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Thr160 of Axin1 is critical for the formation and function of the β-catenin destruction complex

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ABSTRACT

Upon binding of a Wnt ligand to the frizzled (FZD)-low density lipoprotein receptor related protein 5/6 (LRP5/6) receptor complex, the β-catenin destruction complex, composed of Axin1, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1), is immediately inactivated, which causes β-catenin stabilization. However, the molecular mechanism of signal transduction from the receptor complex to the β-catenin destruction complex is controversial. Here we show that Wnt3a treatment promotes the dissociation of the Axin1-APC complex in glioblastoma cells cultured in serum-free medium. Experiments with the GSK3 inhibitor BIO suggest that Axin1-APC dissociation was controlled by phosphorylation. Introduction of a phosphomimetic mutation into Thr160 of Axin1, located in the APC-binding region RGS, abrogated the interaction of Axin1 with APC. Consistent with these observations, the Axin1 phosphomimetic mutant lost the ability to reduce β-catenin stability and to repress β-catenin/TCF-dependent transcription. Taken together, our results suggest a novel mechanism of Wnt signaling through the dissociation of the β-catenin destruction complex by Axin1 Thr160 modification.

1. Introduction

The Wnt signaling pathway plays a pivotal role in embryonic development, stem cell maintenance, and human disease [1]. The Wnt signaling pathway mainly regulates the stability of β-catenin, a key mediator of the Wnt cascade. β-catenin was first identified as a structural component of the cell–cell adhesion complex that binds directly to the cadherin adhesion molecule [2]. However, β-catenin can also enter the nucleus, bind to the TCF/LEF transcription factors and activate the Wnt transcriptional program. In resting cells, cytosolic β-catenin is captured by the β-catenin destruction complex which consists of Axin1, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) [3]. In this complex, β-catenin is sequentially phosphorylated by CK1 and GSK3, which triggers binding by β-transducin repeat containing protein (β-TrCP) and subsequent degradation through the ubiquitin-proteasome pathway. The Wnt signal is received by a receptor complex composed of frizzled (FZD) and low-density lipoprotein receptor related protein 5/6 (LRP5/6), which antagonizes the β-catenin destruction complex. The molecular mechanism of β-catenin destruction complex inactivation after Wnt stimulation has been extensively studied using cell lines maintained in serum-containing medium [3–6]. However, because serum contains several Wnt-stimulating factors, substantial levels of basal Wnt activity may preclude precise analysis of β-catenin destruction complex regulation. Cells that carry an intact Wnt signaling cascade and that can be maintained in defined medium are ideal. In the present study, we analyzed the molecular mechanism of Wnt-induced inhibition of the β-catenin destruction complex using a serum-free cell culture system.

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2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibody (mAb) to APC was generated as described previously [7]. Mouse mAbs to β-catenin and GSK3β were from BD Biosciences (Billerica, MA, USA). Mouse mAb to the Flag-tag was from Sigma (St. Louis, MO, USA). Mouse mAb to Lamin A/C was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse mAb to α-tubulin was from Merck Millipore (Bedford, MA, USA). Rabbit mAbs to Axin1, β-TrCP1 and LRP6 were from Cell Signaling Technology (Danvers, MA, USA). Rabbit mAb to CK1α was from Abcam (Cambridge, UK). Rabbit polyclonal antibodies to phospho-β-catenin S33/S37/T41 and phospho-LRP6 S1490 were from Cell Signaling Technology.

2.2. Cell culture and transfection

Human glioblastoma cells were cultured on laminin-coated plates in DMEM/F-12 medium containing B27 supplement minus vitamin A (Life Technologies, Carlsbad, CA, USA), EGF and bFGF (20 ng/ml each; Wako Pure Chemical Industries, Osaka, Japan). Normal human neural progenitor (NHNP) cells were from Lonza (Basel, Switzerland) and maintained according to the manufacturer's instructions. 293T cells were cultured in DMEM containing 10% fetal bovine serum. To stimulate Wnt signaling, Wnt3a (R&D Systems, Minneapolis, MN, USA) was added to the medium at a final concentration of 20 ng/ml. To enhance Wnt signaling, R-spondin1 (R&D Systems) was added to the medium at a final concentration of 125 ng/ml. To inhibit GSK3 activity, BIO (Merck Millipore) was added at a final concentration of 5 μM. Transfections were performed using Polyethylenimine "Max" (Polysciences Inc., Warrington, PA, USA) or Lipofectamine RNAiMAX reagent (Life Technologies).

2.3. Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 0.5 mM EDTA, 0.1 mM Na_3VO_4 and protease inhibitors]. Cell lysates were incubated with antibodies for 1 h at 4 °C, then the immunocomplexes were adsorbed to Protein A Sepharose 4 Fast Flow (GE Healthcare, Little Chalfont, UK) for 1 h at 4 °C. After three washes with lysis buffer, bound proteins were fractionated by SDS-PAGE and transferred to a PVDF membrane (Immobilon P, Merck Millipore). The membrane was subjected to immunoblot analysis using alkaline phosphatase- or horse radish peroxidase-conjugated secondary antibodies. The membrane was visualized using the NBT/BCIP colorimetric substrate system (Promega, Madison, WI, USA) or the enhanced chemiluminescence detection system (GE Healthcare).

2.4. Reporter assay

A TCF responsive reporter (pTOP-*tk*-luciferase) and a reporter with mutations in the TCF-binding sites (pFOP-*tk*-luciferase) were used to evaluate the activity of β-catenin/TCF-dependent transcription. Cells were transfected with a luciferase-reporter plasmid together with pRL-TK, which was used to monitor the transfection efficiency. To stimulate Wnt signaling, Wnt3a was added to the medium. Firefly luciferase activity was measured with the Luciferase Reporter Assay System (Promega) and normalized to the value of *Renilla* luciferase activity.

2.5. RNA interference

The Silencer Select siRNA oligonucleotide sequences were as follows: LRP5 (5′-GUACAGGCCCUACAUCAUU-3′), LRP6 (5′-GGUGCUAACCGGAUAGUAU-3′) (Life Technologies). Silencer Select Negative Control siRNA was obtained from Life Technologies.

3. Results and discussion

3.1. Wnt signaling promotes the dissociation of the Axin1-APC complex in glioblastoma cells

We previously established human glioblastoma cell lines maintained in serum-free medium that favors the growth of neural stem/precursor/progenitor cells [8,9]. These cell lines, for example GB2 and GB4, did not show any basal activity of β-

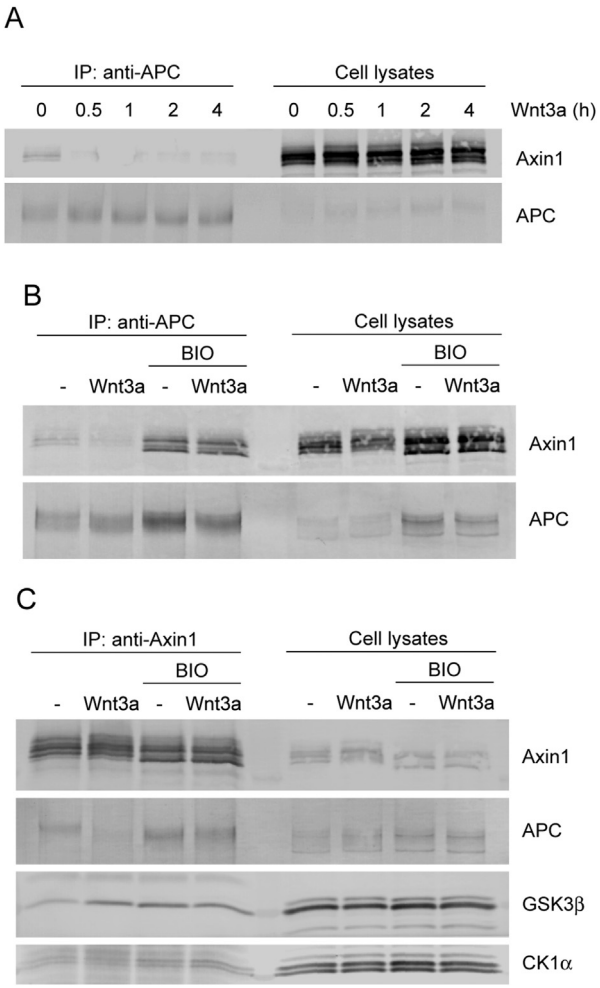

Fig. 1. Wnt signaling promotes the dissociation of the Axin1-APC complex in glioblastoma cells. (A) Wnt3a-dependent dissociation of Axin1 from APC. GB2 cells were cultured in the presence of Wnt3a for the indicated times. Cell lysates were subjected to immunoprecipitation with an anti-APC antibody followed by immunoblotting with antibodies to the indicated proteins. (B) GSK3 activity is critical for dissociation of Axin1 from APC. GB2 cells were pre-treated with the GSK3 inhibitor BIO overnight and then cultured in the presence of Wnt3a for 1 h. Cell lysates were subjected to immunoprecipitation with an anti-APC antibody followed by immunoblotting with antibodies to the indicated proteins. (C) Formation of the β-catenin destruction complex after Wnt3a treatment. GB2 cells were pre-treated with BIO overnight and then cultured in the presence of Wnt3a for 1 h. Cell lysates were subjected to immunoprecipitation with an anti-Axin1 antibody followed by immunoblotting with antibodies to the indicated proteins.

catenin/TCF-dependent transcription evaluated by a TCF responsive reporter, pTOP-*tk*-luciferase, but quickly responded to Wnt3a stimulation (Supplementary Fig. 1). Therefore, we analyzed the formation of the endogenous β-catenin destruction complex after Wnt stimulation in GB2 cells. Time-course experiments showed that Axin1, a rate-limiting factor of the β-catenin destruction complex [10], dissociated from APC immunoprecipitates within 30 min of Wnt3a treatment (Fig. 1A). Similar results were obtained using a different glioblastoma cell line (GB4) and normal human neural progenitor (NHNP) cells (Supplementary Fig. 2). Because changes in the gel mobility of Axin1 were observed after Wnt3a stimulation, we next examined the possibility that Axin1-APC interaction is controlled by post-translational modifications of Axin1. We tested the effect of the GSK3 inhibitor BIO on Axin1-APC dissociation as Axin1 is known to be a substrate for GSK3 [11]. In BIO-treated cells, Wnt3a treatment no longer abrogated the association between Axin1 and APC (Fig. 1B). Reciprocal immunoprecipitation with an Axin1 antibody showed that Axin1 associated with all other components of the β-catenin destruction complex such as APC, GSK3β and CK1α in unstimulated GB2 cells (Fig. 1C). However, upon Wnt3a addition, APC was found to be dissociated from Axin1 immunoprecipitates while both GSK3β and CK1α still associated with Axin1. Together, these results suggest that Wnt signaling promotes the dissociation of the Axin1-APC complex in a phosphorylation-dependent manner.

3.2. Thr160 of Axin1 is critical for the formation of the Axin1-APC complex

Several phosphorylation sites mapped to the β-catenin-binding region of Axin1 have already been characterized [6,12]. However, no phosphorylation in the APC-binding domain RGS has been reported. We found several consensus substrate sequences for GSK3 located in the RGS domain and focused on Thr160 in human Axin1 because phosphorylation of this residue was found in mass spectrometry data [13,14]. We generated mutant forms of Axin1 in which Thr160 is converted to Ala (T160A: phosphoresistant) or Asp (T160D: phosphomimetic) (Fig. 2A and B). These mutants were expressed in 293T cells and tested for the ability to interact with components of the β-catenin destruction complex (Fig. 2C). Notably, the Axin1 phosphomimetic T160D mutant was unable to interact with APC but retained its association with GSK3β and CK1α. Moreover, β-catenin was barely detectable in the Axin1 T160D mutant complex. Furthermore, β-TrCP1 showed reduced affinity to the Axin1 T160D mutant compared to wild-type Axin1. In contrast, no clear difference was observed between wild-type Axin1 and the

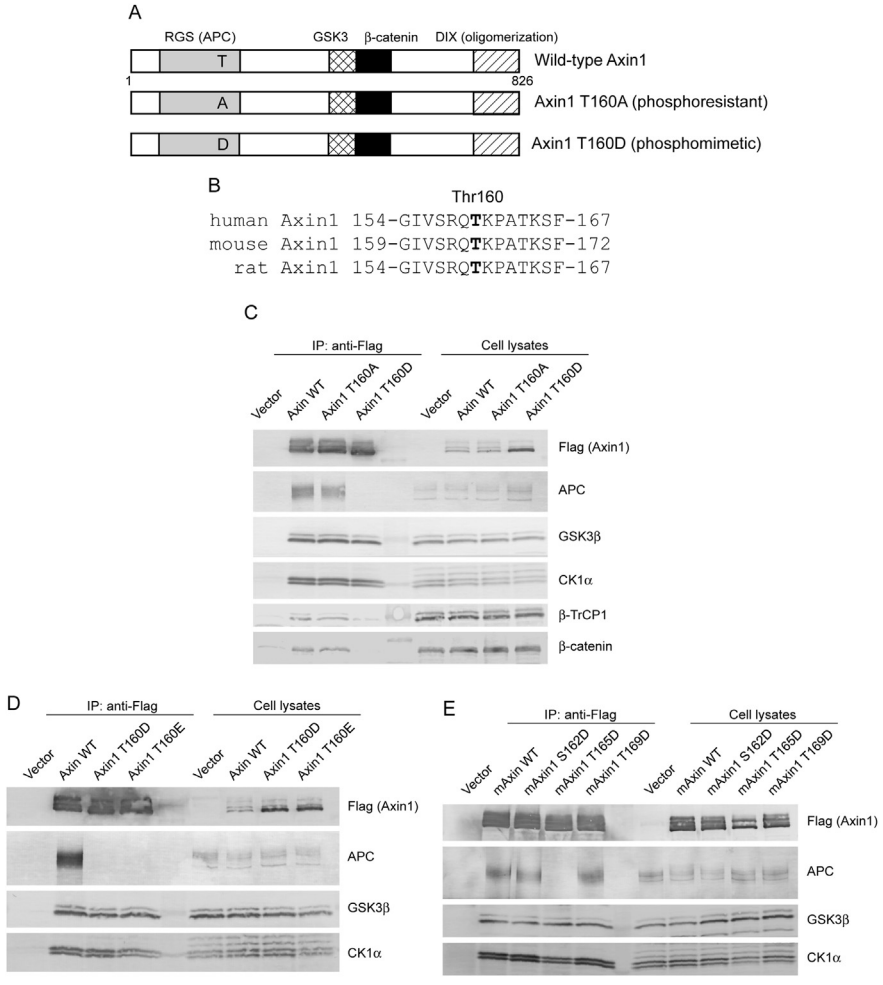

Fig. 2. Thr160 of Axin1 is critical for the formation of the Axin1-APC complex. (A) Schematic representations of wild-type Axin1 and Axin1 mutants. (B) Alignment of sequences flanking the Thr160 site in Axin1 proteins from human, mouse and rat. Thr160 (Thr165 in mouse) is indicated in bold. (C) The Axin1 phosphomimetic T160D mutant fails to interact with APC, β-catenin and β-TrCP1. 293T cells were transfected with Flag-tagged wild-type Axin1, Axin1 T160A or T160D mutants. Cell lysates were subjected to immunoprecipitation with an anti-Flag antibody followed by immunoblotting with antibodies to the indicated proteins. (D) The Axin1 phosphomimetic T160E mutant fails to interact with APC. 293T cells were transfected with Flag-tagged wild-type Axin1, Axin1 T160D or T160E mutants and analyzed as described for (C). (E) Mouse Thr165 functionally corresponds to human Thr160. 293T cells were transfected with Flag-tagged wild-type mouse Axin1, Axin1 S162D, T165D or T169D mutants and analyzed as described for (C).

Axin1 phosphoresistant T160A mutant. Interestingly, phosphatase treatment showed that the Axin1 T160D mutant was hypo-phosphorylated (Supplementary Fig. 3). These results were confirmed by generating another phosphomimetic Axin1 mutant, in which Thr160 is converted to Glu (T160E). The Axin1 T160E mutant was also found to interact with GSK3β and CK1α but not with APC (Fig. 2D). Furthermore, conversion of Thr165 in mouse Axin1, which corresponds to Thr160 in human Axin1 (Fig. 2B), into Asp abrogated the ability to associate with APC but not with either GSK3β or CK1α (Fig. 2E). By contrast, mutations in residues Ser162 and Thr169 in mouse Axin1 did not show any effect on Axin1-APC interaction (Fig. 2E). Together, these results suggest that the formation of the Axin1-APC complex is regulated by Axin1 Thr160 phosphorylation.

3.3. Phosphorylation of LRP6 promotes the dissociation of the Axin1-APC complex

The Wnt co-receptor LRP5/6 is reported to be immediately phosphorylated after Wnt stimulation [15]. This modification generates a docking site for Axin1, which is thought to be involved in inhibition of the β-catenin destruction complex. We therefore tested whether phosphorylation of LRP5/6 is critical for Axin1-APC dissociation. Time-course experiments confirmed that LRP6 phosphorylation was increased within 30 min of Wnt3a treatment of GB2 cells (Fig. 3A). β-catenin phosphorylation was inversely correlated with LRP6 phosphorylation (Fig. 3A). Intriguingly, knockdown of LRP5/6 completely inhibited Axin1-APC dissociation after Wnt3a stimulation (Fig. 3B). These results prompted us to investigate the possibility that Wnt-dependent Axin1-APC dissociation is regulated by LRP5/6 phosphorylation. We tested this hypothesis using the constitutively active LRP6 ΔN mutant, which lacks the extracellular domain, and is reported to be constitutively phosphorylated [15]. Overexpression of the LRP6 ΔN mutant induced dissociation of APC from Axin1 (Fig 3C). By contrast, the Axin1 phosphoresistant T160A mutant was insensitive to the expression of the LRP6 ΔN mutant, suggesting that Thr160 is critical for Wnt-induced Axin1-APC dissociation regulated by LRP6. Together, these results suggest that Wnt-induced phosphorylation of LRP6 causes Axin1-APC dissociation, probably by promoting phosphorylation of Axin1 at Thr160.

3.4. Phosphomimetic mutation at Thr160 in Axin1 abolishes the function of the β-catenin destruction complex

Finally, we investigated the biological significance of Axin1 Thr160 phosphorylation in the regulation of Wnt/β-catenin signaling. Flag-tagged β-catenin was expressed in 293T cells to evaluate LRP6-dependent stabilization of β-catenin (Fig. 4A). As previously reported [15], co-expression of the LRP6 ΔN mutant results in β-catenin stabilization. Overexpression of wild-type Axin1 overcame this effect whereas the Axin1 phosphomimetic T160D mutant was unable to do so. Reporter assays using pTOP-*tk*-luciferase confirmed these results as Wnt3a-induced transcription was repressed by wild-type Axin1 but not by the Axin1 T160D mutant (Fig. 4B).

In this report, we proposed a novel transduction mechanism of Wnt/β-catenin signaling. Wnt-induced phosphorylation of Axin1 at Thr160 may cause the dissociation of the β-catenin destruction complex, thereby activating Wnt/β-catenin signaling. Two previous studies have investigated the mechanisms of inactivation of the β-catenin destruction complex after Wnt stimulation [5,6]. One showed that Wnt-induced dissociation of β-TrCP from the β-catenin destruction complex is critical for β-catenin stabilization [5]. Consistent with this, the Axin1 phosphomimetic T160D mutant showed reduced ability to interact with β-TrCP1 (Fig. 2C). The other report showed that Wnt-induced de-phosphorylation of Axin1 causes dissociation of β-catenin from Axin1 [6]. This is compatible with our observation that the Axin1 T160D mutant which lacked the ability to bind to β-catenin is hypophosphorylated (Supplementary Fig. 3). Thus, phosphorylation of Axin1 at Thr160 might be involved in these molecular processes.

A great deal of effort has been made to target the Wnt/β-catenin pathway with small molecules. Axin1 may be an ideal target as it is considered to be a rate-liming factor of the β-catenin destruction complex. Recently, HLY78, a chemical compound that targets Axin1,

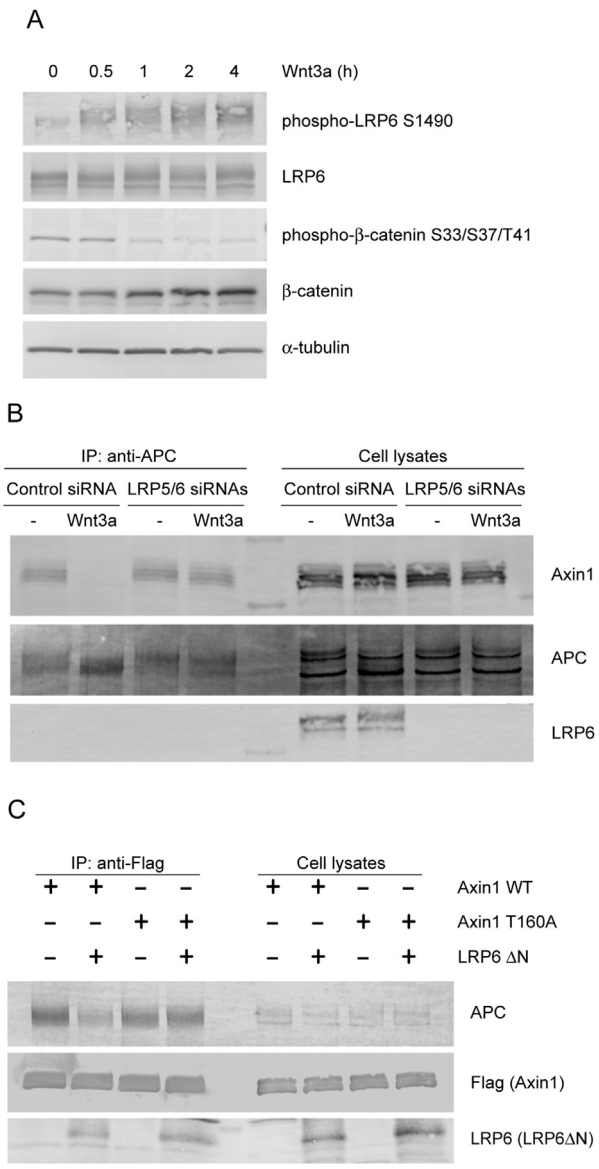

Fig. 3. Phosphorylation of LRP6 promotes the dissociation of the Axin1-APC complex. (A) Wnt3a treatment promotes LRP6 phosphorylation. GB2 cells were treated with Wnt3a for the indicated times. Cell lysates were subjected to immunoblotting with antibodies to the indicated proteins. (B) LRP5/6 is required for Wnt-dependent Axin1-APC dissociation. GB2 cells were transfected with siRNAs targeting LRP5/6 and cultured in the presence of Wnt3a for 1 h. Cell lysates were subjected to immunoprecipitation with an anti-APC antibody followed by immunoblotting with antibodies to the indicated proteins. (C) Thr160 of Axin1 is critical for the Wnt-induced dissociation of the Axin1-APC complex. 293T cells were transfected with Flag-tagged wild-type Axin1 or the Axin1 T160A mutant along with the LRP6 ΔN mutant. Cell lysates were subjected to immunoprecipitation with an anti-Flag antibody followed by immunoblotting with antibodies to the indicated proteins.

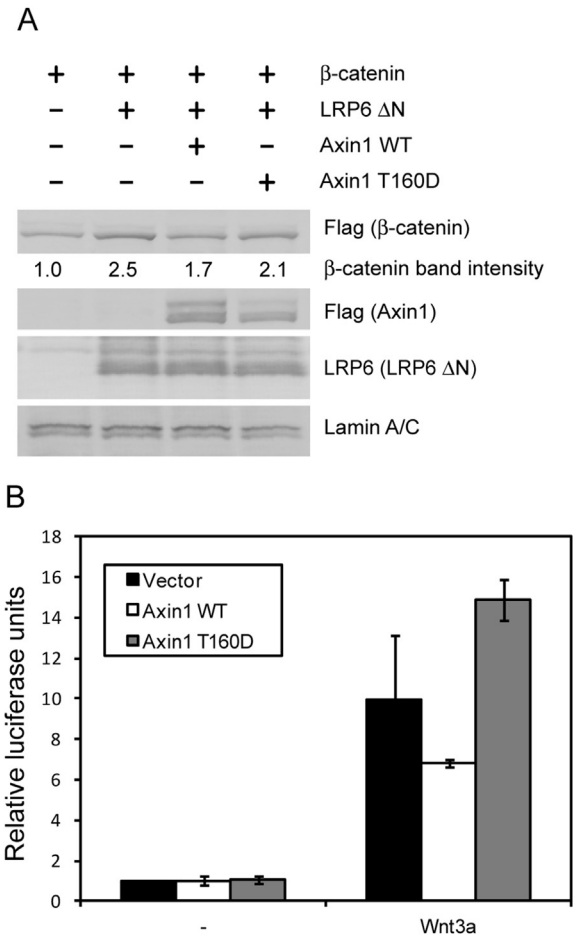

Fig. 4. A phosphomimetic mutation at Thr160 in Axin1 abolishes the function of the β-catenin destruction complex. (A) The Axin1 phosphomimetic T160D mutant fails to reduce β-catenin stability in cells expressing constitutively active LRP6 ΔN mutant. 293T cells were transfected with Flag-tagged β-catenin and the LRP6 ΔN mutant along with wild-type Axin1 or the Axin1 T160D mutant. Nuclear extracts were subjected to immunoblotting with antibodies to the indicated proteins. The relative intensity of the β-catenin band normalized for Lamin A/C is indicated. (B) The Axin1 phosphomimetic T160D mutant fails to repress β-catenin/TCF-dependent transcription. GB2 cells were transfected in triplicate with pTOP-*tk*-luciferase, pRL-TK and a plasmid encoding wild-type Axin1 or the Axin1 T160D mutant. After 24 h, cells were treated with Wnt3a, or left untreated, and cultured for an additional 8 h. Luciferase reporter activity relative to the transfection control is shown. Error bars represent the s.d. ($n = 3$).

was identified [16]. HLY78 efficiently activates the Wnt cascade by targeting the DIX domain of Axin1. Based on our results, compounds that modify Axin1 Thr160 phosphorylation may be able to control the intensity of Wnt signaling. Compound screening using a phospho-specific antibody should be performed in the future.

Conflict of interest

The authors have declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.118.

Transparency document

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