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A Zebrafish Chemical Suppressor Screening Identifies Small Molecule Inhibitors of the Wnt/β-catenin Pathway

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SUMMARY

Genetic screening for suppressor mutants has been successfully used to identify important signaling regulators. Using an analogy to genetic suppressor screening, we developed a chemical suppressor screening method to identify inhibitors of the Wnt/β-catenin signaling pathway. We used zebrafish embryos in which chemically induced β-catenin accumulation led to an "eyeless" phenotype and conducted a pilot screening for compounds that restored eye development. This approach allowed us to identify geranylgeranyltransferase inhibitor 286 (GGTI-286), a geranylgeranyltransferase (GGTase) inhibitor. Our follow-up studies showed that GGTI-286 reduces nuclear localization of β-catenin and transcription dependent on β-catenin/T cell factor in mammalian cells. In addition to pharmacological inhibition, GGTase gene knockdown also attenuates the nuclear function of β-catenin. Overall, we validate our chemical suppressor screening as a method for identifying Wnt/β-catenin pathway inhibitors and implicate GGTase as a potential therapeutic target for Wnt-activated cancers.

INTRODUCTION

Genetic screening in model organisms has uncovered the molecular mechanisms for diverse biological processes, such as cell cycle in *Saccharomyces cerevisiae* (Hartwell, 1991), programmed cell death in *Caenorhabditis elegans* (Metzstein et al., 1998), and embryonic pattern formation in *Drosophila melanogaster* (Nüsslein-Volhard and Wieschaus, 1980). Such genetic screening has been further extended to screening for genetic modifiers, such as dominant suppressors or enhancers, facilitating identification of the signaling components and characterization of the pathways. Some of the most successful examples of modifier gene screening are identifications of components of *Drosophila* receptor tyrosine kinase signaling pathways, such as Drk, Src homology 3/Src homology 2/Src homology 3 adaptor (Simon et al., 1993), and Sos, a guanine nucleotide exchange factor (Simon et al., 1991), which activate a downstream guanosine triphosphatase (GTPase), Ras1. Downstream components of Ras1, such as Raf, mitogen-activated protein kinase kinase, and mitogen-activated protein kinase, were also identified as enhancers or suppressors in a *Drosophila* rough eye system, in which eye development was forced to be abnormal by genetic methods (Karim et al., 1996).

The zebrafish *Danio rerio* has become a widely used model organism in vertebrate genetics and in developmental biology along with the frog *Xenopus laevis*; also, zebrafish are highly amenable to chemical genetic studies (Zon and Peterson, 2005). Because of their small body size, embryos can be fit into 96-well plates and are particularly suitable for in vivo phenotype-based screens for small molecule compounds. Identified chemicals become important pharmacological probes in analyzing the underlying molecular mechanisms of the phenotypes and can ultimately provide new therapeutics. Phenotype-based screens with zebrafish anatomically similar to mammals can contribute to several aspects of the drug development process, such as disease modeling and toxicology.

The evolutionarily conserved Wnt/β-catenin pathway is essential for body axis formation during vertebrate embryogenesis (McMahon and Moon, 1989) and for segment polarity control during *Drosophila* larval development (Babu, 1977; Nüsslein-Volhard and Wieschaus, 1980). Wnt/β-catenin signaling plays crucial roles in cancer and stem cell homeostasis as well. Loss of function in the adenomatous polyposis coli (*Apc*) gene results in β-catenin accumulation, which leads to constitutive activation of the Wnt pathway and epithelial cell transformation (Kinzler et al., 1991). More than 80% of sporadic colon cancers are associated with *Apc* mutation and 10% with *β-catenin* mutation, both of which lead to Wnt pathway activation (Klaus and Birchmeier, 2008). Because of the key role of aberrant Wnt pathway activation in many types of cancers, such as colorectal and breast cancers, the development of Wnt pathway inhibitors has gathered attention (Barker and Clevers, 2006).

Regulating β-catenin levels is a critical event in the Wnt/β-catenin pathway. In the absence of the Wnt ligand, cytoplasmic β-catenin is maintained at low levels by its constitutive degradation, which is controlled primarily by association with the β-catenin destruction complex containing glycogen synthase kinase 3 (GSK3), casein kinase 1α (CK1α), APC, and Axin. Within this

complex, β-catenin is phosphorylated by GSK3 and CK1α and is targeted for degradation by the ubiquitin-proteasome system. In the presence of the Wnt ligand, β-catenin destruction is stopped, leading to β-catenin accumulation in the nucleus and transcriptional activation through the β-catenin/T cell factor (TCF) complex (Cselenyi et al., 2008; Kofron et al., 2007; Tolwinski et al., 2003; Yamamoto et al., 1999).

In zebrafish embryos, the ectopically activated Wnt canonical pathway during gastrulation leads to an "eyeless" phenotype, a phenotype lacking a forebrain and eyes (Heisenberg et al., 1996, 2001; Kim et al., 2000; van de Water et al., 2001). Similar phenotypes have also been observed among other species (Ciani and Salinas, 2005; Wilson and Houart, 2004). Pharmacological inhibition of GSK3 with either LiCl or 6-bromoindirubin-3′-oxime (BIO), a small molecule GSK3 inhibitor, copies the eyeless phenotype (Stachel et al., 1993; Atilla-Gokcumen et al., 2006). Using an analogy to genetic suppressor screening, we developed a phenotype-based chemical suppressor screening method, in which restoration of the eye development in eyeless zebrafish embryos treated with BIO is monitored, to identify Wnt canonical pathway inhibitors. First, we performed a pilot screening on chemical libraries composed of known target compounds and classified inhibitors of AKT, geranylgeranyltransferase (GGTase), calcium-activated potassium channel, and telomerase as chemical suppressors of the eyeless phenotype. Second, these compounds diminished β-catenin/TCF-dependent transcriptional activity in mammalian cells. Third, we found that gene knockdown of the GGTase I beta subunit reduced β-catenin/TCF-dependent transcriptional activity as well as pharmacological inhibition. Finally, the pharmacological inhibition in β-catenin/TCF-dependent transcriptional activation was reversed by a phospho-mimicking β-catenin mutant, S191D, that imitates phosphorylation by c-Jun N-terminal kinase (JNK), a Rac1 effector kinase. Thus, our simple chemical suppressor screening with zebrafish has been validated as a method for identifying Wnt/β-catenin pathway inhibitors, and GGTase has been shown to be a potential therapeutic target for Wnt-activated malignant cancers. Our method may allow small laboratories to conduct chemical genetic screening without the necessity of maintaining mutant animals and genotyping.

RESULTS

Known Wnt/β-catenin Pathway Inhibitors Suppress Chemically Induced Eyeless Phenotype in Zebrafish Embryos

The evolutionally conserved Wnt canonical pathway regulates body axis formation among diverse species and is also involved in human diseases such as colorectal and breast cancers. To develop a chemical genetic screening platform in vertebrates, we used zebrafish embryos because of their small and transparent bodies and sensitivity to chemical compounds. In zebrafish embryos, aberrant β-catenin accumulation in the nucleus leads to body axis defects, resulting in an eyeless phenotype (Heisenberg et al., 1996, 2001; Kim et al., 2000; van de Water et al., 2001). In our system, the eyeless phenotype was induced by abnormal β-catenin accumulation with pharmacological GSK3 inhibition by a GSK3 inhibitor, BIO. Effects of test compounds were evaluated to determine whether eye development was restored (Figure 1A). When eye development was restored to normal and no obvious developmental defect was observed, the compound was classified as a positive candidate. When a developmental defect was observed, the test compound was classified as a negative candidate because possible adverse effects were suspected (Figure 1A).

To verify whether the chemical suppressor screening works as a screening system for Wnt pathway inhibitors, known inhibitors were subjected to the assay system. Pyrvinium (Thorne et al., 2010), ICG-001 (Emami et al., 2004), inhibitor of Wnt response 1 (IWR-1) (Chen et al., 2009), and XAV939 (Huang et al., 2009) at least partially rescued eye development, but IWR-1 and XAV939 treatments resulted in developmental malformation, suggesting signs of their potential toxicity in vivo (Figure 1B). Next, we performed a pilot screening with a chemical library composed of known target compounds (Figure 2A). Among 282 test compounds, geranylgeranyltransferase inhibitor 286 (GGTI-286), Akt inhibitor IV, dequalinium, and β-rubromycin were classified as positive candidates inducing restored eye development without obvious developmental abnormality (Figure 2B).

Chemical Suppressors of the "Eyeless" Phenotype Inhibit the Wnt Canonical Pathway in Mammalian Cells

Next, Wnt canonical pathway inhibition of these four compounds was also tested in mammalian cell lines to exclude possible species-specific effects. Using two cell lines (Chinese hamster ovary [CHO] and human embryonic kidney 293 [HEK293] cells), the effects of the candidate compounds on the Wnt canonical pathway were analyzed by luciferase reporter assay using a β-catenin/TCF-responsible TOPFlash reporter plasmid (Veeman et al., 2003). In CHO cells, all four candidates significantly reduced β-catenin/TCF-dependent transcriptional activity (Figure 2C). The decrease in the transcriptional activity was observed only in HEK293 cells treated with Akt inhibitor IV, dequalinium, and β-rubromycin, not in cells treated with GGTI-286 (Figure S1A available online). Because Akt inhibitor IV showed cytotoxicity at 10 μM, a 1 μM dose was tested and diminished the transcriptional activity to levels comparable with those seen with other compound treatments (Figure 2C; Figure S1A). HEK293 cells are less sensitive to GGTI-286 in comparison with CHO cells. Because β-catenin is a well-known component of cadherin-dependent adherens junctions, we focused on differences in expression levels of cadherins between these two cell lines. Knockdown of endogenous N-cadherin made HEK293 cells sensitive to GGTI-286 (Figure S1B). Conversely, forced expression of exogenous E-cadherin made CHO cells insensitive to GGTI-286 (Figure S1C). Although sensitivity to GGTI-286 may depend on cadherin expression levels, these data indicate that all four candidate compounds inhibit the Wnt canonical pathway in mammalian cells.

Target molecules of the candidates are GGTase I (a target of GGTI-286), Akt (a target of Akt inhibitor IV), calcium-activated potassium channels (targets of dequalinium), and telomerase (a target of β-rubromycin) (Figure 2D). Because Akt promotes β-catenin/TCF-dependent transcriptional activity by directly phosphorylating β-catenin (Fang et al., 2007), Akt inhibition reduces the transcriptional activity. Elevated calcium ion level antagonizes Wnt/β-catenin signaling (Slusarski et al., 1997), so

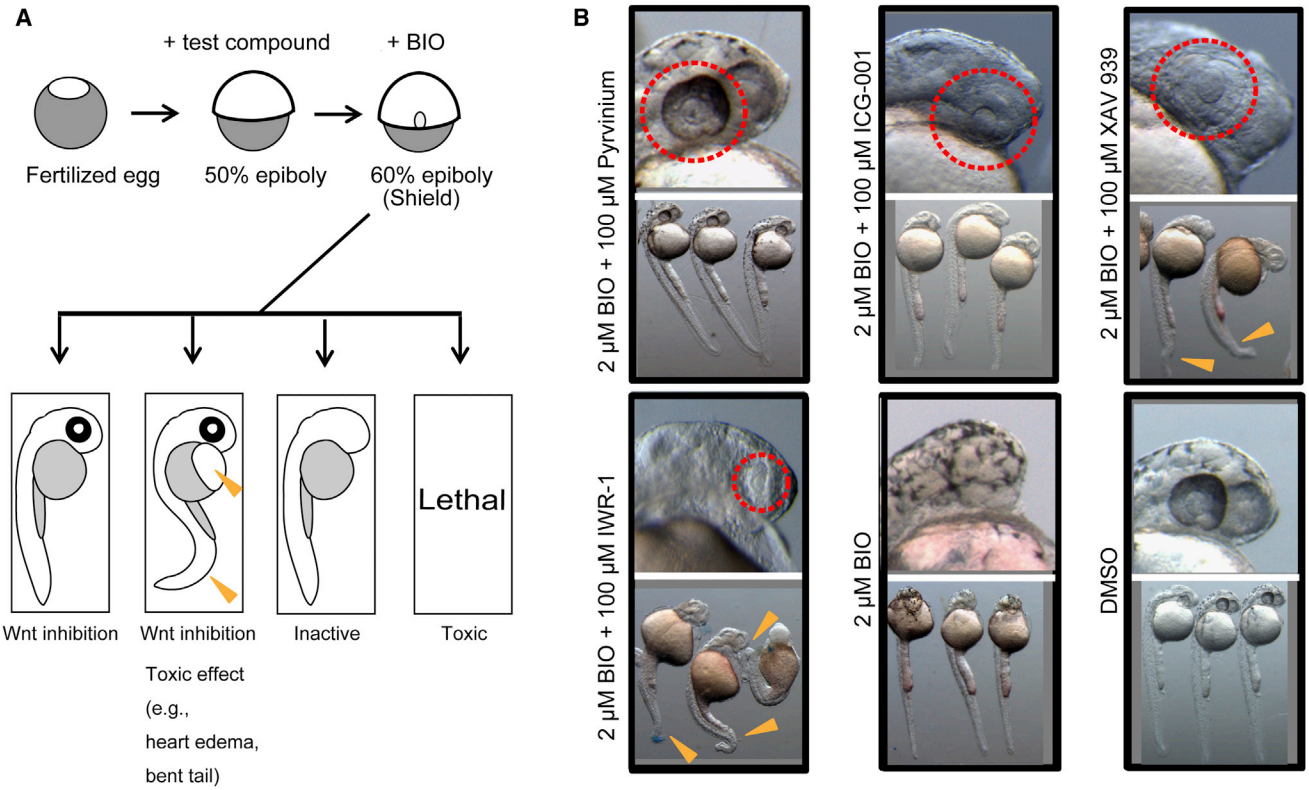

Figure 1. A Zebrafish Assay for Chemical Suppressors Confirms Inhibitory Activities of Known Inhibitors of the Wnt Pathway

(A) A schematic diagram of the zebrafish chemical suppressor screening is shown. Chemical suppressors of the eyeless phenotype were identified. Embryos were pretreated with test compounds at 50% epiboly and treated with BIO (a GSK3 inhibitor) at the shield stage, then incubated for 18 to 24 hr. Effects of the compounds were evaluated under a dissecting microscope. When eye development was restored to normal, the test compound was classified as a positive candidate for Wnt pathway inhibitors. When a developmental defect was observed, the adverse effect might reflect potential toxicity of the compound. Arrowheads indicate sites of toxic effects (e.g., heart edema, bent tail).

(B) Known Wnt pathway inhibitors restored eye development in the presence of BIO. Pyrvinium (a casein kinase activator), ICG-001 (an inhibitor of interaction between β-catenin/TCF and CREB-binding protein), XAV939 (a tankylase inhibitor), and IWR-1 (an Axin stabilizer) at least partially reversed the BIO-induced eyeless phenotype at 100 μM (dotted lines). Treatments of XAV939 and IWR-1 resulted in malformation in tails (arrowheads). Images were taken at 30 hpf.

dequalinium may eventually diminish the Wnt canonical pathway by increasing cytoplasmic calcium ion concentration (Kuum et al., 2012). Telomerase associates and collaborates with the β-catenin/TCF complex to promote downstream gene expression (Park et al., 2009). Consequently, telomerase inhibition results in reduced β-catenin function in the nucleus. Thus, our zebrafish whole-animal screening system works reasonably well to identify Wnt/β-catenin pathway inhibitors.

GGTase Inhibition Suppresses the Wnt Canonical Pathway In Vivo and in Cells

Among target molecules of the four positive compounds, GGTase has not been reported as a direct regulator of the Wnt canonical pathway. Therefore, we attempted to confirm the specific activity of GGTase inhibitors in suppressing this pathway. GGTases are members of a protein prenyltransferase family that includes farnesyltransferase (FTase), GGTase I, and GGTase II. They posttranslationally attach farnesyl or geranylgeranyl groups to the C-terminal proximal cysteine residues of their distinct substrate proteins (Zhang and Casey, 1996). Therefore, inhibition of different prenylating enzymes should lead to specific outcomes of the chemical rescue assay in the zebrafish system. To confirm specific contributions of GGTase I, the inhibitory effect of GGTI-286 on the Wnt canonical pathway was compared with that of FTase inhibitor 276 (FTI-276) (Figure S2A). FTI-276 did not restore eye development and β-catenin/TCF-dependent transcription, in contrast to the reproducible suppression of Wnt/β-catenin signaling by GGTI-286 in zebrafish embryos (Figure 3A) or in CHO cells (Figure 3B). Higher concentrations of GGTI-286 reduced posterior structures in zebrafish embryos without BIO treatment (Figure S2B). Native agonist Wnt3A-induced transcriptional activation of the β-catenin/TCF complex was also diminished by GGTI-286 (Figure 3C) and its derivative GGTI-2147 (Figure 3D) in MDA-MB-231 cells, a human breast cancer cell line without cadherin expression (Figure S2E). However, GGTI-286 did not inhibit serum-induced activator protein 1 transcriptional activity (Figure S2C). Cyclin D1 and Axin2, endogenous target gene products of β-catenin/TCF, were decreased in a similar range of inhibitory concentration of Rac1 membrane localization, a GGTase-dependent process (Figures 3E and 3F). These data indicate that GGTase inhibitors specifically reduce the Wnt canonical pathway in vivo and in cells. Colon cancer cell lines with endogenous E-cadherin were less sensitive to GGTI-286 than

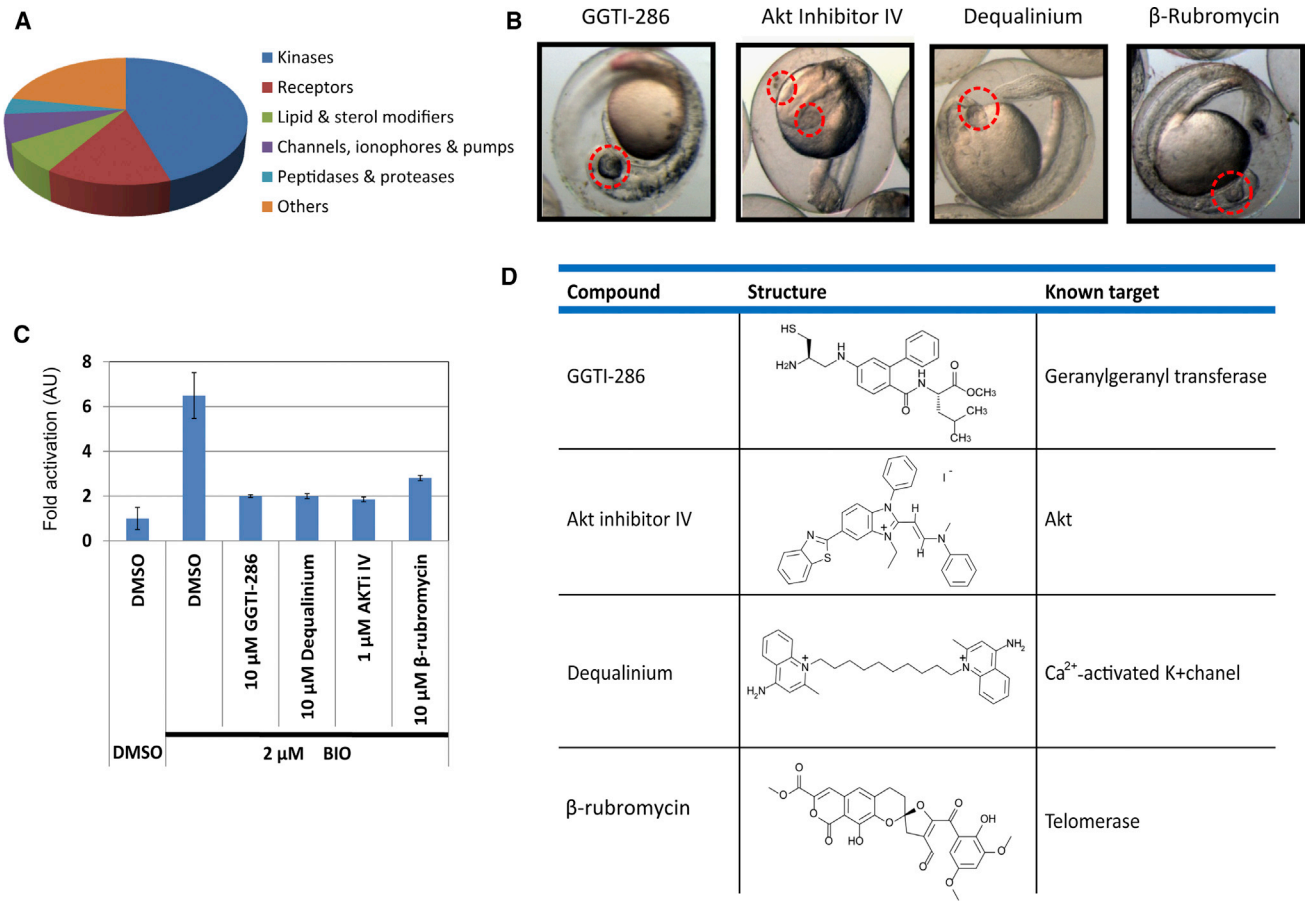

Figure 2. A Zebrafish Chemical Screening Identifies Inhibitors against GGTase, Akt, Telomerase, and K^+ Channel as Chemical Suppressors of the Wnt/β-catenin Pathway

(A) Compounds from SCADS chemical libraries were classified into subcategories of the target molecules.

(B) The 20 μm GGTI-286, Akt inhibitor IV (AKTiIV), dequalinium, and β-rubromycin rescued the BIO-induced eyeless phenotype. Images were taken at 30 hpf.

(C) GGTI-286, AKTiIV, dequalinium, and β-rubromycin reduced β-catenin/TCF-dependent transcriptional activity in CHO cells. Cells were transiently cotransfected with Super 8 x TOPFlash for monitoring β-catenin/TCF-dependent transcriptional activity and with pRL-CMV for normalizing transfection efficiency. The cells were pretreated with 10 μM compounds for 30 min to 6 hr and treated with 2 μM BIO for 18 hr. Cells were lysed, assayed for firefly luciferase activity, and then assayed for *Renilla* luciferase activity. Normalized relative luciferase activities are shown as fold activation to DMSO-treated cells. Values are ± SEM (n = 3). AU, arbitrary units.

(D) Structures and known target molecules of the positive compounds are summarized.

cadherin-free MDA-MB-231 cells (Figure 3E; Figures S2D and S2E).

Because small molecule compounds might show off-target effects, we asked whether GGTase I is involved in the Wnt canonical pathway using small interfacing RNA (siRNA) knockdown against the protein GGTase I β subunit gene, *pggt1b*. Reduction in *pggt1b* gene expression resulted in a decrease in the protein GGTase I β subunit (PGGT1B) expression level and in Wnt-induced β-catenin/TCF transcriptional activity (Figure 4A). Reconstitution with exogenous *pggt1b* reversed siRNA-mediated inhibition in the β-catenin/TCF transcriptional activity (Figure 4B). In vivo *pggt1b* gene knockdown was also performed using two antisense morpholino oligomers (MOs) against *pggt1b* gene (Cao et al., 2009; Eisa-Beygi et al., 2013). BIO treatment expands the expression of *goosecoid* gene, a downstream target gene of the Wnt canonical pathway. However, *pggt1b* gene knockdown retracted the BIO-induced expansion of *goosecoid* expression (Figure 4C). These data indicate that GGTase I participates in the Wnt signaling pathway.

GGTase Inhibition Reduces Nuclear Localization of β-catenin

To understand the mechanism of Wnt pathway suppression by GGTase inhibition, the effects of GGTI-286 on nuclear localization of β-catenin were analyzed by cell fractionation. Upon BIO treatment, β-catenin accumulated in both the cytoplasmic and the nuclear fractions in CHO cells. In the presence of GGTI-286, whereas the cytoplasmic β-catenin amount was largely unchanged, the nuclear β-catenin amount was greatly reduced (Figure 5A), suggesting that GGTI-286 inhibited the nuclear localization of β-catenin. The inhibition in nuclear localization of β-catenin was also confirmed by immunocytochemistry in MDA-MB-231 cells. Wnt3A-induced nuclear localization of β-catenin was reduced by GGTI-286 treatment (Figure 5B).

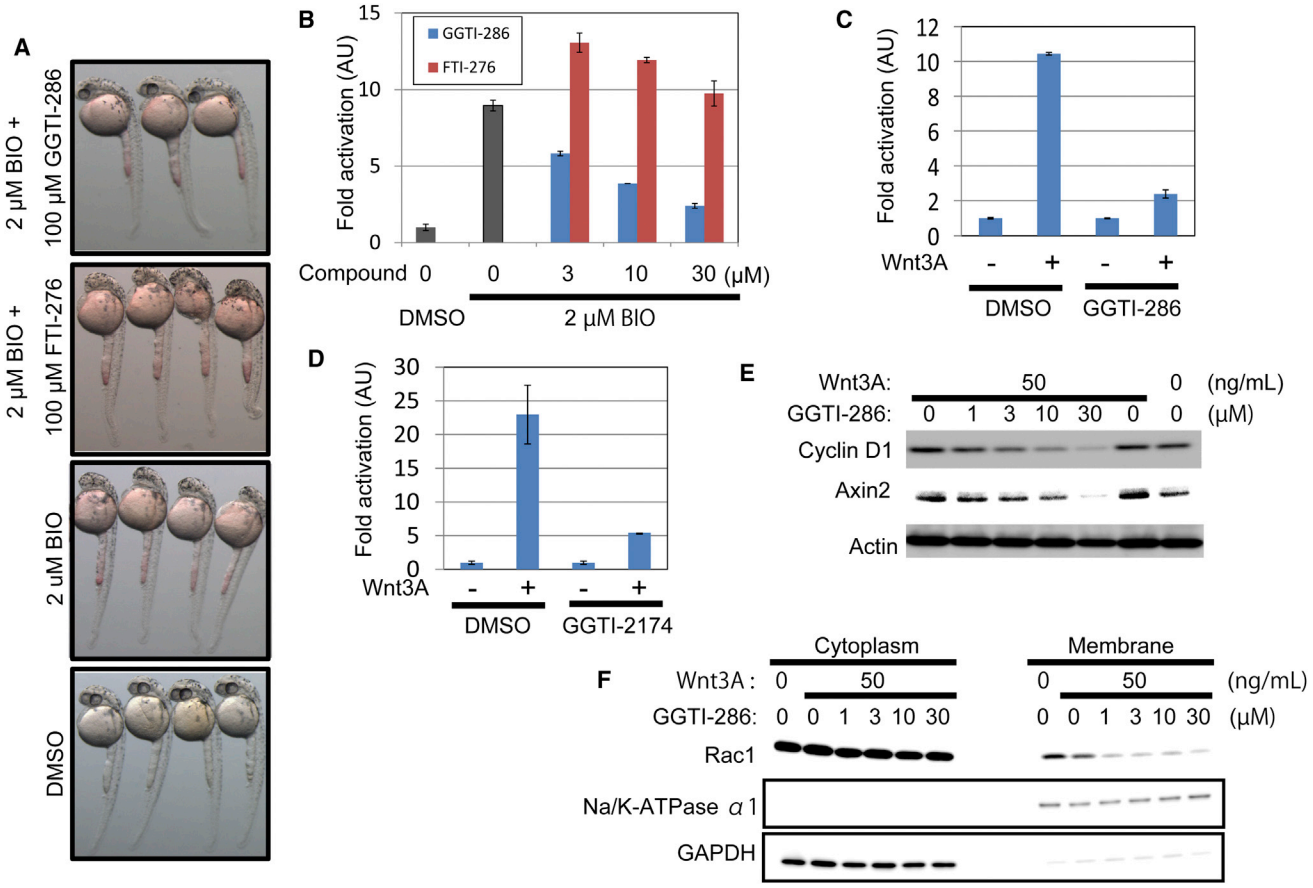

Figure 3. GGTIs Specifically Inhibit the Wnt Canonical Pathway

(A) GGTI-286 rescued the eyeless phenotype in zebrafish embryos, but FTI-276, a farnesyltransferase inhibitor, did not. Embryos were pretreated with 100 μM GGTI-286 or 100 μM FTI-276 at 50% epiboly, treated with 2 μM BIO at the shield stage, and then incubated for another 24 hr. Images were taken at 30 hpf.

(B) GGTI-286 inhibited β-catenin/TCF-dependent transcriptional activity, but FTI-276 did not. CHO cells were transiently transfected with the TOPFlash reporter plasmids. The cells were pretreated with 3, 10, or 30 μM GGTI-286 or FTI-276 for 2 hr and treated with 2 μM BIO for 18 hr. Firefly and *Renilla* luciferase activities were measured. Normalized relative luciferase activities are shown as fold activation to DMSO-treated cells.

(C and D) GGTI-286 (C) or GGTI-2174 (D) inhibited Wnt3A-induced transcriptional activation of the β-catenin/TCF complex. MDA-MB-231 cells were transiently transfected with the reporter plasmids. The cells were pretreated with 10 μM GGTI-286 or 30 μM GGTI-2174 for 6 hr and treated with 50 ng/ml Wnt3A for 18 hr. Firefly and *Renilla* luciferase activities were measured. Normalized relative luciferase activities are shown as fold activation to the absence of Wnt3A. Values are ± SEM (n = at least 3).

(E) GGTI-286 diminished expression levels of cyclin D1 and Axin2 in MDA-MB-231 cells. The cells were pretreated with 1 to 30 μM GGTI-286 for 6 hr and treated with 50 ng/ml Wnt3A for 18 hr. Cell lysates were analyzed by Western blotting with antibodies against cyclin D1, Axin2, and actin.

(F) GGTI-286 reduced membrane localization of Rac1 in MDA-MB-231 cells. The cells were pretreated with 1 to 30 μM GGTI-286 for 6 hr and treated with 50 ng/ml Wnt3A for 18 hr. Cell lysates were fractionated into the cytoplasmic and membrane fractions by ultracentrifuge and analyzed by Western blotting with antibodies against Rac1, Na, K-ATPase α1 subunit, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

These data suggest that GGTI-286 suppresses the Wnt canonical pathway by reducing the nuclear localization of β-catenin.

To further rationalize the identification of GGTase inhibitors as Wnt signaling chemical suppressors, we hypothesized possible crosstalk between GGTase I and the Wnt canonical pathway. GGTase I attaches a geranylgeranyl group to the C terminus of Rho family GTPases, such as RhoA, Rac1, and Rac3 (Allal et al., 2000; Joyce and Cox, 2003). Protein prenylation, such as geranylgeranylation and farnesylation, is an essential process for proper localization and functions of GTPases (Zhang and Casey, 1996). Rac1 has been reported to promote nuclear localization of β-catenin through phosphorylation at Ser191 by a downstream effector kinase, JNK (Wu et al., 2008). Therefore, GGTI-286 may inhibit nuclear localization of β-catenin through reduction in geranylgeranylation of Rac and in phosphorylation of β-catenin Ser191. Nuclear localization and transcriptional activity of β-catenin became resistant to GGTI-286 in cells expressing a phospho-mimicking β-catenin, S191D (Figures 6A and 6B). These data support a model in which GGTI-286 inhibits β-catenin accumulation in the nucleus by interfering with Rac and β-catenin Ser191 phosphorylation (Figure S3).

DISCUSSION

In the present study, we developed an in vivo chemical suppressor screening method to identify inhibitors of the Wnt/β-catenin

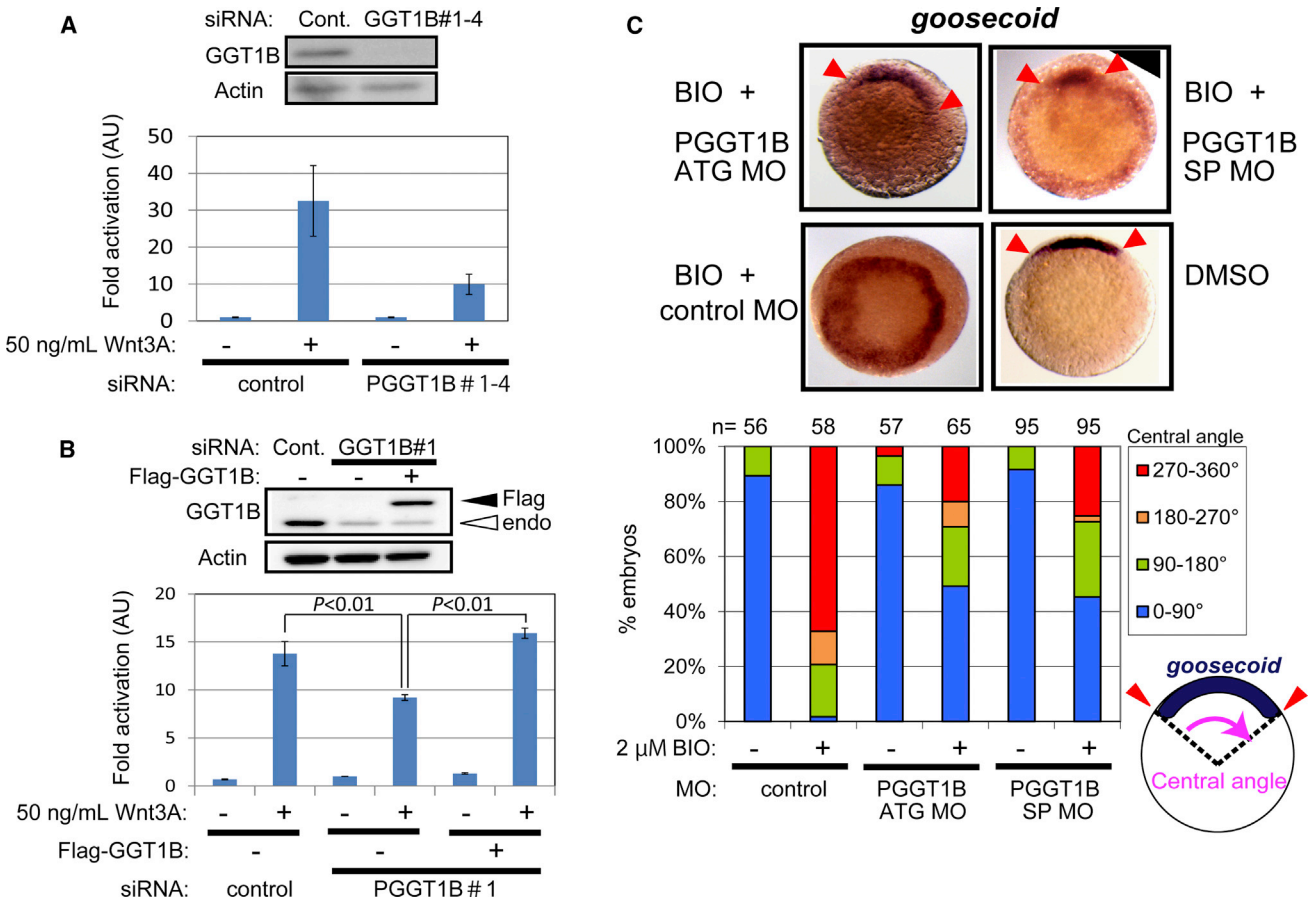

Figure 4. GGTase Is Required for Wnt/β-catenin Signaling

(A) Knockdown of the β subunit of PGGT1B reduced Wnt3A-induced transcriptional activation of the β-catenin/TCF complex. MDA-MB-231 cells were transfected with a mixture of four siRNAs for PGGT1B and the reporter plasmids. The cells were treated with 50 ng/ml Wnt3A for 18 hr. Cont., control.

(B) Reconstitution of PGGT1B with Flag-tagged PGGT1B reversed PGGT1B knockdown-mediated reduction in transcriptional activation of β-catenin/TCF. MDA-MB-231 cells were transfected with a siRNA targeting the 3′ untranslated region (3′ UTR) of *pggt1b* gene, an expression vector for Flag-tagged PGGT1B deleted the 3′ UTR, and the reporter plasmids. The cells were treated with 50 ng/ml Wnt3A for 18 hr. Fold activation to the absence of Wnt3A is shown. Values are ± SEM (n = at least 3). PGGT1B expression levels were analyzed by Western blotting with antibodies against PGGT1B and actin.

(C) Knockdown of PGGT1B inhibited BIO-induced *goosecoid* expression in zebrafish embryos. Embryos were injected with antisense MOs that target translation (ATG MO) or splicing (SP MO) of the zebrafish *pggt1b* gene at the one-cell stage and treated with 30 μM BIO or DMSO for 8 min at 32- to 64-cell stage. At 50% epiboly, embryos were fixed and subjected to in situ hybridization with an antisense *goosecoid* probe. Embryos are animal pole view, dorsal up if it can be distinguished. Arrowheads indicate tips of the *goosecoid* expression area. The central angles of *goosecoid* expression area were measured and scored into four classes: 0° to 90°, 90° to 180°, 180° to 270°, and 270° to 360° (n = at least 56).

signaling pathway using an analogy to genetic suppressor screening in model organisms. Candidate compounds were identified by monitoring suppression of the eyeless phenotype in embryos with chemically induced β-catenin accumulation. A pilot screening identified GGTI-286 as a chemical suppressor of the Wnt/β-catenin pathway in addition to known Wnt pathway inhibitors. We found that GGTI-286 diminished β-catenin/TCF-dependent transcriptional activity by reducing nuclear localization of β-catenin in mammalian cells. Genetic suppression of GGTase I confirmed functional interaction between GGTase I and the Wnt canonical pathway. Thus, our chemical suppressor screening with zebrafish embryo has been validated as a method for identifying Wnt/β-catenin pathway inhibitors and has classified GGTase I as a potential therapeutic target for β-catenin-dependent human diseases.

Our simple chemical suppressor screening with zebrafish embryo identified candidate compounds of Wnt/β-catenin pathway inhibitors. We developed a chemical suppressor screening method that is a phenotype-based assay with restriction in the target signaling pathway. In the present study, the screening identified inhibitors of AKT, calcium-activated potassium channel, and telomerase (Figure 2) in addition to known Wnt/β-catenin pathway inhibitors (Figure 1B). Identification of inhibitors of a negative regulator of GSK3 (Cross et al., 1995), a calcium ion level modulator (Kuum et al., 2012), and a transcriptional coactivator of β-catenin (Park et al., 2009) reinforces verification of our screening for chemical suppressors against the eyeless phenotype as a Wnt/β-catenin pathway inhibitor screening. However, these results do not necessarily mean that modulating functions of known targets are the sole mechanisms of Wnt/β-catenin

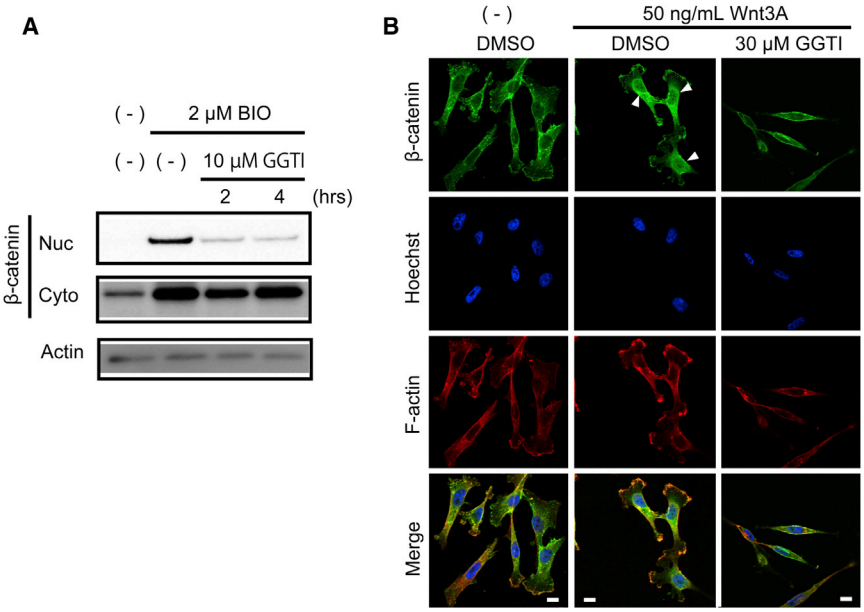

Figure 5. GGTI-286 Inhibits Nuclear Accumulation of β-catenin

(A) GGTI-286 diminished β-catenin nuclear accumulation. CHO cells were pretreated with 10 μM GGTI-286 for 2 or 4 hr, treated with 2 μM BIO for 2 hr, and then mechanically lysed and fractionated. The nuclear (Nuc) or cytoplasmic (Cyto) fractions of β-catenin were analyzed by Western blotting with an antibody against β-catenin. An anti-actin antibody was used for the loading control.

(B) GGTI-286 reduced Wnt3A-induced nuclear accumulation of β-catenin. MDA-MB-231 cells were pretreated with 30 μM GGTI-286 for 4 hr, treated with 50 ng/ml Wnt3A for 18 hr, and then stained with an anti-β-catenin antibody, Hoechst33342, and phalloidin. Fluorescent images were observed under a confocal microscope. Scale bars represent 10 μm.

inhibition. For example, an AKT inhibitor may affect translation of β-catenin. Another example is telomerase inhibition, which causes only modest homeotic phenotypes in *Tert* knockout mice in comparison with the more severe phenotypes in embryonic stem cells or in *Xenopus* embryos (Park et al., 2009). Although the difference in severities among systems can be explained by developmental compensation in germline knockout, the importance of telomerase for the Wnt/β-catenin pathway has not yet been confirmed in our system. These issues will be addressed in future studies. Although a phenotype-based assay expands the variety of therapeutic targets, target identification is generally challenging and sometimes unsuccessful. However, the chemical suppressor screening efficiently enriched known inhibitors of regulators of the Wnt/β-catenin signaling pathway upregulated by pharmacological GSK3 inhibition, just like genetic modifier screening, in which a signaling pathway of interest is genetically manipulated. Because our method requires only wild-type zebrafish and a GSK3 inhibitor but does not need mutants or transgenic fish lines, which necessitate genotyping and large-scale fish facilities, application of the chemical suppressor screening may provide opportunities for phenotype-based screening to small-scale laboratories.

The zebrafish chemical suppressor screening repositioned GGTase inhibitors (GGTIs) as Wnt/β-catenin pathway inhibitors. The BIO-induced eyeless phenotype in zebrafish embryos was chemically suppressed by GGTI-286 (Figures 2B and 3A). Furthermore, GGTI-286 and GGTI-2147 inhibited BIO- or Wnt3A-induced β-catenin/TCF transcriptional activation and endogenous target gene expression (Figures 2C and 3B–3E), and GGTI-286 reduced nuclear localization of β-catenin in cadherin-free cells (Figure 5). On the other hand, HEK293 and colon cancer cells did not respond to GGTI-286 (Figures S1A and S2D). Sensitivity to GGTIs seems to depend on cadherin expression (Figure S1). β-catenin is a well-known component of cadherin-dependent adherens junctions. Perturbation of cytoskeletal integrity by GGTI-mediated inhibition in Rho family GTPases may decrease the cadherin-bound β-catenin, and increasing the cytoplasmic pool of β-catenin may desensitize to GGTI-286. Protein-prenylating enzymes, such as FTase and GGTase, have already been recognized as potential therapeutic targets for human cancers (Berndt et al., 2011). Inhibition of the phosphatidylinositol-3-OH kinase/AKT pathway (Dan et al., 2004) and accumulation of p27Kip1 in the nucleus (Kazi et al., 2009) have been reported as target pathways for GGTase I. Although blockage of these pathways may contribute to growth suppression of cancer cells, our studies indicate that GGTIs neutralize activated Wnt/β-catenin signaling. Thus, inhibition in the Wnt/β-catenin pathway can be added to the pharmacological activities of GGTIs as well as known mechanisms.

The catalytic subunit of GGTase I functionally interacts with the Wnt/β-catenin pathway. In addition to pharmacological inhibition of GGTase I, *pggt1b* gene knockdown also attenuated the Wnt/β-catenin signaling. Knockdown of *pggt1b* reduced Wnt3A-induced β-catenin/TCF transcriptional activation in a breast cancer cell line, MDA-MB-231 (Figures 4A and 4B). Injection of antisense MOs against *pggt1b* also resulted in reduction in *goosecoid* expression, which was expanded by BIO (Figure 4C). These data indicate that PGGT1B is a component of regulatory systems for Wnt/β-catenin signaling. Furthermore, a pseudophosphorylated β-catenin (S191D) reversed GGTI-286-mediated inhibition of nuclear localization of β-catenin and β-catenin/TCF transcriptional activation (Figure 6). GGTase I posttranslationally modifies Rho GTPases such as RhoA (Yoshida et al., 1991) and Rac1 (Kinsella et al., 1991) and is essential for proper functions of these GTPases (Zhang and Casey, 1996). JNK, a downstream kinase of Rac, phosphorylates β-catenin S191 and promotes nuclear localization of β-catenin (Wu et al., 2008). This suggests that reduced S191 phosphorylation resulting from the blockage of GGTase is a cause of Wnt/β-catenin pathway inhibition by GGTIs (Figure S3). Phosphorylation of β-catenin S675 by p21-activated kinase (Zhu et al., 2012) also promotes the nuclear function of β-catenin. Although the PAK-dependent phosphorylation stabilizes the cytoplasmic β-catenin, the level of cytoplasmic β-catenin was largely unaffected by GGTI-286 (Figure 5A). Therefore,

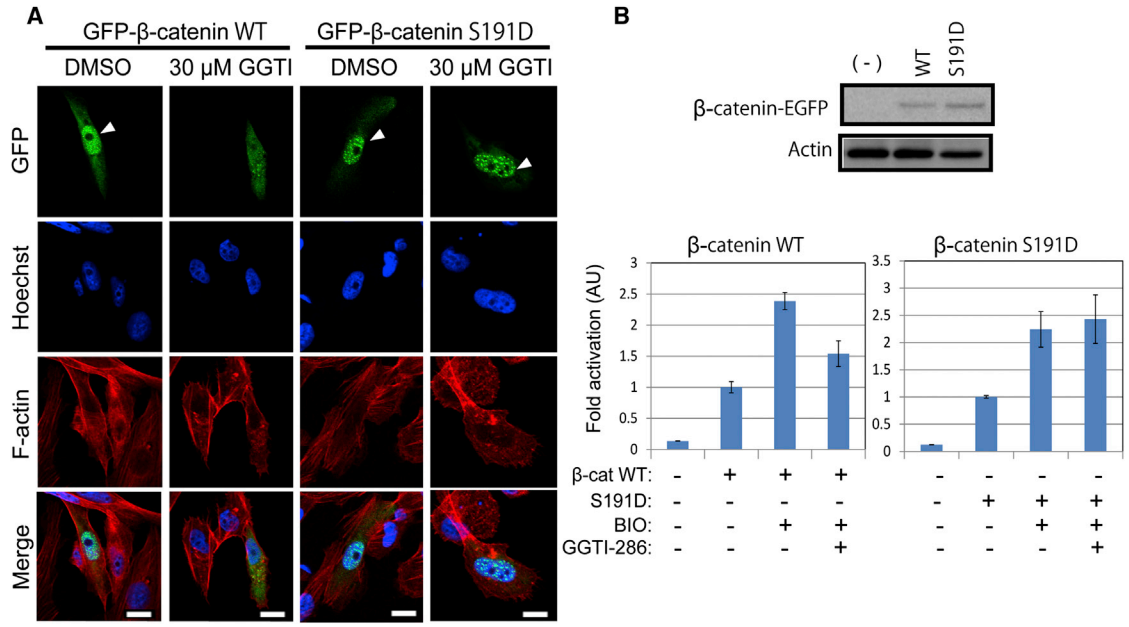

Figure 6. β-catenin S191D, a Phospho-Mimicking Mutant of β-catenin, Overcomes GGTI-286-mediated Inhibition in Wnt/β-catenin Signaling
(A) β-catenin S191D, a pseudophosphorylated mutant of β-catenin Ser191, reversed GGTI-286-mediated inhibition of β-catenin nuclear localization. CHO cells were transiently transfected with expression vectors for EGFP-fusion β-catenin wild-type (WT) or S191D. The cells were treated with 30 μM GGTI-286 for 18 hr and stained with Hoechst33342 and phalloidin. Fluorescent images were observed under a confocal microscope. Scale bars represent 10 μm.
(B) β-catenin S191D reversed GGTI-286-mediated inhibition of β-catenin/TCF-dependent transcriptional activation. CHO cells were transiently transfected with expression vectors for β-catenin WT or S191D and the reporter plasmids. The cells were treated with 2 μM BIO for 18 hr. Luciferase activities were measured. Normalized relative luciferase activities are shown as fold activation to the absence of BIO. Values are ± SEM (n = at least 3).

relative sensitivity to GGTIs of β-catenin S191 phosphorylation seems to be higher than that of S675 phosphorylation. Our data indicate that GGTase functionally interacts with the Wnt canonical pathway and suggest GGTase as a potential therapeutic target for β-catenin-dependent malignant cancers that lack cadherin expression.

Our chemical suppressor screening is a 96-well plate-formatted vertebrate whole-animal screening. The system may be useful not only for evaluating pharmacologic effects but also for predicting possible toxicity. GGTI-2418 is a GGTI that significantly inhibits the growth of breast tumors in animal models (Kazi et al., 2009) and was advanced to a phase I study (O'Dwyer et al., 2010). Although we do not know the exact reason for the discontinuation of the clinical study, GGTI-2418 is very well tolerated, with minimal side effects, at least according to an interim report of the phase I study (O'Dwyer et al., 2010). Zebrafish whole-animal screening may become a powerful method to select less toxic compounds from "safety unknown" chemical libraries. Applying the system to other target pathways expands the possibility of vertebrate chemical genetics and can accelerate the discovery of lead compounds and drug development with toxicity information in vertebrates.

SIGNIFICANCE

Screening for genetic modifiers, such as dominant suppressors or enhancers, in model organisms has identified and characterized important signaling pathways. Likewise, a phenotype induced by a chemical compound can be suppressed or enhanced by another compound. In this study, we developed a screening method to identify chemical suppressors of the chemically induced Wnt/β-catenin pathway in a vertebrate model organism, zebrafish. Although a phenotype-based assay expands the variety of therapeutic targets, target identification of a chemical inhibitor is generally challenging and sometimes unsuccessful. Because our chemical suppressor screening restricts the target signaling by upregulating the Wnt/β-catenin pathway with a GSK3 inhibitor, targets of all the "hit" compounds were placed in the Wnt/β-catenin pathway, resembling a genetic modifier screening that identifies genes functioning in a signaling pathway of interest. Because our method requires only wild-type zebrafish and does not need mutants or transgenic fish lines, which demand genotyping and large-scale fish facilities, application of this chemical suppressor screening may provide opportunities for phenotype-based screening to small-scale laboratories interested in broad fields of chemical biology and compound screening. Furthermore, the zebrafish whole-animal screening may become a powerful method to select less toxic compounds from "safety unknown" chemical libraries. Applying the system to other target pathways expands the possibility of vertebrate chemical genetics and can accelerate the discovery of seed compounds and drug development with toxicity information in vertebrates. Our zebrafish system for inhibitors of the Wnt/β-catenin pathway exemplifies a successful chemical suppressor screening in vertebrates.

EXPERIMENTAL PROCEDURES

Maintenance of Zebrafish

Zebrafish RIKEN wild-type strain was obtained from the National Bioresource Project of Japan and maintained under a 12-hr day/12-hr night cycle at 28.5°C. Fertilized eggs were obtained by mating adult fish soon after the light was turned on. Embryos were staged according to hours postfertilization (hpf) and morphological criteria. At our university, approval from the institutional committee for animal experiments is not necessary when fish are used as experimental animals.

Cell Culture and Reagents

HEK293, CHO, and MDA-MB-231 cells were grown according to instructions from The American Type Culture Collection (http://www.atcc.org). Wnt-3A was purchased from R&D Systems. Super 8x TOPFlash, Super 8x FOPFlash, and expression vectors for wild-type β-catenin-enhanced green fluorescent protein (EGFP) were obtained from Addgene. An expression vector for the β-catenin S191D-EGFP was generated by site-directed mutagenesis. Gene-specific siRNA oligonucleotides for human GGTase I β subunit (GGT1B) were purchased from Qiagen. Antisense morpholino oligonucleotide for GGT1B (ATG MO: 5′-AAT CCA CCG ACT CAA AAT CCG CCA T-3′ [Cao et al., 2009]; splicing MO: 5′-CAC GCG GTG TGT GGA CTC ACG GTC A-3′ [Eisa-Beygi et al., 2013]) were synthesized by Gene Tools. Pyrvinium pamoate and BIO (Meijer et al., 2003) were purchased from Sigma. ICG-001, XAV939, and IWR-1 were purchased from Tocris Bioscience. GGTI-286 and FTI-276 were purchased from Merck. Inhibitor kits 1 to 3 were provided by the Screening Committee of Anticancer Drugs (SCADS) in the Scientific Support Programs for Cancer Research Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Small Molecule Screening

Five zebrafish embryos at 4.5 hpf were arrayed in each well of round-bottomed 96-well plates in 50 μl of embryo medium (13.7 mM NaCl, 0.5 mM KCl, 25 μM Na_2HPO_4, 44 μM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$). Compounds (in DMSO) from the SCADS inhibitor kits were transferred into the wells with arrayed embryos at the 50% epiboly stage. Embryos were incubated in 50 μl of 40 μM test compounds at 28.5°C for approximately 30 min. At shield stage, 50 μl of 4 μM BIO was added to the wells containing embryos treated with the test compounds. Embryos were incubated in a humidified box at 28.5°C and were screened for rescue of BIO-induced eyeless phenotype at 30 hpf under a dissecting microscope.

Whole-Mount In Situ Hybridization

One-cell stage embryos were injected with 10 ng of control or GGT1B MOs, treated with 30 μM BIO or DMSO for 8 min at 32- to 64-cell stage, and incubated at 28.5°C until 50% epiboly. Whole-mount in situ hybridization was carried out essentially as described previously (Westerfield, 1995). Digoxigenin-labeled RNA probes were transcribed using RNA digoxigenin labeling mix, T7 RNA polymerase, and pBluescript goosecoid (Addgene) as a template plasmid. The central angles of *goosecoid* expression area were measured using ImageJ software.

Cell Fractionation

MDA-MB-231 cells were treated with GGTI-286 or DMSO for 18 hr. Cells were harvested in ice-cold PBS, resuspended in cell disruption buffer containing 10 mM HEPES (pH 7.0), 3.5 mM $MgCl_2$, 100 mM KCl, 3 mM NaCl, 1.25 mM EGTA, 1 mM NaF, 1 mM Na vanadate, and protease inhibitors and disrupted by sonication. Disrupted cells were centrifuged at 500 × *g* for 5 min to remove nuclei and fractionated into S100 and P100 fractions by centrifugation at 100,000 × *g* for 1 hr. S100 and P100 were treated as cytoplasmic and membrane fractions, respectively.

Nuclear Protein Extraction

HEK293 cells were grown in a six-well plate for 24 hr and then treated with GGTI-286 or DMSO for indicated periods. The cells were washed with ice-cold PBS and centrifuged. The cell pellet was resuspended in hypotonic buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1 mM NaF, 1 mM Na vanadate, and protease inhibitors and incubated on ice for 30 min. The cells were then lysed by stroking through 27G needles ten times. After centrifugation (16,000 × *g* for 5 min at 4°C), the supernatant was kept as a cytoplasmic fraction. The cell pellet was washed twice with the hypotonic buffer and resuspended in nuclear extracting buffer (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 1 mM NaF, 1 mM Na vanadate, and protease inhibitors). After incubation at 4°C for 30 min, the extraction mixture was centrifuged (16,000 × *g* at 4°C for 5 min), and the supernatant was isolated as a nuclear fraction.

Western Blotting

The following primary antibodies were used for Western blot: antibodies against GGT1B (1:1,000; Cell Signaling Technology), actin (1:1,000; Sigma), β-catenin (1:1,000; Sigma), cyclin D1 (1:1,000; Cell Signaling Technology), axin2 (1:1,000; Cell Signaling Technology), Rac1 (1:1,000; Millipore), sodium-potassium adenosine triphosphatase (Na,K-ATPase) α1 subunit (1:2,000; GeneTex), GFP (1:1,000; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated antimouse immunoglobulin G (IgG) or antirabbit IgG antibodies were used as secondary antibodies (1:10,000; Amersham Biosciences), and the ECL Plus Western Blotting Detection Kit (Amersham Biosciences) was used for detection.

Luciferase Reporter Assay

Luciferase reporter assays were carried out in six-well plates in the presence of 1% fetal bovine serum. DNA per well was as follows: Super 8x TOPFlash (firefly luciferase), 0.9 μg; pRL-CMV or pRL-SV40 (*Renilla* luciferase) as reference, 0.1 μg; and expression vectors for wild-type or S191D β-catenin, 1 μg. Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample, and then all results were normalized to negative controls. Data were expressed as mean ± SEM from at least three independent experiments.

Statistical Analysis

The error bars represent the SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.02.015.

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REFERENCES

Allal, C., Favre, G., Couderc, B., Salicio, S., Sixou, S., Hamilton, A.D., Sebti, S.M., Lajoie-Mazenc, I., and Pradines, A. (2000). RhoA prenylation is required for promotion of cell growth and transformation and cytoskeleton organization but not for induction of serum response element transcription. J. Biol. Chem. *275*, 31001–31008.

Atilla-Gokcumen, G.E., Williams, D.S., Bregman, H., Pagano, N., and Meggers, E. (2006). Organometallic compounds with biological activity: a very selective and highly potent cellular inhibitor for glycogen synthase kinase 3. Chembiochem *7*, 1443–1450.

Babu, P. (1977). Early developmental subdivisions of the wing disk in Drosophila. Mol. Gen. Genet. *151*, 289–294.

Barker, N., and Clevers, H. (2006). Mining the Wnt pathway for cancer therapeutics. Nat. Rev. Drug Discov. *5*, 997–1014.

Berndt, N., Hamilton, A.D., and Sebti, S.M. (2011). Targeting protein prenylation for cancer therapy. Nat. Rev. Cancer *11*, 775–791.

Cao, P., Hanai, J., Tanksale, P., Imamura, S., Sukhatme, V.P., and Lecker, S.H. (2009). Statin-induced muscle damage and atrogin-1 induction is the result of a geranylgeranylation defect. FASEB J. *23*, 2844–2854.

Chen, B., Dodge, M.E., Tang, W., Lu, J., Ma, Z., Fan, C.W., Wei, S., Hao, W., Kilgore, J., Williams, N.S., et al. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat. Chem. Biol. *5*, 100–107.

Ciani, L., and Salinas, P.C. (2005). WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nat. Rev. Neurosci. *6*, 351–362.

Cross, D.A.E., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785–789.

Cselenyi, C.S., Jernigan, K.K., Tahinci, E., Thorne, C.A., Lee, L.A., and Lee, E. (2008). LRP6 transduces a canonical Wnt signal independently of Axin degradation by inhibiting GSK3's phosphorylation of β-catenin. Proc. Natl. Acad. Sci. USA *105*, 8032–8037.

Dan, H.C., Jiang, K., Coppola, D., Hamilton, A., Nicosia, S.V., Sebti, S.M., and Cheng, J.Q. (2004). Phosphatidylinositol-3-OH kinase/AKT and survivin pathways as critical targets for geranylgeranyltransferase I inhibitor-induced apoptosis. Oncogene *23*, 706–715.

Eisa-Beygi, S., Hatch, G., Noble, S., Ekker, M., and Moon, T.W. (2013). The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) pathway regulates developmental cerebral-vascular stability via prenylation-dependent signalling pathway. Dev. Biol. *373*, 258–266.

Emami, K.H., Nguyen, C., Ma, H., Kim, D.H., Jeong, K.W., Eguchi, M., Moon, R.T., Teo, J.-L., Oh, S.W., Kim, H.Y., et al. (2004). A small molecule inhibitor of β-catenin/cyclic AMP response element-binding protein transcription. Proc. Natl. Acad. Sci. USA *101*, 12682–12687.

Fang, D., Hawke, D., Zheng, Y., Xia, Y., Meisenhelder, J., Nika, H., Mills, G.B., Kobayashi, R., Hunter, T., and Lu, Z. (2007). Phosphorylation of β-catenin by AKT promotes β-catenin transcriptional activity. J. Biol. Chem. *282*, 11221–11229.

Hartwell, L.H. (1991). Twenty-five years of cell cycle genetics. Genetics *129*, 975–980.

Heisenberg, C.P., Brand, M., Jiang, Y.J., Warga, R.M., Beuchle, D., van Eeden, F.J., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., et al. (1996). Genes involved in forebrain development in the zebrafish, Danio rerio. Development *123*, 191–203.

Heisenberg, C.P., Houart, C., Take-Uchi, M., Rauch, G.J., Young, N., Coutinho, P., Masai, I., Caneparo, L., Concha, M.L., Geisler, R., et al. (2001). A mutation in the Gsk3-binding domain of zebrafish Masterblind/Axin1 leads to a fate transformation of telencephalon and eyes to diencephalon. Genes Dev. *15*, 1427–1434.

Huang, S.M., Mishina, Y.M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G.A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., et al. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature *461*, 614–620.

Joyce, P.L., and Cox, A.D. (2003). Rac1 and Rac3 are targets for geranylgeranyltransferase I inhibitor-mediated inhibition of signaling, transformation, and membrane ruffling. Cancer Res. *63*, 7959–7967.

Karim, F.D., Chang, H.C., Therrien, M., Wassarman, D.A., Laverty, T., and Rubin, G.M. (1996). A screen for genes that function downstream of Ras1 during Drosophila eye development. Genetics *143*, 315–329.

Kazi, A., Carie, A., Blaskovich, M.A., Bucher, C., Thai, V., Moulder, S., Peng, H., Carrico, D., Pusateri, E., Pledger, W.J., et al. (2009). Blockade of protein geranylgeranylation inhibits Cdk2-dependent p27Kip1 phosphorylation on Thr187 and accumulates p27Kip1 in the nucleus: implications for breast cancer therapy. Mol. Cell. Biol. *29*, 2254–2263.

Kim, C.-H., Oda, T., Itoh, M., Jiang, D., Artinger, K.B., Chandrasekharappa, S.C., Driever, W., and Chitnis, A.B. (2000). Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. Nature *407*, 913–916.

Kinsella, B.T., Erdman, R.A., and Maltese, W.A. (1991). Carboxyl-terminal isoprenylation of ras-related GTP-binding proteins encoded by rac1, rac2, and ralA. J. Biol. Chem. *266*, 9786–9794.

Kinzler, K.W., Nilbert, M.C., Su, L.K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D., et al. (1991). Identification of FAP locus genes from chromosome 5q21. Science *253*, 661–665.

Klaus, A., and Birchmeier, W. (2008). Wnt signalling and its impact on development and cancer. Nat. Rev. Cancer *8*, 387–398.

Kofron, M., Birsoy, B., Houston, D., Tao, Q., Wylie, C., and Heasman, J. (2007). Wnt11/β-catenin signaling in both oocytes and early embryos acts through LRP6-mediated regulation of axin. Development *134*, 503–513.

Kuum, M., Veksler, V., Liiv, J., Ventura-Clapier, R., and Kaasik, A. (2012). Endoplasmic reticulum potassium-hydrogen exchanger and small conductance calcium-activated potassium channel activities are essential for ER calcium uptake in neurons and cardiomyocytes. J. Cell Sci. *125*, 625–633.

McMahon, A.P., and Moon, R.T. (1989). Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis. Cell *58*, 1075–1084.

Meijer, L., Skaltsounis, A.L., Magiatis, P., Polychronopoulos, P., Knockaert, M., Leost, M., Ryan, X.P., Vonica, C.A., Brivanlou, A., Dajani, R., et al. (2003). GSK-3-selective inhibitors derived from Tyrian purple indirubins. Chem. Biol. *10*, 1255–1266.

Metzstein, M.M., Stanfield, G.M., and Horvitz, H.R. (1998). Genetics of programmed cell death in C. elegans: past, present and future. Trends Genet. *14*, 410–416.

Nüsslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. Nature *287*, 795–801.

O'Dwyer, P.J., Gallagher, M., Nguyen, B., Waddell, M.J., and Chiorean, E.G. (2010). Phase I accelerated dose-escalating safety and pharmacokinetic (PK) study of GGTI-2418, a novel geranylgeranyltransferase I inhibitor in patients with refractory solid tumors. Ann. Oncol. *21*, ii42.

Park, J.-I., Venteicher, A.S., Hong, J.Y., Choi, J., Jun, S., Shkreli, M., Chang, W., Meng, Z., Cheung, P., Ji, H., et al. (2009). Telomerase modulates Wnt signalling by association with target gene chromatin. Nature *460*, 66–72.

Simon, M.A., Bowtell, D.D., Dodson, G.S., Laverty, T.R., and Rubin, G.M. (1991). Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell *67*, 701–716.

Simon, M.A., Dodson, G.S., and Rubin, G.M. (1993). An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to sevenless and Sos proteins in vitro. Cell *73*, 169–177.

Slusarski, D.C., Yang-Snyder, J., Busa, W.B., and Moon, R.T. (1997). Modulation of embryonic intracellular Ca2+ signaling by Wnt-5A. Dev. Biol. *182*, 114–120.

Stachel, S.E., Grunwald, D.J., and Myers, P.Z. (1993). Lithium perturbation and goosecoid expression identify a dorsal specification pathway in the pregastrula zebrafish. Development *117*, 1261–1274.

Thorne, C.A., Hanson, A.J., Schneider, J., Tahinci, E., Orton, D., Cselenyi, C.S., Jernigan, K.K., Meyers, K.C., Hang, B.I., Waterson, A.G., et al. (2010). Small-molecule inhibition of Wnt signaling through activation of casein kinase 1α. Nat. Chem. Biol. *6*, 829–836.

Tolwinski, N.S., Wehrli, M., Rives, A., Erdeniz, N., DiNardo, S., and Wieschaus, E. (2003). Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3β activity. Dev. Cell *4*, 407–418.

van de Water, S., van de Wetering, M., Joore, J., Esseling, J., Bink, R., Clevers, H., and Zivkovic, D. (2001). Ectopic Wnt signal determines the eyeless phenotype of zebrafish masterblind mutant. Development *128*, 3877–3888.

Veeman, M.T., Slusarski, D.C., Kaykas, A., Louie, S.H., and Moon, R.T. (2003). Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr. Biol. *13*, 680–685.

Westerfield, M. (1995). The Zebrafish Book. (Eugene, Oregon: University of Oregon).

Wilson, S.W., and Houart, C. (2004). Early steps in the development of the forebrain. Dev. Cell *6*, 167–181.

Wu, X., Tu, X., Joeng, K.S., Hilton, M.J., Williams, D.A., and Long, F. (2008). Rac1 activation controls nuclear localization of β-catenin during canonical Wnt signaling. Cell *133*, 340–353.

Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S., and Kikuchi, A. (1999). Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3β regulates its stability. J. Biol. Chem. *274*, 10681–10684.

Yoshida, Y., Kawata, M., Katayama, M., Horiuchi, H., Kita, Y., and Takai, Y. (1991). A geranylgeranyltransferase for rhoA p21 distinct from the farnesyltransferase for ras p21S. Biochem. Biophys. Res. Commun. *175*, 720–728.

Zhang, F.L., and Casey, P.J. (1996). Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. *65*, 241–269.

Zhu, G., Wang, Y., Huang, B., Liang, J., Ding, Y., Xu, A., and Wu, W. (2012). A Rac1/PAK1 cascade controls β-catenin activation in colon cancer cells. Oncogene *31*, 1001–1012.

Zon, L.I., and Peterson, R.T. (2005). In vivo drug discovery in the zebrafish. Nat. Rev. Drug Discov. *4*, 35–44.

