP5 compared with MEFs do not require LRP6 for Wnt1-responsiveness. A, evaluation of LRP expression patterns in HC11 cells. The relative mRNA expression of LRP5 and LRP6 was compared for MEFs and HC11 cells using qRT-PCR analysis (normalized to housekeeping genes YWHAZ and hypoxanthine-guanine phosphoribosyltransferase). B, relative LRP expression in HC11 cells. Wild type MEFs and HC11 cells were harvested and analyzed for their LRP expression (using β -actin as a loading control) as for Fig. 1. C, RNAi-mediated knockdown of LRP5 and LRP6. HC11 cells were transduced with lentiviral shRNA constructs targeting *lrp5*, *lrp6*, or a scrambled control (as for Fig. 2E). qRT-PCR analysis shows the specificity and efficiency of the shRNA constructs. D, evaluation of the requirement for LRP5 and/or LRP6 in response to Wnt3a and Wnt1 in HC11 cells. Knockdown cells (prepared as for C) were transfected with superTOP-FLASH, *Renilla* luciferase, and either mWnt3a or mWnt1. Cells were lysed 36 h post-transfection, and superTOP-FLASH reporter activity was measured using a luciferase assay. Results are shown from triplicate wells and are representative of three independent experiments. UT, untransduced; RLU, relative luminescence units. Error bars show standard deviations.

Interestingly, Axin1 did not pull through with LRP5, suggesting that LRP5-6 complexes did not contain this signaling molecule (irrespective of Wnt induction). Anti-Axin1 immunoprecipitation almost quantitatively retrieved Axin1 from the bound fraction (Fig. 7D). Interestingly, Axin1 immunoprecipitates contained LRP6 regardless of Wnt treatment (*i.e.* these are constitutively complexed), and there was no LRP5 present in that fraction, confirming the result from LRP5 immunoprecipitation. Anti-phospho-LRP antibody pulled through LRP6/Axin1 and also LRP6/LRP5 (Fig. 7E).

These data provide evidence in support of several close range interactions, namely LRP6-Axin1 and LRP5-LRP6 in untreated cells and phospho-LRP6-Axin1 and phospho-LRP6-LRP5 in Wnt-treated cells. Furthermore, these data suggest that binding of Axin1 to LRP6 does not require treatment with an exogenous canonical Wnt ligand (data are summarized in Fig. 8, A and B).

DISCUSSION

In this study, we aimed to resolve a paradox that arose from our analysis of the phenotype of *lrp5*^{-/-} mouse mammary

glands. Here, we show that both LRP5 and LRP6 are required to respond to a group of Wnt ligands that includes Wnt1, Wnt9b, and Wnt10b. For MEFs and MECs, unless both LRP5 and LRP6 are present, the signal generated is low. In contrast, Wnt3a can signal through either receptor alone, although LRP6 is more efficient (39). The concentrations of Wnt3a and Wnt9b used for some of these studies were chosen to generate similar Wnt signaling responses (TOP-FLASH/Axin2 transactivation). When Wnt3a was used at 20 ng/ml and Wnt9b was used at 400 ng/ml, these outputs matched. By this means, we hoped to reduce the effects of variable ligand affinity and stability on functional receptor interactions. Furthermore, the gland hyperplasia induced by Wnt1 or Wnt3a (expressed by the same vector construct) was approximately similar, suggesting that the potency issues that plague Wnt studies *in vitro* are unlikely to underlie the requirement for two instead of one LRP species.

A dual requirement explains why *lrp5*^{-/-} mice are resistant to Wnt1-mediated tumorigenesis (24). Also, because ductal mammary stem cell activity is dependent upon Wnt signaling

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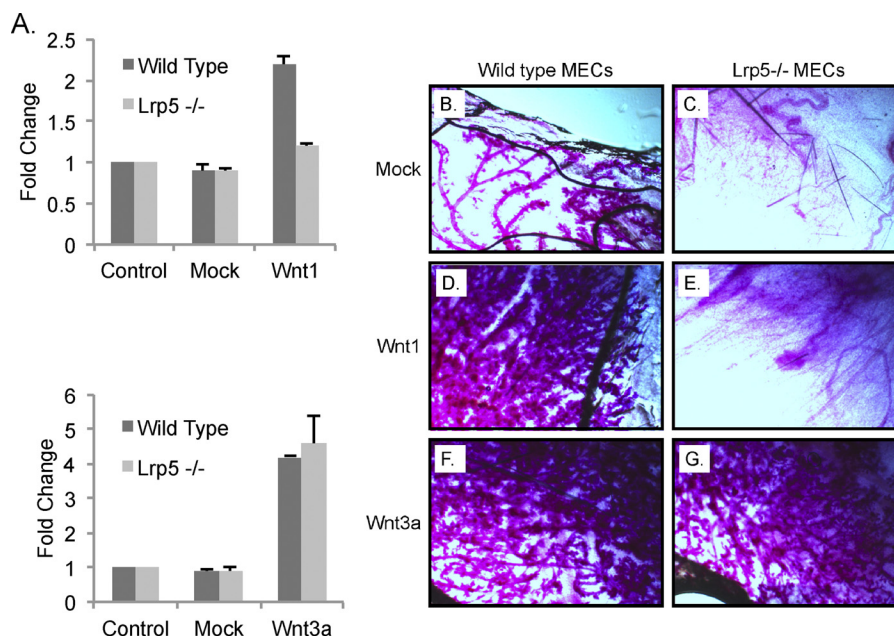


FIGURE 6. Wnt1 class of ligands requires LRP5 to induce hyperplasia *in vivo* in mammary glands, whereas Wnt3a does not. A, Wnt1- and Wnt3a-induced transcriptional activation of Axin2 expression. Wild type and *lrp5*^{-/-} MECs were transduced with lentiviral expression vectors for mWnt1 or mWnt3a (or a mock lentiviral vector) in suspension. For evaluation of expression, some cells were transferred to culture, and RNA was assayed for Axin2 mRNA expression 48 h later (as for Fig. 2B). B–G, analysis of ductal hyperplasia in outgrowths from wild type and *lrp5*^{-/-} MECs expressing mWnt1 or mWnt3a. MECs (50,000) transduced with mock or mWnt1- or mWnt3a-expressing lentiviral constructs were transplanted to cleared fat pads (see “Experimental Procedures”), and development of mammary trees was evaluated 8 weeks later. Fat pads were harvested and stained with carmine red (trees are dark against the clear fat pad background; *n* = 3). Error bars show standard deviations.

and specifically upon LRP5 (despite expression of LRP6), we propose that the Wnt ligand normally responsible for maintaining mammary stem cells falls into this LRP5-LRP6 dual-dependent group of ligands. Wnt ligands have classically been characterized into canonical and non-canonical ligands based on their functionality and ability to activate the canonical Wnt pathway (48–50). There are 10 Wnt ligands expressed in the mammary gland (Wnts 2, 4, 5a, 5b, 6, 7b, 9a, 9b, 10b, and 16), and several are specifically associated with either basal or luminal mammary epithelial cells or with stromal cell types (51). We tested Wnt9b and -10b in this study and showed that they had the same dual LRP dependency as Wnt1. Wnt1 and Wnt3a are not usually expressed in the mouse mammary gland, although they were identified as oncogenic loci for mouse mammary glands using a retroviral integration screen (52). Interestingly, Wnt10b also emerged from this unbiased screen. This information may also be helpful for studies of Wnt-dependent stem cells *in vitro*. Published work from another group showed that Wnt3a (identified as “pan-active” irrespective of LRP expression by our work) was successfully used to maintain stem cell function *in vitro* (200 ng/ml changed daily) (53). Another culture system devised to maintain intestinal stem cells included a Wnt relative, an R-Spondin (RSpo1), at 500 ng/ml (54). There are no data yet to describe whether selective LRP species are required by Lgr-RSpo complexes in mammary glands.

We assumed that results of functional receptor testing derived from the study of MEFs could be unique to only this cell type. Therefore, we corroborated key data obtained from cultured MEFs for MECs, which are our principal cell type of interest. Wnt1 required both LRP5 and LRP6 *in vitro* (by Axin2 expression analysis) and *in vivo* in an MEC outgrowth assay

(whereas Wnt3a induced outgrowth and hyperplasia regardless of the absence of LRP5). The outgrowth assay typically depends upon ductal stem cell activity; thus, there was no outgrowth of the stem cell-deficient MEC populations extracted from *lrp5*^{-/-} mammary glands (Fig. 6C). Interestingly, providing Wnt3a ectopically to *lrp5*^{-/-} MECs generated hyperplastic outgrowth following transplantation *in vivo* (Fig. 6G). This observation suggests that stemness can be induced *de novo* by Wnt exposure.

The currently accepted model of receptor activation is shown in Fig. 8C. Typically, Wnt ligands are proposed to associate with an LRP and Fzd species to generate a β -catenin/TCF signal (5, 6). The fact that both LRP5 and LRP6 are required to respond to the Wnt1 class of ligands shows that these receptors are not functionally equivalent. It implies that they may physically interact through heterodimerization. Earlier studies of receptor activation showed that overexpressed LRP6 receptors homodimerize and polymerize into “signalosomes” (16, 55, 56). More recent data support a role for the homodimerization of endogenous LRP6 in augmenting canonical Wnt activity (40). Using a novel immunoprecipitation assay, we provide data for the heteropolymerization of LRP5 and LRP6. Thus, LRP6 was pulled through with LRP5 with high efficiency in the IFAST assay (Fig. 7C), and this complex was present regardless of the presence of ectopic Wnt ligand. Interestingly, LRP5-6 was not pulled through with Axin1, whereas LRP6 alone was. In fact, Axin1 complexation with LRP6 was measured by this assay regardless of Wnt ligand treatment, whereas most studies would suggest that this complex forms as a result of receptor activation (9). However, none of the previous studies were able to study the receptor complexes at endogenous levels, and this

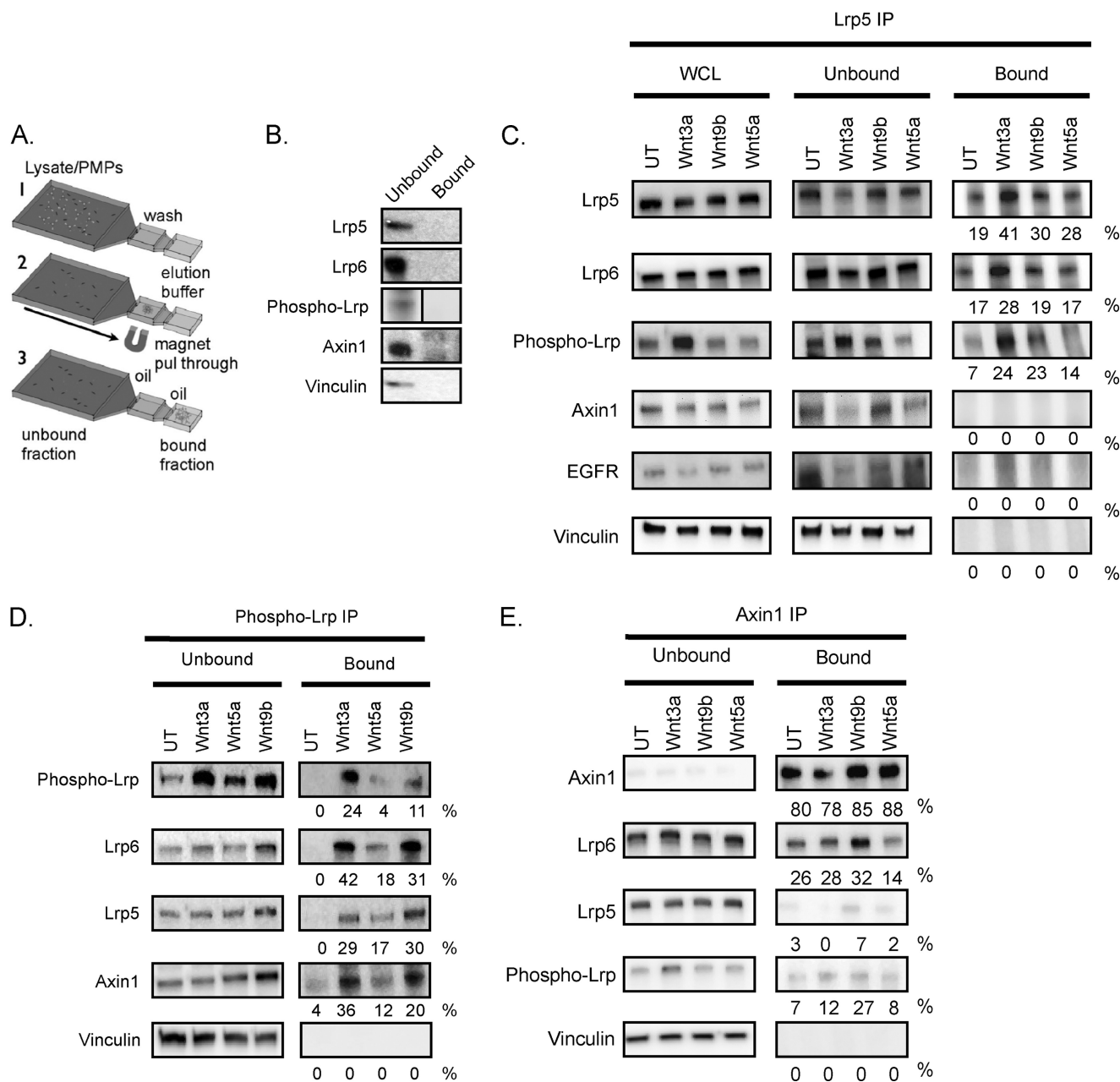


FIGURE 7. Evidence for LRP5-LRP6 complexes. *A*, the IFAST method for immunoprecipitation (36). Microchannel devices contain 3 wells connected by oil. Antibody, paramagnetic particles (PMPs), and cell lysate are combined in the input well (left-hand side), and complexes are pulled through into the wash well and the final elution well (right-hand side) by means of a magnet (according to the "Experimental Procedures"). *B*, specificity control. Cell lysate (300 μ g; wild type MEFs) was incubated with non-immune IgG and paramagnetic particles and processed. Lanes are labeled "Unbound" (input after pull-through of paramagnetic particles) and "Bound" (output after elution). *C*, evaluation of co-immunoprecipitation with LRP5. MEFs were treated with no ligand (UT), Wnt3a (20 ng/ml), Wnt9b (400 ng/ml), or Wnt5a (40 ng/ml) for 2 h, lysed, and immunoprecipitated with anti-LRP5 (1.2 μ g) and paramagnetic particles. The analysis of whole cell lysate (WCL; before IP) is shown on the left-hand side together with unbound and bound fractions. The efficiency of pull-through was calculated as described under "Experimental Procedures" (and presented here as a percentage). The same or parallel blots were probed for LRP6, phospho-LRP6, Axin1, and two specificity controls, EGFR receptor (EGFR) and vinculin. *D*, evaluation of co-immunoprecipitation with Axin1. *E*, evaluation of co-immunoprecipitation with phospho-LRP. Results shown are representative of three separate MEF batches.

may explain why our results differ. Furthermore, even when cells were Wnt-treated, the LRP5-6 heteromer did not associate with Axin1, and neither did Axin1 associate with LRP5. Axin1 association with LRP5 (overexpressed human LRP5 with a carboxyl-terminal domain tag) has been shown to occur only when an inhibitory interaction from the extracellular domain is relieved (58). This association is therefore known to be highly context-dependent. Although it can happen *in vitro*, perhaps it only rarely happens *in vivo*. MacDonald *et al.* (39) have recently

shown that the phosphorylation and Axin1 binding properties of LRP6 can be mimicked in the low Axin1-binding LRP5 if gap sequences were introduced between the canonical phosphorylation sites (PPSPXP). Our data suggest that Axin1 association is not required for activation of the heteromer, or perhaps LRP5-Axin1 is so different from the better characterized LRP6-Axin1 complex that it cannot be measured by the same assays. The interactions that were demonstrated by the IFAST experiments are summarized in Fig. 8, *A* and *B*.

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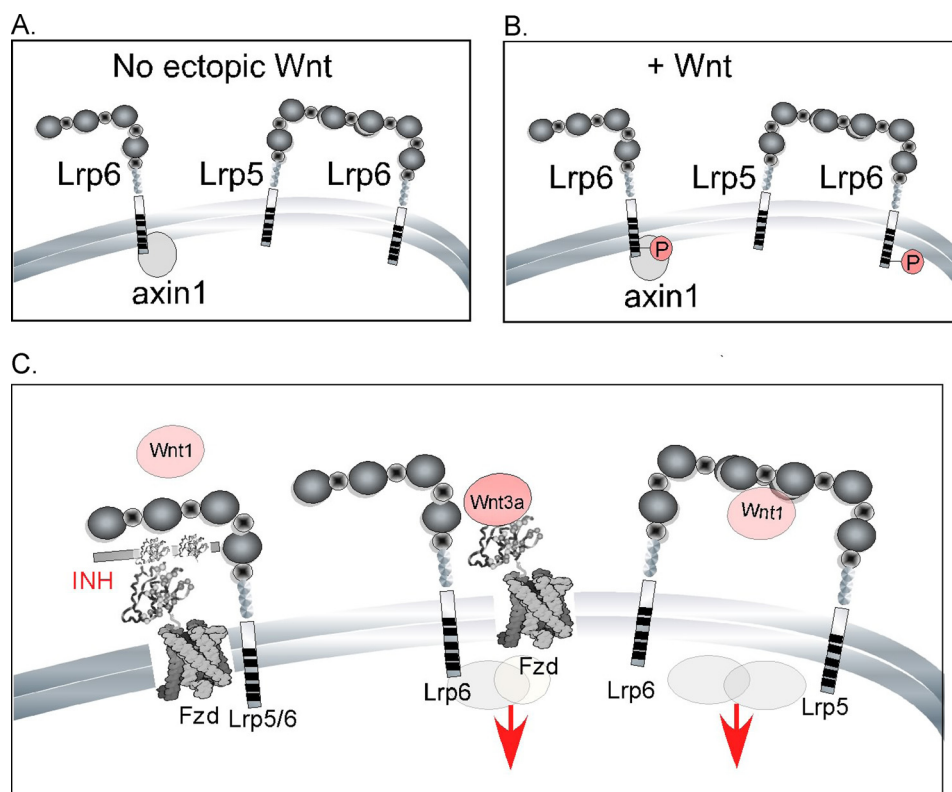


FIGURE 8. Model of LRP interactions. *A* and *B*, evidence for interactions derived from IFAST IP. Proteins associated into complexes are shown in the uninduced (basal level) MEFs (*A*) and for MEFs treated with Wnt3a (*B*). *C*, a model depicting LRP signaling activities in response to different Wnt ligands. *Left-hand side*, binding of Wnt1/Wnt9b ligands is not effective when LRP5 or LRP6 are present alone. Shown here is one potential explanation: the presence of an inhibitor (*INH*) in the adult mammary gland/MEF culture that blocks assembly of LRP-Fzd complexes by E1-E2 ligands. Under the same conditions, Wnt3a (shown here binding at a separate domain, E3-E4) can effectively signal via LRP6 (or LRP5; *center*). Signaling by the Wnt1 class of ligands requires complexation of LRP5 and LRP6 (*right-hand side*). This complex is proposed to be inhibitor-resistant and may have different co-receptor/signaling component associations.

A model is presented as Fig. 8C to summarize our conclusions and propose an explanation for our data points as follows. Overexpression of either LRP5 or LRP6 overcomes the need for both receptors to be present (Figs. 4 and 5) either in normal mammary epithelial cell lines or in MEFs with LRP species overexpressed. Thus, this dual requirement does not reflect binding of ligand to a specific receptor or an obligate requirement for both to be present to generate a response. Instead, we propose that there is a cell type-specific restriction of Wnt ligand efficacy most easily explained if there is a limiting amount of an inhibitor present that is out-competed by higher concentrations of receptor. The identification of an inhibitor-resistant LRP5 species ($\Delta 666-809$) as a common mutation arising in breast cancer points to the importance of constitutively expressed Wnt inhibitors as a physiological suppressor for Wnt signaling (59). Along similar lines, Wnt inhibitors such as secreted Frizzled-related proteins (sFRPs) (60) and Wnt inhibitory factor (WIF1) (61) have been shown to be epigenetically silenced in tumors.

Interestingly, our description of two groups of Wnt ligands based on this functional assay correlates with recently published subgroups of Wnt ligands distinguished by their interactions with specific ligand binding regions on the LRP6 receptor (40, 41). Thus, Wnt1 was shown to interact with the E1-E2 domains of LRP6, whereas Wnt3a interacts with the E3-E4 domains. We have incorporated this into the model to propose that if an inhibitor effectively competes for E1-E2 Wnt ligand

binding sites on the receptor (but not E3-E4) in ternary complexes of Fzd species then Wnt3a activity would not be restricted. Using this model, an E1-E2 ligand could only induce a β -catenin/TCF signal when both receptor types are present to form an inhibitor-resistant heteromer of LRP5 and LRP6 (Fig. 8C). The implication is that there is an extracellular inhibitor in MECs and MEFs that restricts Wnt1/9b/10b ligand activity unless both receptors are present. Various Wnt inhibitors have been found in mammary gland (51, 60, 61), and we are currently working to test this model. An obvious candidate is Dkk1 given recent structural data mapping the binding domain to E1 of LRP6 (62, 63). We have no information yet about the role of Fzd in these complexes.

We propose that overexpression of LRP receptors overcomes the physiological regulation and restrictions of β -catenin/TCF signaling by decreasing the effectiveness of secreted inhibitors, allowing all Wnt ligands in the extracellular milieu to signal effectively and expanding the responsive cells to include those that do not express both LRP species. This model also predicts that overexpression of LRP receptors would create a highly effective gain of function for Wnt signaling during tumorigenesis. Indeed, this has been observed in triple negative breast tumor cohorts (that include the majority of basaloid tumors), which were shown to express 2–5 \times higher levels of *lrp6* mRNA (42, 43). In parallel with this, one-third of basaloid breast tumors show significant activation of a Wnt signaling reporter (nuclear β -catenin) (64). Furthermore, inhibition of LRP6 in

the basaloid breast cancer cell line MDA-MB231 inhibits TOPFLASH Wnt reporter activity (10–100×) together with the endogenous Axin2 reporter (2×) and tumorigenic physiologies such as growth (2×), colony formation (5×), and tumor growth *in vivo* (65). LRP receptor levels appear to be limiting to Wnt signaling, and overexpression of LRP6 induces significant hyperplasia in mouse mammary glands (57). These data all support LRP6 as a viable target for breast cancer therapy and provide rationale for the development of extracellular inhibitors of Wnt signaling aimed at inhibiting LRP6 function (40, 41).

In summary, we have shown that several key Wnt ligands require expression of both LRP5 and LRP6 receptors to generate a β -catenin/TCF Wnt signal. For normal mammary glands, Wnt responder activity is limited to the basal cell subpopulation, consistent with their co-expression of LRP5 and LRP6 (27). For a basaloid breast tumor model, tumor stem cell activity is associated with LRP5-expressing cells (34). The study presented here describes why LRP5 can act as a gatekeeper for Wnt responses in both stem cells and tumor cells, enabling Wnt responsiveness under conditions where LRP6 alone is not effective.

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