Chapter 1
Zebrafish Wnt/β-Catenin Signaling Reporters Facilitate Understanding of In Vivo Dynamic Regulation and Discovery of Therapeutic Agents

Tohru Ishitani and Juqi Zou

Abstract Wnt/β-catenin signaling plays multiple roles in embryogenesis, organogenesis, and adult tissue homeostasis, and its dysregulation is linked to numerous human diseases such as cancer. Although strict spatiotemporal regulation must support the multi-functionality of Wnt/β-catenin signaling, detailed mechanisms remain unclear. In addition, Wnt/β-catenin signaling is a potential drug target candidate and several inhibitors have been identified by in vitro screening, but none have yet been incorporated into clinical practice. Recent studies using reporter zebrafish lines have gradually improved our understanding of in vivo dynamic regulation of Wnt/β-catenin signaling and have facilitated the discovery of new chemicals that can reduce Wnt/β-catenin signaling and cancer cell viability with few side effects. Here, we describe several new mechanisms supporting the spatiotemporal regulation of Wnt/β-catenin signaling and new small molecule inhibitors, discovered using zebrafish reporters. In addition, we discuss the potential of zebrafish signaling reporters in both developmental biology and pharmaceutical sciences.

Keywords Wnt/β-catenin signaling · reporter · modifier · chemical inhibitor · anti-cancer drug

1.1 Introduction

Wnt/β-catenin signaling is an evolutionarily conserved system that controls cell proliferation, fate specification, differentiation, survival, and death during embryogenesis, organogenesis, and adult tissue homeostasis (Clevers 2006; Clevers and Nusse 2012; Logan and Nusse 2004). Dysregulation of this signaling system is

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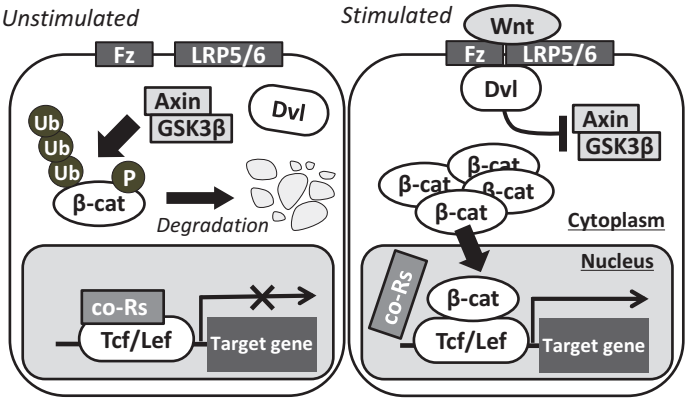

Fig. 1.1 The Wnt/β-catenin signaling pathway. In unstimulated conditions, levels of cytoplasmic β-catenin are kept low by a destruction complex including GSK3β and Axin. Tcf/Lef represses the expression of target genes by interacting with transcriptional co-repressors (co-Rs). Binding of Wnt to the receptor Frizzled (Fz) and its co-receptor LRP5/6 activates Dvl, and then Dvl promotes dissociation of the β-catenin destruction complex and consequently stabilizes cytoplasmic β-catenin. As a result, β-catenin accumulates and enters the nucleus, where it forms complexes with Tcf/Lef that activate gene expression. *Ub* ubiquitin, *P* phosphorylation, *β-cat* β-catenin

linked to various human diseases, including cancer, obesity, diabetes, osteoporosis, schizophrenia, and autism (Clevers 2006; Clevers and Nusse 2012; De Ferrari and Moon 2006; Logan and Nusse 2004). This system transduces its signal by controlling the levels of cytoplasmic β-catenin protein (Fig. 1.1). In unstimulated cells, cytoplasmic β-catenin are maintained at low levels by a destruction complex that includes glycogen synthase kinase 3β (GSK3β) and Axin. GSK3β phosphorylates β-catenin at the N-terminal region, thereby promoting its ubiquitination by the E3 Ub ligase β-TrCP and the subsequent proteasomal degradation (Clevers 2006; Clevers and Nusse 2012; Logan and Nusse 2004). The Tcf/Lef family of transcription factors represses the expression of Wnt/β-catenin target genes by interacting with transcriptional co-repressors, such as histone deacetylase 1 (HDAC1) and Groucho (Arce et al. 2009). Wnt/β-catenin signaling is activated when the secreted glycoprotein Wnt binds to the cell surface receptor Frizzled (Fz) and its co-receptor LRP5/6. This Wnt-bound receptor complex then recruits the cytoplasmic protein Dishevelled (Dvl), which in turn promotes the dissociation of the β-catenin degradation complex. This series of events results in the stabilization of cytoplasmic β-catenin. The increased β-catenin concentration drives its migration into the nucleus where it forms complexes with Tcf/Lef, which activates gene expression. This core Wnt/β-catenin signaling process has been revealed through extensive investigation using invertebrate models, mammalian cell culture, and *Xenopus* early embryos over the past three to four decades. In addition, knockout mouse analyses have contributed to our understanding of the role Wnt/β-catenin signaling plays in animal development and disease. However, the spatiotemporal dynamics of Wnt/β--catenin signaling and its regulatory mechanisms in living animals remains unclear though recent studies using Wnt/β-catenin signaling reporter zebrafish lines have

gradually improved our understanding. Here, we introduce Wnt/β-catenin signaling reporter zebrafish lines and review several studies in which new mechanisms supporting the spatiotemporal regulation of Wnt/β-catenin signaling were revealed using these reporter lines. Furthermore, we also demonstrate how reporter lines may be useful in the exploration of new anti-cancer drugs.

1.2 Wnt/β-Catenin Signaling Reporter Zebrafish Lines and Their Properties

Zebrafish are one of the most suitable animals for live imaging because of their optical clarity and rapid development. A transgenic zebrafish line carrying the Wnt/β--catenin signaling reporter top:GFP (original name: TOPdGFP)—which contains four copies of consensus Tcf/Lef binding sites, a *c-fos* minimal promoter, and a d2EGFP reporter gene (Fig. 1.2; Dorsky et al. 2002)—has proved to be a useful tool for understanding the regulation of in vivo Wnt/β-catenin signaling. In fact, new domains in which Wnt/β-catenin signaling is activated and new mechanisms that regulate Wnt/β-catenin signaling have been discovered using this reporter (some of which are described in Sect. 1.3). However, given that fine reporter activity has only been observed in some Wnt/β-catenin signaling-active sites in living top:GFP-transgenic fish using a fluorescence stereomicroscope (Fig. 1.2), attempts have been made to improve Wnt/β-catenin signaling reporters. We generated OTM:d2EGFP (original name: Tcf/Lef-miniP:dGFP), which comprises a destabilized green fluorescent protein (d2EGFP) driven by a promoter containing six copies of Tcf/Lef binding sites and a minimal promoter (miniP) derived from Promega pGL4 (Fig. 1.2; Shimizu et al. 2012). Moro et al. (2012) also generated two new reporters, 7xTCF-Xla.Siam:GFP and 7xTCF-Xla.Siam:nlsmCherry, which express GFP or monomeric Cherry proteins with nuclear localization signals (nlsmCherry) under the control of seven multimerized TCF responsive elements upstream of the minimal promoter of the *Xenopus* direct β-catenin target gene, *siamois* (Fig. 1.2). Results showed that both reporters were activated in almost all cells in which Wnt/β-catenin signaling is known to be active in zebrafish, and they also revealed further new developmental roles of Wnt/β-catenin signaling, including in the formation of the brain-blood vessel network and gill filaments (Moro et al. 2012; Shimizu et al. 2012). These reporters are useful but should be used separately. 7xTCF-Xla.Siam:GFP and 7xTCF-Xla.Siam:nlsmCherry are suitable for searching for new Wnt/β-catenin signaling-active cells/areas because they express fluorescence strongly; the half-lives of GFP and mCherry are very long (more than 24 h), so fluorescence of 7xTCF-Xla.Siam:GFP and 7xTCF-Xla.Siam:nlsmCherry can be detected in cells where Wnt/β-catenin signaling was activated in the past. On the other hand, OTM:d2EGFP is suitable for the detection of dynamic changes during Wnt/β-catenin signaling in vivo as the half-life of the d2EGFP protein, the fluorescent reporter in OTM:d2EGFP, is relatively short (approximately 2 h). In addition, to detect “highly” dynamic signaling changes, we recently generated “the third

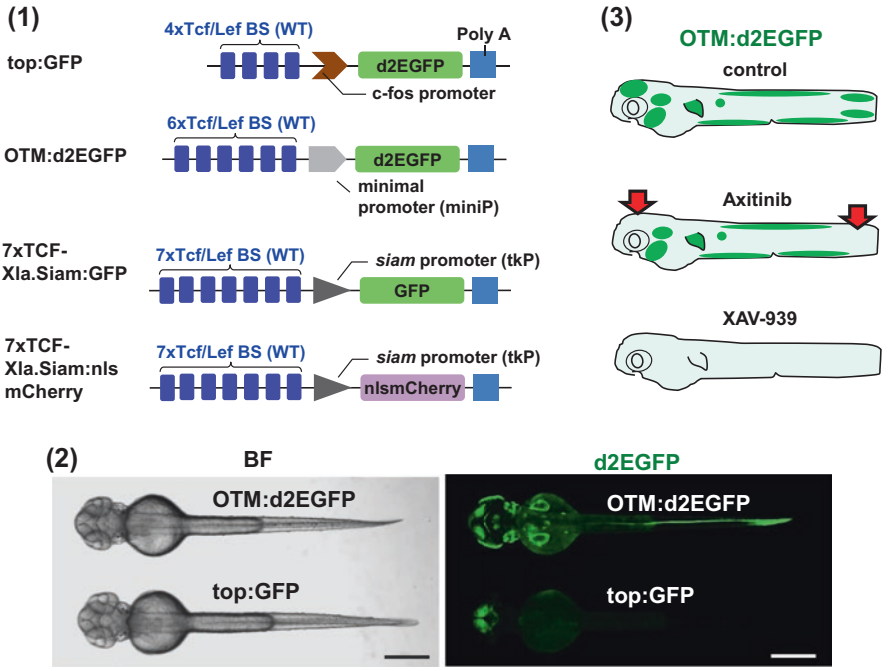

Fig. 1.2 Zebrafish Wnt/β-catenin signaling reporters. (1) Schematic diagrams of Wnt/β-catenin signaling-reporter constructs. Tcf/Lef BS: consensus sequence of the Tcf/Lef-binding site; PolyA: polyadenylation sequence. (2) Comparison of reporter activity in OTM:d2EGFP- and top:GFP transgenic zebrafish embryos. Dorsal views of transgenic embryos, with the anterior side to the left. Cells expressing d2EGFP were visualized by fluorescence microscopy (right panel). Bright-field (BF) images are shown in the left panel. Scale bar, 200 μm. (3) Effects of chemical inhibitors on OTM:d2EGFP reporters. Reporter activity was shown as green. Axitinib reduces OTM:d2EGFP expression in the midbrain and tail (red arrows), while XAV-939 completely blocks it in whole embryos

generation of *in vivo* Wnt/β-catenin signaling reporter"—OTM:Eluc-CP—which expresses destabilized emerald luciferase in response to Wnt/β-catenin activation. Using this, we can detect the dynamic change of Wnt/β-catenin signaling activity during brain anterior-posterior patterning (Akieda et al. in preparation). OTM:Eluc-CP will facilitate rigorous analysis of the spatiotemporal dynamics of Wnt/β-catenin signaling.

1.3 Studies Using the Reporter Zebrafish Revealed New Regulatory Mechanisms of Wnt/β-Catenin Signaling

Mechanisms supporting the spatiotemporal dynamics of Wnt/β-catenin signaling in living animals remain unclear, but recent studies with reporter zebrafish, described within this section, have gradually improved our understanding.

1.3.1 Reck: A New Wnt Regulator in Plasma Membrane

The G protein-coupled receptor Gpr124 promotes Wnt7-mediated β-catenin signaling and controls central nervous system (CNS) angiogenesis (Posokhova et al. 2015; Zhou and Nathans 2014). Vanhollebeke et al. (2015) discovered that Reck (reversion-inducing-cysteine-rich protein with Kazal motifs), a GPI-anchored extracellular protein, cooperates with Gpr124 to activate Wnt/β-catenin signaling in zebrafish CNS angiogenesis. Knockdown of Gpr124 and Reck using morpholino antisense oligonucleotides (MO) reduced 7xTCF-Xla.Siam: GFP reporter activity in zebrafish CNS endothelial cells and induced identical CNS-specific vascular defects. Reck interacted with Gpr124 in the plasma membrane and synergistically promoted Wnt7-mediated β-catenin signaling. In addition, live imaging analyses of genetically mosaic zebrafish revealed that Gpr124- and Reck-dependent Wnt/β--catenin signaling is specifically required for endothelial tip cells during sprouting angiogenesis in the CNS. Interestingly, knockdown of Reck and Gpr124 specifically affected CNS angiogenesis but did not produce gross morphological phenotypes, indicating that Gpr124 and Reck are Wnt/β-catenin signaling modulators specifically required for CNS angiogenesis.

1.3.2 Hipk2 and Nephrocystin-4: New Dvl Modulators

Homeodomain-interacting protein kinase 2 (Hipk2) was identified as a positive regulator of Wnt/β-catenin signaling using biochemistry and *Drosophila* genetics (Lee et al. 2009). However, the mechanism by which Hipk2 promotes Wnt/β-catenin signaling and its physiological significance was unclear but is explained by our recent study using OTM:d2EGFP. MO-mediated knockdown of Hipk2 reduced OTM:d2EGFP activity and the protein levels of Dvl, which is a core regulator of Wnt/β-catenin signaling. Consequently, Wnt/β-catenin signaling-mediated brain anterior-posterior (AP) patterning and tail development in zebrafish embryos were disturbed. Interestingly, these defects were rescued by injection of Hipk2 wild-type or kinase-negative mutant mRNA (Shimizu et al. 2014), suggesting that Hipk2 promotes Dvl protein levels and Wnt/β-catenin signaling in a kinase activity-independent manner, and this regulation contributes to brain and tail development. Biochemical analyses revealed that Hipk2 promotes the binding of protein phosphatase 1c (PP1c) to Dvl and thus the consequent dephosphorylation of Dvl, which prevents Itch ubiquitin E3 ligase-mediated Dvl ubiquitination and degradation to stabilize Dvl (Fig. 1.3; Shimizu et al. 2014). Consistent with this, knockdown of PP1c also reduced OTM:d2EGFP activity; Hipk2 MO-induced reduction of OTM:d2EGFP activity was rescued by injection of the Dvl 3A mutant, in which PP1c-dephosphorylation sites were substituted to alanine (Shimizu et al. 2014). Thus, a new post-translational modification of Dvl and its roles in Wnt signal transduction and embryogenesis were revealed by the reporter zebrafish studies.

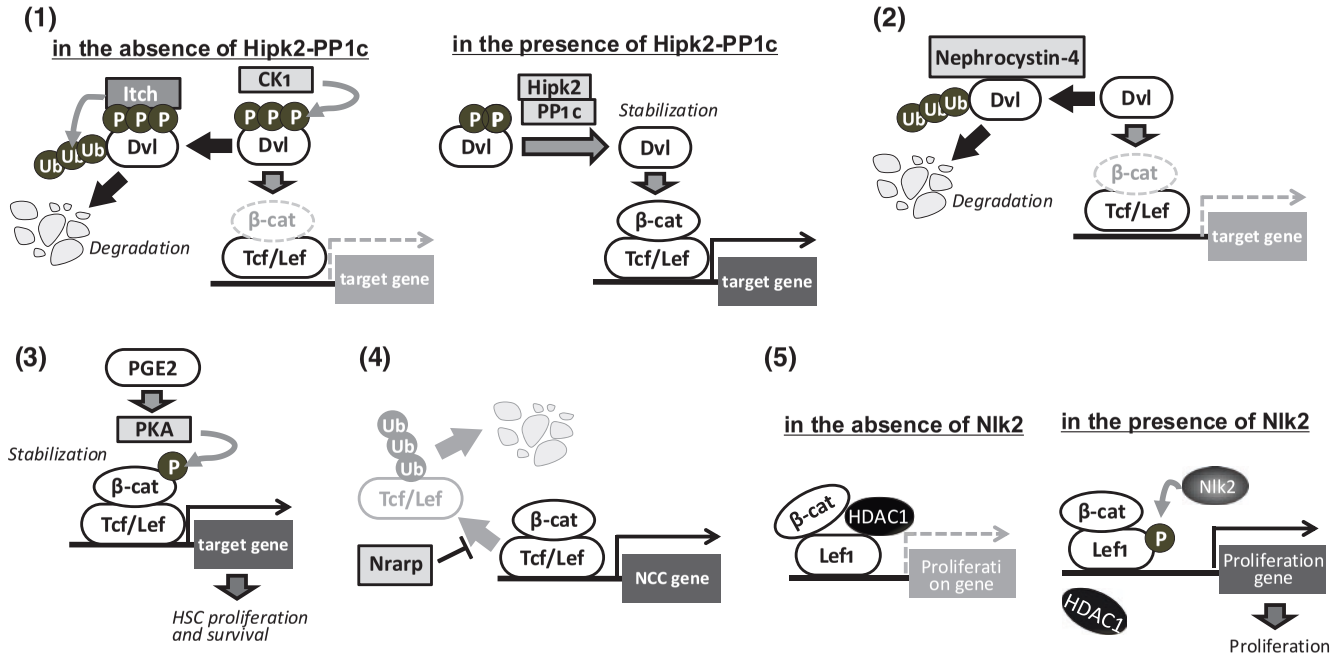

Fig. 1.3 New regulatory mechanisms of Wnt/β-catenin signaling revealed using the reporter zebrafish. (1) In the absence of Hipk2-PP1c activity, Dvl is phosphorylated by CK1 and consequently ubiquitinated by Itch and then degraded via proteasome. As a result, the Wnt/β-catenin target gene is inactivated. In the presence of Hipk2-PP1c activity, Hipk2 promotes the binding of PP1c to Dvl and PP1c-mediated Dvl dephosphorylation and consequently stabilizes Dvl. Stabilized Dvl efficiently transduces Wnt signaling and induces target gene expression. (2) Nephrocystin-4 binds to and promotes the ubiquitination and degradation of Dvl. (3) PGE2 promotes PKA-mediated phosphorylation and stabilization of β-catenin and thereby stimulates HSC proliferation and survival. (4) Nrarp blocks the ubiquitination of Lef1 and consequently promotes Lef1 stabilization and Lef1-mediated Wnt/β-catenin signaling. (5) In the absence of Nlk2 activity, HDAC1 binds to Lef1 and inhibits Lef1-mediated transcription. When Nlk2 is activated, Nlk2 phosphorylates Lef1 thereby preventing HDAC1-mediated Lef1 inhibition and allowing NPCs to proliferate

The *NPHP4* gene encoding nephrocystin-4 is associated with nephronophthisis, which is a hereditary nephropathy characterized by interstitial fibrosis and cyst formation. Burckle et al. (2011) examined the molecular and cellular functions of Nephrocystin-4 using zebrafish as a model. Injection of Nephrocystin-4 MO enhanced top:GFP activity in the pronephric duct and produced pronephric cysts; Nephrocystin-4 MO-mediated pronephric cyst formation was enhanced by co-injection with the MO against prickle2 gene encoding a negative regulator of Dvl. In the mammalian kidney cell line (MDCK), exogenous Nephrocystin bound to Dvl and reduced its protein level and Wnt/β-catenin reporter activity (Burckle et al. 2011). Based on these findings, it was concluded that Nephrocystin-4 represses the Wnt-β-catenin pathway via Dvl degradation (Fig. 1.3) and contributes to morphogenesis of zebrafish pronephros.

1.3.3 Simplet and PGE-PKA Axis: New β-Catenin Modulators

Simplet was first isolated as a gene expressed in the developing CNS of medaka fish (Deyts et al. 2005) although molecular function is unclear. Using reporter fish, Kizil et al. (2014) showed that Simplet is required for Wnt/β-catenin signaling as it positively regulates β-catenin nuclear localization; injection of *simplet* MO prevented nuclear accumulation of β-catenin. Activation of top:GFP and 7xTCF-Xla.Siam:nlsmCherry activities and expression of Wnt/β-catenin target genes, *cdx4*, *tbx6*, and *gbx1*, were also prevented in the tail bud stage of zebrafish embryos but not in 85% epiboly stage embryos; loss of Wnt/β-catenin signaling also produced axial defects (Kizil et al. 2014). Simplet localized into the nucleus. Overexpression of Simplet promoted β-catenin nuclear localization but not in nuclear-localization-signal (NLS)-deleted mutant SimpletΔNLS positive cells; overexpression of this mutant blocked β-catenin nuclear localization and transcriptional activity. Biochemical analyses revealed that Simplet proteins interact directly with β-catenin (Kizil et al. 2014). These results suggest that Simplet associates with β-catenin to promote its nuclear localization and transcriptional activity and plays an essential role in zebrafish axial development.

Wnt/β-catenin signaling has been implicated in the regulation of hematopoietic stem cells (HSCs); the bioactive lipid prostaglandin E2 (PGE2) also regulates the induction and engraftment of HSCs (Reya et al. 2003; Trowbridge et al. 2006). Therefore, Goessling et al. (2009) focused on the relationship between PGE2 and HSCs and discovered that PGE2 promotes Wnt/β-catenin signaling in HSC regulation. Treatment of zebrafish embryos with PGE2 enhanced top:GFP activity in HSCs and Wnt/β-catenin signaling-mediated HSC proliferation and survival, while treatment with indomethacin, which suppresses PGE2 production, reduced them. This suggests that PGE2 positively regulates Wnt/β-catenin signaling in HSCs. Furthermore, treatment with the PKA chemical activator forskolin reversed indomethacin-induced HSC reduction, while treatment with the PKA chemical inhibitor H89 blocked PGE2-induced HSC formation, suggesting that PGE2 regu-

lates HSCs via PKA. By carrying out assays using hematopoietic mouse ES cells, Goessling et al. (2009) confirmed that the relationship between PGE2, PKA, and Wnt/β-catenin signaling is also conserved in mammals. In addition, it was found that PGE treatment promoted the phosphorylation of β-catenin at Ser-675, which is mediated by PKA, and stabilizes β-catenin in mouse ES cells; indomethacin reduced this phosphorylation and β-catenin protein levels (Goessling et al. 2009). Thus, PGE2-PKA axis stimulates β-catenin stability and the consequent Wnt/β-catenin signaling activity through β-catenin phosphorylation to promote HSC formation (Fig. 1.3).

1.3.4 Nrarp and NLK: New Tcf/Lef Modifiers

We discovered the essential roles of Lef1 post-translational modification in embryogenesis using top:GFP reporter zebrafish and showed that proper control of Lef1 ubiquitination is involved in the development of neural crest cells (NCCs) (Fig. 1.3; Ishitani et al. 2005). NCCs are pluripotent progenitors induced within the neuroepithelium in vertebrate embryos. They migrate to target destinations throughout the embryo and differentiate into diverse cell types, including sensory neurons, glia, smooth muscle, cranial cartilage, bone cells, endocrine cells, and pigment cells. Wnt/β-catenin signaling is known to regulate induction, migration, and differentiation of NCCs (Yanfeng et al. 2003) and furthermore, Ishitani et al. (2005) found that Nrarp (Notch-regulated ankyrin repeat protein), a small protein containing two ankyrin repeats, promotes Wnt/β-catenin signaling activity in NCCs by blocking Lef1 ubiquitination. Nrarp was expressed in migrating neural crest cells; Nrarp knockdown, using MO, reduced top:GFP reporter activity in migrating NCCs and induced defects in NCC migration and differentiation (Ishitani et al. 2005), which suggests that Nrarp contributes to NCC development through positive regulation of Wnt/β-catenin signaling in migrating NCCs. Biochemical analyses also showed that Nrarp blocks the ubiquitination-proteasome-dependent degradation of Lef1 and consequently stabilizes it, which promotes Wnt/β-catenin signaling in migrating NCCs (Ishitani et al. 2005). This was the first discovery of ubiquitination of Tcf/Lef family proteins.

Lef1 phosphorylation is also essential for midbrain development (Fig. 1.3). Previous reports showed that MAP kinase-related nemo-like kinase (NLK) phosphorylates the Tcf/Lef family of transcription factors and activates Wnt/β-catenin signaling in *Caenorhabditis elegans* (Ishitani et al. 1999; Meneghini et al. 1999) though the physiological role of NLK-mediated Tcf/Lef regulation in vertebrates was not explained. We found that knockdown of zebrafish NLK (Nlk2) reduced top:GFP reporter activity and proliferation of neural progenitor cells in the developing midbrain, without gross morphological defects (Ota et al. 2012). This suggests that Nlk2 acts as a midbrain-specific Wnt/β-catenin activator and promotes cell proliferation in the midbrain of zebrafish. Biochemical studies revealed that Nlk2 phosphorylates Lef1 at the conserved Thr residue, which promotes Lef1 tran-

scriptional activity by blocking the interaction of Lef1 with HDAC1(Ota et al. 2012). Consistent with this finding, the Nlk2 knockdown-induced reduction in top:GFP activity was reversed by co-knockdown of HDAC1 (Ota et al. 2012). Thus, the midbrain-specific regulation of Wnt/β-catenin signaling was revealed using reporter fish.

It is noteworthy that inhibition of Reck, Gpr124, Nephrocystin-4, PGE2, Nrarp, and/or Nlk2 induces defects in specific tissues, which suggests they are cell/tissue type-specific Wnt/β-catenin signaling modifiers, but not general Wnt/β-catenin signaling regulators. Such cell/tissue type-specific modifiers should support the spatiotemporal dynamics of Wnt/β-catenin signaling, which enables Wnt/β-catenin signaling to play diverse roles in animal development and homeostasis.

1.4 Utility of the Wnt/β-Catenin Signaling Reporter Zebrafish for Drug Discovery

Wnt/β-catenin signaling regulates stem cell fates, and dysregulation of Wnt/β-catenin signaling causes various human diseases, including cancer, mental disorders, osteoporosis, and obesity. Therefore, chemical inhibitors against Wnt/β-catenin signaling have potential as regenerative medicines and therapeutic agents; several chemical inhibitors of Wnt/β-catenin signaling, such as XAV-939, IWR-1, IWP-2, and ICG-001, have already been identified. XAV-939 and IWR-1 reduce β-catenin protein levels by promoting Axin protein stabilization, and IWP-2 also blocks Wnt secretion (Chen et al. 2009). ICG-001 blocks β-catenin binding to a histone acetyltransferase CREB-binding protein (CBP) and consequently prevents CREB-mediated β-catenin activation (Teo et al. 2005). Importantly, treatment with these chemical inhibitors eliminates not only Wnt/β-catenin signaling activity in mammalian cells but also the activities of Wnt/β-catenin signaling reporters (7xTCF-Xla.Siam:GFP and OTM:d2EGFP) in zebrafish (Moro et al. 2012; Shimizu et al. 2012). Therefore, reporter fish were used for identification of new chemicals that can inhibit Wnt/β-catenin signaling in vivo. In this section, we describe three chemicals, the in vivo activities of which were characterized using Wnt/β-catenin signaling reporter fish.

1.4.1 9-Hydroxycanthin-6-one Promotes β-Catenin Degradation by Activating GSK3β

Ohishi et al. (2015) screened plant extracts using reporter assays in HEK293 cells and identified the β-carboline alkaloid 9-hydroxycanthin-6-one as a new Wnt/β-catenin inhibitor. Although the direct target molecules of 9-hydroxycanthin-6-one

remain unclear, treatment with 9-hydroxycanthin-6-one activated GSK3β-mediated phosphorylation and the consequent degradation of β-catenin. To confirm in vivo activity of this inhibitor, Ohishi et al. (2015) used top:GFP Wnt/β-catenin reporter fish. Treatment with 9-hydroxycanthin-6-one reduced top:GFP activity and expression of endogenous Wnt/β-catenin target genes, including *mitf* and *zic2a*, and partially rescued the eyeless phenotype induced by treatment with BIO, a GSK3β specific inhibitor (Ohishi et al. 2015), which suggests that this inhibitor can block Wnt/β-catenin signaling via GSK3β regulation in vivo.

1.4.2 PMED-1 Blocks β-Catenin Binding to CREB

Because XAV-939, IWR-1, and 9-hydroxycanthin-6-one affect the activity of the Wnt/β-catenin core signaling system, which contributes to the homeostasis of various tissues, these inhibitors may not only affect abnormal tissues but may also damage healthy ones. In contrast, pharmacological inhibition of the cell type-specific modulators may enable cell type-specific Wnt/β-catenin signaling regulation and contribute to disease treatment with few side effects. Two histone acetyltransferases, CREB and p300, interact with β-catenin to activate β-catenin-mediated transcription although the binding of each results in distinct effects. CBP-β-catenin complexes positively regulate the expression of genes promoting cell proliferation while p300-β-catenin complexes are not involved in cell proliferation (Teo and Kahn 2010). Interestingly, ICG-001 specifically inhibits β-catenin binding to CBP, but not to p300, and blocks β-catenin-mediated cell proliferation. In addition, ICG-001 is selectively cytotoxic to colon carcinoma cells because treatment with ICG-001 kills SW480 and HCT116 colon cancer cells, while it has no effect on CCD-841Co normal colonic epithelial cells (Teo et al. 2005). Therefore, ICG-001 is thought to be usable for cancer treatment with few side effects. Delgado et al. (2014) searched for chemicals that possess similar activity to ICG-001 by using in silico analysis and zebrafish reporter assays. To identify compounds structurally similar to ICG-001, they screened the ZINC 10 database (http://zinc.docking.org/subsets/lead-like) and identified PMED-1 as the lead compound, with ≥70% similarity to ICG-001. Similar to ICG-001, PMED-1 blocked the interaction of β-catenin with CBP, but not with p300, and reduced the viability of hepatocellular carcinoma (HCC) cells; it has no toxicity in human normal hepatocytes (Fig. 1.4; Delgado et al. 2014). Results also showed that PMED-1 can block Wnt/β-catenin signaling in vivo using OTM:d2EGFP reporter zebrafish. Interestingly, the OTM:d2EGFP activity in PMED-1-treated zebrafish embryos was strongly inhibited from 5 to 15 h after treatment but restored after 24 h; OTM:d2EGFP activity still continued to be suppressed 24 h after treatment in XAV939-treated zebrafish embryos, which indicates that the half-life of Wnt/β-catenin inhibitory activity of PMED-1 is shorter than that of XAV939. Thus, it is possible to evaluate the effect on Wnt/β-catenin signaling activity and its duration in vivo of a new Wnt/β-catenin inhibitor using reporter fish.

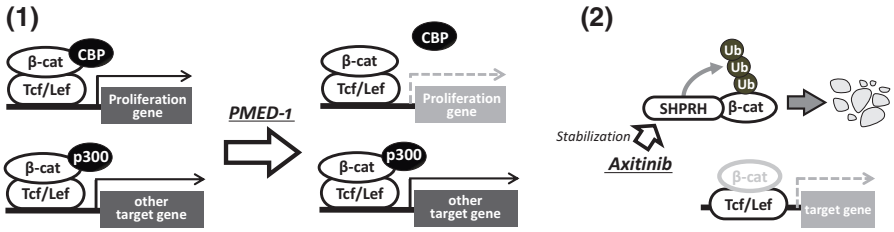

Fig. 1.4 Chemical inhibitors against Wnt/β-catenin signaling, which were characterized by reporter fish analyses. (1) PMED-1 blocked the interaction of β-catenin with CBP but not with p300. (2) Axitinib binds to and stabilizes the E3 ubiquitin ligase, SHPRH. Axitinib-stabilized SHPRH promoted the ubiquitination and degradation of nuclear β-catenin

1.4.3 Axitinib Promotes β-Catenin Degradation in Nucleus

Most Wnt/β-catenin pathway mutations in cancer patients are observed in the β-catenin gene and the APC gene, which encodes a component of the β-catenin destruction complex. Therefore, it is important to develop drugs that target downstream of the destruction complex. Recently, Qu et al. (2016) identified axitinib as such a drug; 460 Food and Drug Administration (FDA)-approved drugs were screened to find chemicals capable of inhibiting Wnt/β-catenin signaling activation induced by treatment with BIO, a GSK3β specific inhibitor, in HEK293 cells. Qu et al. (2016) also confirmed that axitinib inhibits in vivo Wnt/β-catenin signaling in OTM:d2EGFP zebrafish. Interestingly, axitinib reduced OTM:d2EGFP activity in the developing midbrain and tail but not in the developing ear, lateral line primordia, pectoral fin, fin fold, or cranial NCCs (Fig. 1.2; Qu et al. 2016); XAV-939 completely eliminated OTM:d2EGFP activity in the whole body (Fig. 1.2; Shimizu et al. 2012). Results suggest that axitinib inhibits Wnt/β-catenin signaling in specific cells but not in all cells. Consistent with this idea, axitinib reduced the activities of Wnt/β-catenin signaling and proliferation in colon cancer cells but not in normal intestinal tissues (Qu et al. 2016), indicating that axitinib may be usable for colon cancer treatment with few side effects. Furthermore, biochemical analyses revealed that axitinib binds to and stabilizes the E3 ubiquitin ligase SHPRH (SNF2, histone-linker, PHD and RING finger domain-containing helicase). Axitinib-stabilized SHPRH promoted the ubiquitination and degradation of nuclear β-catenin, which was independent of the β-catenin destruction complex including APC and GSK3β (Fig. 1.4; Qu et al. 2016). Thus, a new Wnt/β-catenin signaling inhibitor and its mechanism of action were elucidated.

1.5 Conclusions

Numerous molecules that regulate Wnt/β-catenin signaling have been discovered previously using invertebrate models, mammalian cell culture, and *Xenopus* early embryos. It was believed that most were “general regulators” that participate in the

control of Wnt/β-catenin signaling in all cells/tissues, but their physiological roles in vertebrates were unclear. However, recent studies using reporter zebrafish lines have revealed cell/tissue-type specific Wnt/β-catenin signaling modifiers, such as Reck, Gpr124, Nephrocystin-4, Nrarp, and Nlk2, which must complicate the spatio-temporal pattern of Wnt/β-catenin signaling activity in order to play multiple roles in animal development and homeostasis. It is also possible that parts of previously identified regulators may also be cell/tissue-type-specific modifiers. Future studies on previously and newly identified Wnt regulators using reporter fish will facilitate further understanding of cell/tissue type-specific Wnt/β-catenin signaling regulation and thereby make clear the whole picture of Wnt/β-catenin signaling regulation in living animals.

Reporter zebrafish lines will also help the discovery of new anti-cancer drugs that have few side effects. The chemicals that control the activity of Wnt/β-catenin core signaling systems may affect the homeostasis of healthy tissues, while chemical inhibitors against cell/tissue-specific modulators may enable cancer tissue-specific regulation. It is worth noting that axitinib inhibits OTM:d2EGFP reporter activity in a part of Wnt/β-catenin-active cells in zebrafish embryos and also reduces the activity of Wnt/β-catenin signaling and proliferation in colon cancer cells but not in normal intestinal tissues (Qu et al. 2016). This indicates that axitinib acts as a Wnt/β-catenin inhibitor in specific cells and may be able to reduce colon cancer cell activity without causing severe side effects. It also suggests that such cell/tissue-specific reporter inhibition could be used as an index for safe Wnt/β-catenin inhibitors that can be employed for cancer therapy in anti-cancer drug screening.

In addition to Wnt/β-catenin signaling, other cell signaling pathways, including TGF-β/BMP and Shh, are activated repeatedly and play multiple roles in animal development and homeostasis, and dysregulation of these pathways is involved in tumorigenesis. In addition, reporter fish lines that visualize various signaling pathways have been generated (Casari et al. 2014; Laux et al. 2011; Schwend et al. 2010). Therefore, a similar strategy can be implemented to investigate the in vivo regulatory mechanisms of other cell signaling pathways and their control agents. Thus, cell signaling reporter zebrafish are a useful tool for both investigating the mechanisms of dynamic signaling regulation and for identifying new drugs controlling particular signaling pathways in specific cells/tissues.

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