Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974

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Edited by Marc de la Roche, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, and accepted by the Editorial Board October 31, 2013 (received for review July 31, 2013)

Wnt signaling is one of the key oncogenic pathways in multiple cancers, and targeting this pathway is an attractive therapeutic approach. However, therapeutic success has been limited because of the lack of therapeutic agents for targets in the Wnt pathway and the lack of a defined patient population that would be sensitive to a Wnt inhibitor. We developed a screen for small molecules that block Wnt secretion. This effort led to the discovery of LGK974, a potent and specific small-molecule Porcupine (PORCN) inhibitor. PORCN is a membrane-bound O-acyltransferase that is required for and dedicated to palmitoylation of Wnt ligands, a necessary step in the processing of Wnt ligand secretion. We show that LGK974 potently inhibits Wnt signaling in vitro and in vivo, including reduction of the Wnt-dependent LRPS phosphorylation and the expression of Wnt target genes, such as AXIN2. LGK974 is potent and efficacious in multiple tumor models at well-tolerated doses in vivo, including murine and rat mechanistic breast cancer models driven by MMTV–Wnt1 and a human head and neck squamous cell carcinoma model (HN30). We also show that head and neck cancer cell lines with loss-of-function mutations in the Notch signaling pathway have a high response rate to LGK974. Together, these findings provide both a strategy and tools for targeting Wnt-driven cancers through the inhibition of PORCN.

Wnt inhibition | β-catenin | HNSCC

Wnt signaling is one of a key oncogenic pathway in multiple cancers (1, 2). On binding to its receptors LRP5/6 and Frizzled (FZD) at the plasma membrane, Wnt ligand triggers the disruption of the β-catenin degradation machinery (consisting of AXIN2, GSK3β, APC, and others), leading to the accumulation of β-catenin in the cytoplasm (3). Elevated levels of β-catenin ultimately lead to its translocation into the nucleus to form a complex with LEF/TCF and drive downstream gene expression (3). Dysregulation of Wnt signaling (1) can occur through mutations of downstream components, such as APC and β-catenin, which are well-documented in colon cancer (1). In addition, overexpression of Wnt ligands or costimulants, such as R-Spondin 2/3 (RSPO2/3), or silencing of Wnt inhibitor genes has been reported in various cancers (1, 4). Mutations of pan-Wnt pathway components, such as AXIN1/2 or the RSPO coreceptors RNF43/ZNFR3, may potentially play key roles in pancreatic, colon, and hepatocellular carcinoma (4–6). The Wnt gene was originally identified as an oncogene in murine mammary tumors 30 y ago (2) and confirmed to be a key oncogenic pathway in many studies, including an unbiased insertional mutagenesis screen, with Wnt1, Wnt3, and Wnt3A comprising 38% of all unbiased hits (7). In human breast cancer, overexpression of Wnt or silencing of Wnt inhibitor genes has been observed in up to one-half of breast cancer samples (8). Cytoplasmic and nuclear β-catenin have also been correlated with triple-negative and basal-like breast cancer subtypes (9, 10), and Wnt signaling has also been implicated in cancer-initiating cells in multiple cancer types (11–14). Wnt pathway signaling activity is dependent on Wnt ligand. During the biosynthesis of Wnt ligands, Wnt undergoes posttranslational acylation (palmitoylation) that is mediated by Porcupine (PORCN), a membrane-bound O-acyltransferase (3, 15). PORCN is specific and dedicated to Wnt posttranslational acylation, which is required for subsequent Wnt secretion (16). Loss of PORCN leads to inhibition of Wnt ligand-driven signaling activities in KO mouse models (17, 18). In humans, loss-of-function (LoF) mutations in the PORCN gene cause focal dermal hypoplasia, an X-linked dominant disorder associated with a variety of congenital abnormalities in both heterozygotes and those individuals with mosaicism for the PORCN gene. This phenotype is consistent with the role of the Wnt signaling pathway during embryogenesis and development (15).

Significance

Targeting the Wnt pathway in cancer is an attractive therapeutic approach. However, success has been limited because of the lack of effective therapeutic agents and the lack of biomarkers to define the patient population that would benefit from such a therapy. Herein, we report the discovery of LGK974, a drug that targets Porcupine, a Wnt-specific acyltransferase. We show that LGK974 potently inhibits Wnt signaling, has strong efficacy in rodent tumor models, and is well-tolerated. We also show that head and neck cancer cell lines with loss-of-function mutations in the Notch signaling pathway have a high response rate to LGK974. Together, these findings provide a strategy and tools for targeting Wnt-driven cancer.


This article is a PNAS Direct Submission. M.J.R is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1314239110/-/DCSupplemental.
Given the key role of Wnt signaling in cancer, targeting this pathway has been an attractive therapeutic approach. However, success has been limited because of the lack of effective therapeutic agents for targets in the Wnt pathway and the lack of a defined patient population that would be sensitive to a Wnt inhibitor. Herein, we describe a cellular high-throughput screen for small molecules that block Wnt secretion. This effort led to the discovery of LGK974, a potent and specific PORCN inhibitor. We show that LGK974 potently inhibits Wnt signaling in vitro and in vivo and has strong efficacy in tumor models in vivo. We also show that head and neck squamous cell carcinoma cell lines with LoF mutations in the Notch signaling pathway have a high response rate to LGK974. These findings provide a path forward to target Wnt-driven cancer through the inhibition of PORCN.

Results
Cellular High-Throughput Screen for Wnt Secretion Inhibitors. To identify small-molecule Wnt signaling inhibitors, a cellular Wnt pathway-based screen was performed against ~2.4 million compounds. In this assay, Wnt-secreting cells, a stable L-cell line overexpressing Wnt3A, were cocultured with the Wnt reporter cells, a TM3 cell line harboring a luciferase reporter gene (19) driven by Wnt-responsive promoter elements. The level of the luciferase reporter gene readout was used as an indicator for Wnt ligand-driven, LEIP/TCP-dependent transcriptional activities. Compounds showing the greatest activity in the screen were reconfirmed and further triaged through a set of secondary assays as follows. To distinguish the hits from functioning in a parallel signaling pathway, a Hedgehog (HH) coculture assay analogous to the Wnt assay was set up as a counterscreen. The HH pathway bears much similarity to the Wnt pathway, including biosynthesis and posttranslational modification of the ligand, receptor stimulation, and downstream transcriptional activation (20). To distinguish hits that function upstream or downstream of Wnt secretion, Wnt3A conditioned medium (CM) was added back into the cell culture medium in the context of the Wnt reporter cells. Specific Wnt secretion inhibitors should be active in the Wnt coculture assay but not the HH coculture or Wnt3A CM assays. These compounds were further characterized to elucidate their potential targets, including the two known dedicated nodes required for Wnt secretion: Wntless and PORCN. (3, 15).

GNF-1331 (Fig. S1A), one of the chemical classes identified from the screen, was selected for tritium labeling and subsequent evaluation in a radioligand binding assay. As shown in Fig. LA, [3H]-GNF-1331 bound well to the membrane preparations from 293T cells transfected with PORCN but not to the membrane preparations from cells transfected with Wntless or a vector control, suggesting that GNF-1331 specifically interacted with PORCN. Furthermore, the specific interaction between GNF-1331 and PORCN could be competed off by unlabeled GNF-1331 (Fig. L4).

Inhibition of PORCN by LGK974 Blocks Wnt Signaling in Vitro. Medicinal chemistry optimization of GNF-1331 was carried out to improve potency and pharmacokinetic properties. This effort led to the discovery of LGK974 (Fig. 1B), a highly specific and potent PORCN inhibitor. LGK974 effectively displaced [3H]-GNF-1331 with an IC50 of 1 nM in the PORCN radioligand binding assay (Fig. 1C and Fig. S1B). LGK974 also potently inhibited Wnt signaling in the aforementioned Wnt coculture assay with an IC50 of 0.4 nM (Fig. 1D). This inhibitory effect was rescued by the addition of exogenous Wnt3A CM (Fig. 1E). Additionally, LGK974 showed no major cytotoxicity in cells up to 20 μM (Fig. S1C). To further confirm the activity of LGK974 in blocking PORCN-dependent Wnt secretion, 293A cells were transfected with HA-tagged Wnt3A and treated with various doses of LGK974. As shown in Fig. 1F and Fig. S1D, LGK974 potently decreased levels of HA-Wnt3A in the supernatant with slightly increased levels of HA-Wnt3A in the cell lysate, suggesting that Wnt3A secretion was substantially inhibited by LGK974 in a dose-dependent manner. In Wnt-responsive cells, secreted Wnts cause phosphorylation of the Wnt coreceptor LRP6. In L-Wnt3A cells, a mouse cell line overexpressing Wnt3A, LGK974, strongly blocked Wnt-dependent phosphorylation of LRP6 (Fig. S1E).

Inhibition of PORCN by LGK974 Blocks All Wnts Tested. Based on genetic KOs, PORCN is known to affect posttranslational palmitoylation of all Wnt ligands (3, 15). Indeed, the residues around the universal Wnt palmitoylation site, Ser209, are conserved among all 19 Wnts (Fig. S1F), and the CHGxSGC palmitoylation motif was not identified in any other protein throughout the proteome (http://www.genome.jp/tools/motif/MOTIF2.html). To test if LGK974 can recapitulate the consequences of genetic loss of PORCN on Wnt processing, we tested a set of canonical Wnts, including Wnt1, -2, -3, -3A, -6, -7A, and -9A, in Wnt-dependent tumor fragments derived from MMTV-Wnt1 transgenic mice, the CHG×SGC palmitoylation motif was not identified in any other protein throughout the proteome (http://www.genome.jp/tools/motif/MOTIF2.html). To test if LGK974 can recapitulate the consequences of genetic loss of PORCN on Wnt processing, we tested a set of canonical Wnts, including Wnt1, -2, -3, -3A, -6, -7A, and -9A, in Wnt-dependent reporter assays. As shown in Fig. S1G, LGK974 showed comparable inhibitory activities against all tested Wnts, which is consistent with the genetic loss of PORCN phenotype.

LGK974 Induces Tumor Regression at a Well-Tolerated Dose in a Wnt-Driven Murine Tumor Model. Given the pivotal role of Wnt signaling in tissue stem cell renewal, achieving an acceptable therapeutic index with targeted inhibition of Wnt signaling poses an important clinical challenge (3). The discovery of the potent, selective, and orally bioavailable PORCN inhibitor LGK974 enabled us to investigate this issue using a well-established Wnt-dependent murine breast tumor model, the mouse mammary tumor virus (MMTV)-driven Wnt1 model (2). In a murine MMTV-Wnt1 tumor model using s.c. implanted tumor fragments derived from MMTV-Wnt1 transgenic mice,
LGK974 inhibited both analysis, respectively. As shown in Fig. 2 (phospho-LRP6) Wnt signaling events were examined by quantification with LGK974 in a murine MMTV-Wnt3 model (Fig. S2).

To correlate the observed antitumor activity with the inhibition of Wnt signaling, both distal (AXIN2 mRNA) and proximal (phospho-LRP6) Wnt signaling events were examined by quantitative RT-PCR through TaqMan assays and Western blot analysis, respectively. As shown in Fig. 2B and C, a dose of 3 mg/kg LGK974 inhibited both AXIN2 expression and phospho-LRP6 (pLRP6) levels 7 h after the last dose to a greater degree than the inhibition seen at 3 h. Both AXIN2 expression and pLRP6 levels were back to baseline levels 24 h after treatment, suggesting that sustained pathway inhibition was not required to achieve antitumor activities. Consistent with our results, a recent publication using a different PORCN inhibitor from our patent (21) also showed good efficacy in this model at a well-tolerated dose (22).

**LGK974 is Well-Tolerated in Wnt-Dependent Tissues at the Efficacious Dose in Rats.** To further examine the effect of LGK974 on normal tissue homeostasis, we carried out a set of rat efficacy and toxicology studies. We confirmed that LGK974 induced tumor regression when dosed at 3 mg/kg per day in a rat MMTV-Wnt1 xenograft tumor model (Fig. 3A). Toxicology studies were performed on nontumour bearing rats at 3 and 20 mg/kg. As shown in Fig. 3B–D, at the efficacious dose of 3 mg/kg per day for 14 d, LGK974 was well-tolerated without abnormal histopathological findings in Wnt-dependent tissues, including the intestine, stomach, and skin (23–26). When rats were administrated a very high dose of 20 mg/kg per day for 14 d, loss of intestinal epithelium was observed (Fig. S2C), consistent with the concept that Wnt is required for intestinal tissue homeostasis. This study shows a therapeutic window for LGK974, promoting tumor regressions at doses that spare normal tissues.

**Cellular Functional Effects of LGK974 in Human Head and Neck Cancer Cell Lines.** To identify human cancer cell lines that respond to porcupine inhibition, we surveyed cell lines from different cancer types using AXIN2 mRNA expression as a readout for Wnt pathway activity. We defined a responsive cell line as one that achieved greater than 50% AXIN2 mRNA reduction after treatment with 10–100 nM LGK974 for 48 h. We found that head and neck cancer (HNSCC) cell lines were responsive to LGK974, with 31 of 96 HNSCC cell lines showing pathway inhibition on treatment with LGK974 (Table S1), in contrast to colon cancer, brain cancer, small cell lung cancer, lymphoma/leukemia, or colon cancer cell lines, where no responsive lines were identified (Fig. S3A).

To correlate pathway inhibition with cellular function, the human HNSCC cell line HN30 was used for additional in vitro and in vivo characterization. LGK974 potently reduced Wnt-dependent AXIN2 mRNA levels in HN30 cells with an IC50 of 0.3 nM (Fig. 4A). It strongly attenuated HN30 colony formation, albeit with a right-shifted IC50 vs. the IC50 seen for pathway inhibition in vitro (Fig. 4B). Similar AXINX2 mRNA reduction and colony formation attenuation effects were also observed with the aforementioned structurally different PORCN inhibitor, GNF-1331 (Fig. S3B and C). The reduced colony formation caused by Fig. 2. LGK974 inhibits Wnt signaling in vivo and induces tumor regression in a mechanistic MMTV-Wnt1 tumor model. (A) LGK974 showed a strong efficacy in a Wnt tumor model (MMTV-Wnt) in nude mice. Spontaneous tumors from the MMTV-Wnt1 transgenic mice were implanted in nude mice. LGK974 was dosed at 0.3, 1.0, and 3.0 mg/kg per day for 13 d. LGK974 induced robust tumor regression at 1.0- and 3.0-mg/kg doses. (B) A PD study was performed in the murine MMTV-Wnt tumor model. LGK974 significantly inhibited AXIN2 expression 7 h after the last dose, and the effect diminished 24 h after the dose. (C) In the same PD study, pLRP6 expression level showed a very similar pattern to the PD response of AXIN2. LGK974 inhibitory effect peaked at 7 h after the last dose and diminished 24 h after the last dose. Fig. 3. LGK974 is well-tolerated in Wnt-dependent tissues at an efficacious dose in rats. (A) LGK974 induced tumor regression when dosed at 3 mg/kg per day in a rat MMTV-Wnt1 xenograft tumor model. (B) Intestine, (C) skin/hair follicle, and (D) stomach tissues from rats treated with vehicle control or LGK974 at the dose of 3 mg/kg per day were subjected to H&E staining. (Magnification: intestine, 40×; hair follicle and stomach, 20×. Scale bars: intestine, 500 μm; hair follicle and stomach, 100 μm.)
LGK974 could be partially reversed with overexpression of dominant β-catenin (Fig. S3D), which suggests that LGK974 functions, at least in part, through inhibiting the β-catenin–dependent activities. To further confirm whether the cellular effect of LGK974 was caused by the inhibition of PORCN-dependent Wnt signaling activities, shRNA experiments were performed. shRNA against PORCN potently knocked down PORCN expression levels (Fig. S3E) and substantially inhibited both the expression of the Wnt target gene AXIN2 (Fig. S3F) and the colony formation of HN30 cells in vitro (Fig. S3G), consistent with the LGK974 data.

Efficacy of LGK974 in a Mouse Model of Wnt-Dependent Human HNSCC Cell Line. To test the antitumor activity of LGK974, a mouse xenograft model of the HNSCC HN30 cell line was established. As shown in Fig. 4C, LGK974 had strong efficacy when administered one time per day. Changes in tumor volume for each of the treated T and C groups were measured and used to calculate growth delay, which was expressed by the T/C ratio. After 14 d of treatment, a dose of 0.1 mg/kg per day led to moderate tumor growth delay (T/C: 60%), a dose of 0.3 mg/kg per day significantly inhibited tumor growth (T/C: 26%), and doses of 1.0 and 3.0 mg/kg resulted in substantial tumor regression (T/C: −31% and −50%, respectively) (Fig. 4C). The regimen was well-tolerated, and there was no significant body weight loss in the mice. A similar result was obtained with twice daily treatment in this model with −44% T/C at a dose of 0.5 mg/kg two times per day (Fig. S3F).

Like in the MMTV-Wnt1 model, both AXIN2 mRNA and pLRP6 levels were used as pharmacodynamic markers to link with the observed antitumor activity in the HN30 mouse xenograft model. After a single 3-mg/kg dose LGK974, the level of AXIN2 mRNA expression in tumors was reduced by ~60–95% between 5 and 10 h postdose, and the effect started to diminish at 16 h in correlation with decreasing drug concentrations (Fig. 4D). A time delay was observed between the peak drug concentration (at 1 h) and the maximum AXIN2 mRNA inhibition (at 10 h). Additionally, as shown in Fig. 4E, pLRP6 levels in the HN30 tumors were substantially reduced in a time-dependent manner. The maximum effect was achieved at 7–10 h postdose, and the pLRP6 levels were largely back to baseline levels by 24 h.

The delayed pharmacokinetic (PD) are presumably caused by the mechanism of PORCN inhibition, which blocks the secretion of newly formed Wnts while having no effect on preexisting Wnts. The time needed for preexisting Wnts to become unavailable or inactive to their receptors is presumably the reason for the delay between the peak time of drug exposure and PD effects. This pharmacokinetic, PD, and efficacy relationship is consistent with aforementioned results from the MMTV-Wnt1 model. Sustained pathway inhibition is not required for inducing tumor regression in the HN30 xenograft model, which may yield a safety margin by differentiating tumor cells from normal cells, such as Wnt-dependent stem cells.

To examine other Wnt-related genes that might be regulated by treatment with LGK974 in vivo, a TaqMan GeneCard analysis revealed down-regulation of several other known Wnt target genes, including LEF1 and NDK1, as well as Wnt ligands, including Wnt3 and Wnt9B (Fig. 4F). Comparing the gene expression patterns between HN30 tumors and normal oropharynx tissues using this GeneCard analysis (Fig. 4G), Wnt ligands, including Wnt3, 7A, 10A, and 11, were substantially overexpressed in HN30 tumors, whereas the known Wnt inhibitory genes, such as SFRP2, FRZB, SFRP4, and DKK2, were substantially down-regulated, which together, is consistent with the hypothesis that Wnt signaling aberrations might be an underlining driver of this tumor-derived cell line. Using these types of Wnt pathway-related gene signatures may be a useful means to ultimately inform patient selection in support of the clinical development of PORCN inhibitors, like LGK974.

Correlation of Notch Mutations with PORCN Inhibitor Responsiveness. To further understand the mechanism of action in the cells that responded to LGK974 treatment, exome sequencing was performed on 40 HNSCC cell lines, including 25 responsive lines and 15 nonresponsive lines (a responsive cell line was defined as one that achieves greater than 50% AXIN2 mRNA reduction after treatment with 10–100 nM LGK974 for 48 h) (Table S2). Consistent with two independent epidemiologic studies on HNSCC cell lines reported recently (27, 28), TP53, CDKN2A, Notch1/2/3, PTEN, HRAS gene, and PIK3CA were among the top oncogenes or tumor suppressor genes mutated in this set of cell lines (Fig. S44). To correlate genetic changes with LGK974 response, we focused on aggregated LoF mutations, which include stop codon gain, start codon loss, frameshift, and splicing site mutations (details in Material and Methods). One of the most striking results of our study was the correlation of aggregated LoF Notch1 mutations with responsiveness to LGK974. As shown in Fig. 5A and B, five frameshift/nonsense mutations have been identified among the responsive cell lines, with only one Notch1 nonsense mutation among the nonresponsive cell lines. Interestingly, all cells with Notch1 mutations harbored at least one mutant allele that affected the N terminus of Notch1. This result is consistent with the notion that the N terminus of Notch1 is required for its function, with the N-terminal EGF repeats

Fig. 4. Human HNSCC cell line profiling with LGK974. (A) LGK974 inhibited Wnt signaling in HN30 cells with an IC50 of 0.3 nM. (B) LGK974 reduced HN30 cell colony formation in vitro. (C) LGK974 single agent-induced tumor regression in the human HNSCC tumor model HN30 in vivo. (D) A multitime points pharmacokinetic/PD study was done in the HN30 xenograft model. The 3-mg/kg dose inhibited the expression of a Wnt signaling target gene, AXIN2, by ~60–95% between 5 and 10 h after the dose, and the effect was completely absent at 24 h after the dose. (E) LGK974 showed similar inhibitory activities on pLRP6 in Western blot analysis, peaking around 7–10 h after the dose. (F) Using TaqMan GeneCard analysis, LGK974 downregulated the known Wnt target genes, including AXIN2, LEF1, and NDK1, as well as Wnt ligands, including Wnt13 and Wnt19B. (G) Compared with the gene expression patterns of normal human oropharynx tissues, Wnt ligands, including Wnt3, -7A, -10A, and -11, were substantially overexpressed, whereas the known Wnt inhibitory genes, such as SFRP2, FRZB, SFRP4, and DKK2, were substantially down-regulated.
responsible for ligand–receptor interaction (29), such that mutations in this region are more likely to be LoF mutations.

To further characterize the functional consequence of the missense mutations, such as Notch1 C478F, which is identified in the HN30 cells, a Notch reporter gene assay was performed with overexpression of the WT or the C478F mutant (Fig. 5C and Fig. S4B). The C478F mutation is located in the extracellular domain of Notch1, which is presumably critical for Notch receptor and ligand interaction. Indeed, in the presence of the Notch ligand DLL1, the activity of mutant C478F Notch1 was abolished in this Notch reporter gene assay (Fig. 5C).

To determine if restoration of Notch signaling activity can inhibit cell growth in Notch1 mutant cells, HN30 cells were infected with lentivirus expressing either WT full-length Notch1 or constitutively active Notch intracellular domain (NICD). After 17 d of selection with G418, cells infected with Notch1 or NICD showed dramatically reduced total cell numbers compared with the control group (Fig. S5). These data suggest that Notch signaling functions as a tumor suppressor gene in Notch1 mutant cells.

Notch suppresses Wnt signaling in keratinocytes in multiple models (30, 31). Inhibition of Notch signaling through Notch1 KO or expression of dominant negative MAML1 in keratinocytes or skin tissues from genetically engineered mice correlated with reduced total cell numbers compared with the control group (Fig. S5). These data suggest that Notch signaling functions as a tumor suppressor gene in Notch1 mutant cells.

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translocation (45). LoF mutants lose their tumor suppressor function and promote Wnt/β-catenin signaling (45). From our exome sequencing and cell line profiling efforts, we observed an enrichment of LGK974 responders in FAT1 mutant HNSCC cell lines (Fig. S8), which is consistent with the role of FAT1 in Wnt signaling. Furthermore, in mouse transgenic skin tumor models, activating mutation of HRAS substantially increased Wnt signaling, and β-catenin is required in HRAS-driven tumorigenesis (12, 47). Consistently, from our analysis, four of five cell lines with HRAS mutations responded to LGK974 (Fig. S9), supporting the notion that multiple mechanisms are involved in LGK974 responsiveness.

In this report, we described the successful discovery of a potent, selective, and orally bioavailable PORCN inhibitor, LGK974. We showed compelling evidence for inhibition of Wnt signaling in vivo, attenuation of tumor growth in human disease models at well-tolerated doses, and identification of potential genetic markers to enrich for tumors that are responsive to LGK974. These studies lay the foundation for the treatment of Wnt-driven tumors in the clinic.

**Materials and Methods**

All cell lines were cultured in a humidified incubator at 37 °C with 5% (vol/vol) CO2. All compounds were initially dissolved in 100% DMSO and then diluted to the indicated concentrations for studies in vitro. Detailed methods are described in SI Materials and Methods.

**ACKNOWLEDGMENTS.** We gratefully thank Dr. F. Cong for the Wnt reporter gene construct and Wnts/Fzd cDNA expression constructs; T. Bouloume and J. Watson for histology studies; X. Cui, T. Tuntland, N. Englund, A. Schumacher, H. Gao, C. Fryer, D. Han, G. Zhang, J. Cheng, P. Gordon, W. Richmond, M. Warmuth, and M. Yao for assistance and discussions; and Vivicor staff for animal handling. This work was supported by the Novartis Research Foundation.

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