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Role of the Rap2/TNIK kinase pathway in regulation of LRP6 stability for Wnt signaling

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ABSTRACT

The Wnt/β-catenin signaling pathway plays critical roles in early embryonic development, stem cell biology and human diseases including cancers. Although Rap2, a member of Ras GTPase family, is essential for the Wnt/β-catenin pathway during the body axis specification in *Xenopus* embryo, the mechanism underlying its regulation of Wnt signaling remains poorly understood. Here, we show that Rap2 is implicated in control of the stability of Wnt receptor, low-density lipoprotein receptor-related protein 6 (LRP6). Knockdown of Rap2 resulted in the proteasome and/or lysosome-dependent degradation of LRP6 both in the presence and absence of Wnt ligand stimulation. In line with this, constitutively active LRP6 lacking its extracellular domain, which is constitutively phosphorylated and resides in intracellular vesicles, was also degraded in the Rap2-silenced cells. In addition, Rap2 and LRP6 associated physically with each other. Furthermore, we found that TRAF2/Nck-interacting kinase (TNIK), a member of the Ste20 protein family, acts as a downstream effector of Rap2 in control of LRP6 stabilization. Consistently, TNIK could rescue the inhibitory effects of Rap2 depletion on Wnt-dependent gene transcription, reporter activation and neural crest induction. Taken together, these results suggest that Rap2 acts via TNIK to regulate the stability of LRP6 receptor for Wnt/β-catenin signaling.

1. Introduction

Signaling by the Wnt family of secreted glycoproteins controls cell differentiation, cell proliferation, cell polarity and cell fate determination during early embryonic development and later tissue homeostasis [1,2]. As a result, abnormal Wnt signaling leads to a variety of human diseases including cancers, degenerative disorders, and osteoporosis. The best characterized form of Wnt pathway is the highly conserved canonical Wnt/β-catenin signaling [2–5]. In the absence of Wnt stimulation, β-catenin is bound by the multi-protein destruction complex composed of the scaffold proteins Axin and APC, and the serine/threonine kinases, CK1α and GSK3. Within this complex, β-catenin is phosphorylated sequentially by CK1α and GSK3, thereby undergoing the βTrCP-dependent ubiquitination and proteasomal degradation. When Wnt ligands bind to a cell-surface receptor complex consisting of Frizzled and LRP6, LRP6 is phosphorylated at conserved PPPSPxS motifs within its intracellular domain, which is mediated by a membrane-associated GSK3 and CK1γ, and a cytosolic scaffold protein Dishevelled is polymerized, thereby recruiting the Axin-GSK3 complex to the cytoplasmic tail of the LRP6 receptor at the plasma membrane. The binding of the Axin complex to the phosphorylated LRP6 leads to suppression of GSK3-mediated phosphorylation of β-catenin, allowing it to accumulate in the cytosol. Stabilized β-catenin enters into the nucleus, subsequently forming a complex with the DNA-binding factor, Tcf/Lef and activating the transcription of tissue-specific target genes.

Recent evidence shows that the cellular trafficking of Wnt signaling components plays critical roles in the activation and duration of this pathway. Upon Wnt3a stimulation, LRP6 is internalized through a caveolin-mediated endocytic pathway, which is essential for Wnt-dependent accumulation of β-catenin [6]. Wnt-activated LRP6 forms aggregates designated signalosomes, which contain phospho-LRP6, Frizzled, Dishevelled, GSK3 and Axin, at and under the plasma membrane [7]. The entry of LRP6 into an acidic intracellular compartment is required for its phosphorylation, and constitutively active LRP6 lacking its extracellular domain, which is spontaneously aggregating and constitutively phosphorylated, translocates to acidic vesicles in unstimulated cells that colocalize with the markers for the late endosome or multivesicular bodies (MVB) [8,9]. In addition, it has been proposed that the sustained inhibition of GSK3 which is necessary for β-catenin stabilization in Wnt signaling could be achieved by sequestration of the Wnt receptor complexes containing GSK3 into MVBs, which separates this kinase from its cytosolic substrates such as β-catenin [9]. However, it remains unclear whether the

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MVBs containing the Wnt receptor complexes would serve as precursors for lysosomal degradation or recycling back to the cytosol or plasma membrane. Frizzled receptor is also internalized along with LRP6 by Wnt stimulation through the caveolin-mediated pathway [6]. Besides, the cell surface levels of Frizzled are controlled by several regulators including an ER-retention protein, Shisa, which inhibits its trafficking to the plasma membrane [10], and a cell-surface transmembrane E3 ubiquitin ligase, ZNRF3, which promotes the turnover of the receptor [11]. Furthermore, the trafficking of internalized Frizzled into the lysosomal degradation versus recycling pathway is regulated by an ubiquitinylation/deubiquitinylation cycle [12]. These regulations of the membrane levels of Frizzled occur regardless of Wnt stimulation, thus affecting the cell responsiveness to Wnt ligands. Nevertheless, it remains to be further investigated how the cellular levels of LRP6 as well as Frizzled receptors are regulated either in the presence or absence of Wnt activation.

Rap2 is a member of the Ras family of small GTP-binding proteins, which regulates cell differentiation, cell proliferation, cell migration and cytoskeletal rearrangement [13]. This GTPase functions as a binary switch by cycling between GTP-bound active and GDP-bound inactive forms. It has been shown that Rap2 is essential for the Wnt/β-catenin signaling pathway during the specification of the dorsoventral body axis in *Xenopus* development [14], but how it regulates Wnt signaling remains unknown. In this study, we present a role of Rap2 in control of the stability of LRP6 as a possible mechanism underlying its regulation of the Wnt/β-catenin pathway. Moreover, we show that TRAF2/Nck-interacting kinase (TNIK), which belongs to the Ste20 family of protein kinases [15,16], acts as a downstream effector of Rap2 in regulation of LRP6 stability and Wnt-dependent gene transcriptions.

2. Materials and methods

2.1. Xenopus embryo manipulation and animal cap assays

In vitro fertilization, microinjection and embryo culture were performed as described previously [14]. Developmental stages of embryos were determined according to the Nieuwkoop and Faber's normal table of development [17]. For animal cap assay, animal pole explants were dissected at stage 9 from the injected embryos and then cultured to the indicated stages in 1× Modified Ringer's (MR) media containing 10 μg/ml of bovine serum albumin, 50 μg/ml of gentamycin and 5 μg/ml of streptomycin.

2.2. Cell culture and transfections

HEK293T cells were cultured in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin. Cells grown to 70%–80% confluency on 6-well plates were transfected with siRNAs with Lipofectamine RNAiMAX (Invitrogen) using the forward transfection protocol and 48 h later, re-transfected with DNA constructs with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

2.3. Constructs, morpholino oligo and siRNA

The open reading frame of *XTNIK* was amplified by PCR from the *X. laevis* IMAGE cDNA clone MXL1736–98358477 (Open Biosystems) and inserted into the *ClaI*/*XbaI* sites of pCS2+ vector. The complete coding region of *XMINK* was also PCR-amplified from the *X. laevis* IMAGE cDNA clone MXL1736–9507286 (Open Biosystems) and subcloned into the *EcoRI*/*XbaI* sites of pCS2+ vector. The following constructs were described previously: Myc-*LRP6*, Myc-*LRP6ΔN* [18], *XWnt8*, Myc-*XRap2B*, S01234 [14] and *noggin* [19]. Capped mRNAs were synthesized by *in vitro* transcription using the mMessage mMachine kit (Ambion). The anti-sense morpholino oligos (MOs) were purchased from Gene Tools. The sequences of *Rap2A* MO, *Rap2B* MO and control MO were described previously [14]. The synthetic siRNAs (Silencer predesigned siRNA) targeting *Rap2A* or *Rap2B* and negative control siRNA were obtained from Ambion, Inc [20].

2.4. Immunoprecipitation and Western blotting

To show the complex formation of LRP6 and Rap2, HEK293T cells were homogenized in Triton X-100 lysis buffer (20 mM Tris–HCl, 1% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 50 mM NaF, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin). The cell lysates were incubated with anti-LRP6 or anti-Rap2 antibody at 4 °C overnight and subsequently with Protein A Sepharose (Zymed) at 4 °C for 3 h. The immunocomplexes bound to Protein A beads were washed five times with the lysis buffer. Western blotting analysis was performed according to the standard protocol with anti-Myc (1:1000, Santa Cruz), anti-β-actin (1:1000, Santa Cruz), anti-Rap2 (1:500, BD Biosciences), anti-ABC (1:1000, Cell Signaling), anti-phospho-LRP6 (1:1000, Cell Signaling) and anti-LRP6 (1:1000, Santa Cruz) antibodies. Measurement of the intensities of Western blot bands was performed using ImageJ 1.40 g software. Statistical analyses were carried out using Microsoft Excel software.

2.5. RT-PCR analysis

Total RNA was extracted from whole embryos and animal cap explants using TRI Reagent (Molecular Research Center) and treated with RNase-free DNase I (Roche Molecular Biochemicals) to remove genomic DNA. RNA was transcribed using M-MLV reverse transcriptase (Promega) at 37 °C for 1 h. PCR products were analyzed on 2% agarose gels. The numbers of PCR cycles for each primer set were determined empirically to maintain amplification in the linear range. PCR primers used here were previously described: *Siamois* [14]; *Xnr3* [14]; *Snail* [21]; *Slug* [22]; *FoxD3* [21].

2.6. Luciferase reporter assay

Four-cell stage embryos were injected in the animal pole region with the indicated reagents, cultured to stage 11 and then separated into three pools of five whole embryos each for assay in triplicate. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Three independent experiments were performed. Fig. 4B shows a single representative result.

3. Results and discussion

3.1. Knockdown of Rap2 leads to degradation of LRP6 receptor

The absence of Rap2 function has been shown to inhibit the stabilization of β-catenin induced by Wnt signal [14]. Consistent with this, anti-sense morpholino oligo (MO)-mediated knockdown of Rap2 interfered with the increment in the level of active form of β-catenin (ABC) whose phosphorylation by GSK3 at serine 33, serine 37 and threonine 41 residues is blocked in Wnt-stimulated *Xenopus* embryonic cells (Fig. 1A). Intriguingly, we found that the Wnt-induced phosphorylation of LRP6 receptor at serine 1490 was also down-regulated in the Rap2-depleted cells (Fig. 1A). As LRP6 phosphorylation is necessary for the Wnt-dependent dephosphorylation and stabilization of β-catenin [18,23], we initially hypothesized that Rap2 might function as a modulator of the

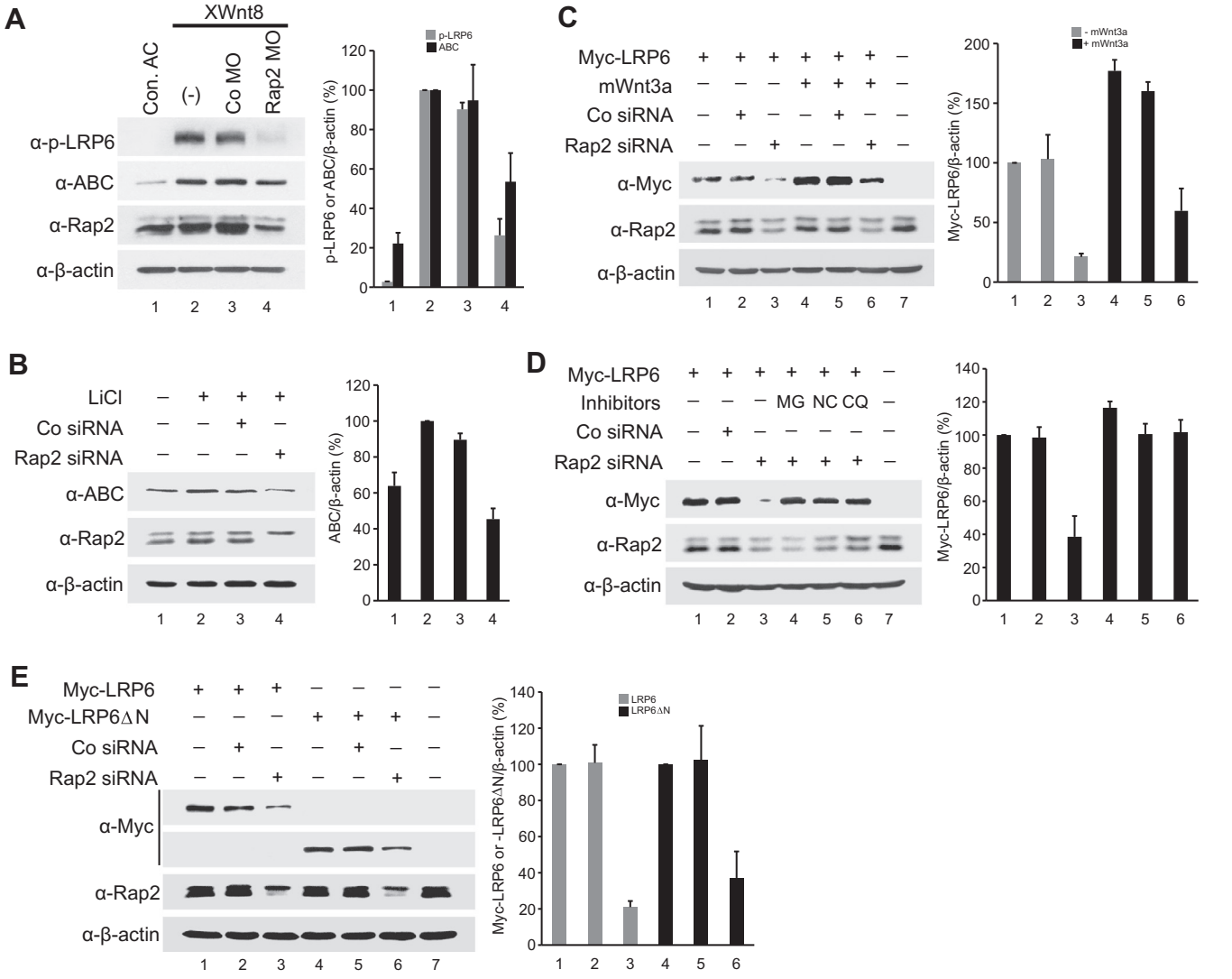

Fig. 1. Rap2 is essential for the stabilization of LRP6. (A) Four-cell stage *Xenopus* embryos were injected in the animal pole region as indicated with *XWnt8* RNA (400 pg), *Rap2* MO (40 ng) and control (Co) MO (40 ng) and then animal cap explants were dissected at stage 9 and cultured to stage 11 for western blotting analysis. *Rap2* MO, a mixture of *Rap2A* MO and *Rap2B* MO. Con. AC, uninjected control animal caps. β-actin serves as a loading control. (B–E) HEK293T cells were transfected with Co siRNA (10 nM) or *Rap2* siRNA (10 nM, a combination of *Rap2A* siRNA and *Rap2B* siRNA). Forty-eight hours after siRNA transfection, cell were transfected with Myc-*LRP6* (C–E) or Myc-*LRP6ΔN* (E) constructs and then treated or not as indicated with LiCl (50 mM, 3 h), mWnt3a (300 ng/ml, 3 h), MG132 (MG, 40 μM), NH_4Cl (NC, 40 mM) or chloroquine (CQ, 100 mM) before harvesting for Western blotting analysis 24 h later. Bar graph in each panel shows quantification of protein levels (normalized to β-actin) from three independent experiments. Error bars indicate the standard error (SE).

former event in the Wnt/β-catenin signaling pathway. However, the silencing of Rap2 could impair the elevation of ABC level by lithium, which is an inhibitor of GSK3, in HEK293T cells (Fig. 1B), suggesting the possibility that Rap2 could regulate the stability of β-catenin independently of LRP6 phosphorylation. Thus, we further examined whether Rap2 is also implicated in control of LRP6 stability. As shown in Fig. 1C, the siRNA-mediated depletion of Rap2 decreased the cellular levels of LRP6 protein both in the presence and absence of Wnt stimulation. Furthermore, the reduction of LRP6 level in the Rap2-silenced cells could be reverted to control levels by treatment of the proteasomal inhibitor MG132 or the lysosomal inhibitors NH_4Cl or chloroquine (Fig. 1D), indicating that the loss of Rap2 function leads to the proteasome- and/or lysosome-dependent degradation of this Wnt receptor. Constitutively active LRP6 lacking its ligand binding domain (LRP6ΔN) is able to activate the Wnt/β-catenin pathway without Wnt stimulation [24]. Notably, we observed that like wild-type LRP6, LRP6ΔN could be destabilized by knockdown of Rap2 in unstimulated cells (Fig. 1E). It has been shown that LRP6 is present predominantly at the plasma membrane in unstimulated cells [6]. Upon Wnt stimulation, it is relocalized to an acidic intracellular compartment [6,8]. LRP6ΔN mutant is also localized to acidic vesicles even in unstimulated cells [8], which colocalize with the markers for the late endosome or multivesicular body (MVB) [9]. Given that knockdown of Rap2 causes LRP6 degradation regardless of ligand stimulation as shown above, it seems possible that LRP6 receptor is in transit constitutively through an endocytic sorting compartment such as MVB, which would serve as a precursor for lysosomal degradation in the absence of Rap2. Taking together, we conclude that Rap2 plays a critical role in maintaining the proper cellular levels of LRP6 receptor.

3.2. Rap2. interacts with LRP6 receptor

To confirm the specific effect of Rap2 on the stabilization of LRP6, we wanted to know whether they might associate physically with each other. For this, we next tested for interaction between endogenous Rap2 and LRP6 in HEK293T cells by performing

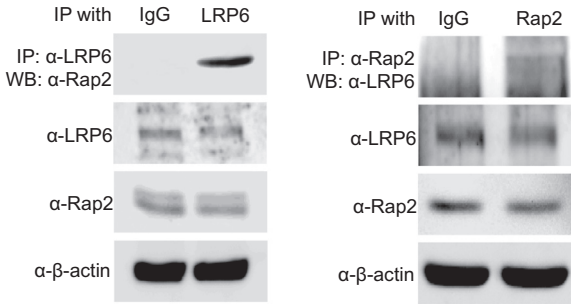

Fig. 2. Rap2 physically interacts with LRP6. HEK293T cell lysates were immunoprecipitated for endogenous LRP6 or Rap2, followed by western blotting analysis to investigate their coprecipitations. IgG, control IgG. IP, immunoprecipitation. WB, Western blotting.

immunoprecipitation analysis. As shown in Fig. 2, Rap2 could be clearly detected in the immunocomplex precipitated with anti-LRP6 antibody and vice versa. Thus, these results support a role of Rap2 as a specific regulator of LRP6 stability.

3.3. TNIK acts as a downstream effector of Rap2 in control of LRP6 stability

TRAF2/Nck-interacting kinase (TNIK) and Misshapen/NIKs (Nck-interacting kinases)-related kinase (MINK), which are members of the Ste20 family of protein kinases, share very high homology within the conserved N-terminal catalytic kinase domain and the C-terminal regulatory domain [15,16,25]. They have been reported as effectors of Rap2 to regulate cell spreading, cell movements or neuronal morphology [16,26–28]. In addition, these kinases are involved in the canonical and/or non-canonical Wnt signaling pathways [28–30]. However, their potential roles as downstream effectors of Rap2 in regulation of Wnt signaling remain to be documented. Thus, we first investigated whether TNIK and/or MINK would function downstream of Rap2 in control of LRP6 stabilization. As shown above, the siRNA-mediated silencing of Rap2 resulted in a marked decrease in the level of LRP6 receptor in HEK293T cells, and this down-regulation could be recovered to some extent by co-transfection of *Xenopus Rap2B* and *TNIK* but not by *MINK* (Fig. 3). These data suggests that TNIK but not MINK might act as an effector of Rap2 in control of LRP6-mediated canonical Wnt signaling.

3.4. TNIK functions downstream of Rap2 in Wnt/β-catenin signaling

We further examined whether TNIK would be an effector of Rap2 in control of canonical Wnt signaling. To this end, we first carried out RT-PCR analysis to see if this kinase could mediate the activity of Rap2 in regulation of Wnt-responsive gene transcription. Overexpression of *Wnt8* RNA induced the ectopic expression of Wnt target genes such as *Siamois* and *Xnr3* in naïve ectodermal tissue (Fig. 4A, lane 4). Of note, co-injection of *Rap2* MO, but not control MO, impeded this *Wnt8*-dependent expression of target genes, which could be reversed to some degree by co-expression of *XRap2B* RNA, which is immune to MO effect, or *TNIK* RNA (Fig. 4A, lanes 5–8). Consistently, depletion of Rap2 also interfered with full activation by Wnt8 of the luciferase reporter which is driven by the β-catenin/Tcf response element contained in *Siamois* promoter (S01234), and this reduction of luciferase activity could be rescued by co-injection of *XRap2B* or *TNIK* RNA (Fig. 4B). Wnt signaling is one of the major pathways critical for neural crest (NC) induction [31]. Thus, we also tested whether TNIK would act downstream of Rap2 in control of Wnt-dependent neural crest formation. NC can be induced ectopically in *Xenopus* ectodermal tissue by injecting both *Wnts* and anti-BMP neuralizing factors such as *noggin* and *chordin* [31]. As shown in Fig. 4C, co-expression of *Wnt8* and *noggin* induced ectopically the expression of NC markers including *Snail*, *Slug* and *FoxD3* (lane 4). Co-injection of *Rap2A* MO, but not control MO, could abrogate the ectopic expression of NC markers (Fig. 4C, lanes 5 and 6) and this inhibitory effect was rescued by co-expression of *XRap2B* or *TNIK* RNA (Fig. 4C, lanes 7 and 8), indicating a role of TNIK as an effector of Rap2 in NC induction. Notably, depletion of Rap2A alone could inhibit NC induction, which might be due to the possibility that zygotic Rap2A has a predominant role over Rap2B at the time of NC formation in *Xenopus* development. Overall, these results suggest that TNIK mediates the activity of Rap2 in the Wnt/β-catenin signaling pathway.

In the current study, we have demonstrated that Rap2 plays a crucial role in the regulation of LRP6 stability. Knockdown of Rap2 caused LRP6 degradation in a proteasome and/or lysosome-dependent manner, which was irrespective of Wnt stimulation. This suggests that LRP6 receptor undergoes a constitutive endocytic trafficking, which might end up in a degradation pathway in the absence of Rap2 function. In support of this, while LRP6 appears to be present mostly at the cell surface without Wnt activation, some portion of the receptor also exhibits localization to the cytoplasmic vesicles [6], indicative of its ligand-independent endocytic trafficking. In addition, Wnt-stimulated LRP6 is endocytosed through a caveolin-dependent pathway and even recycled to the

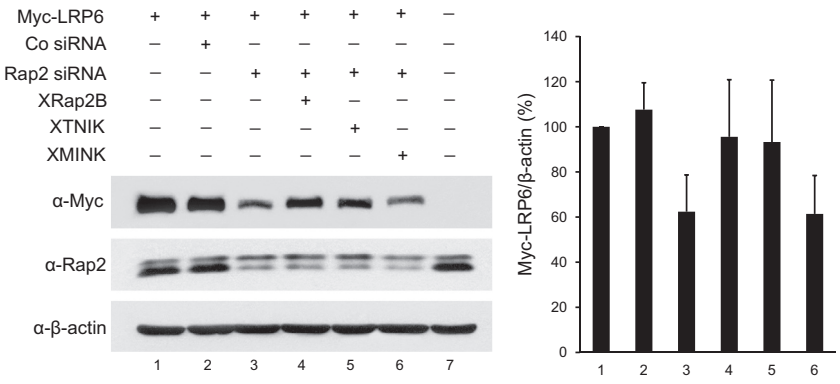

Fig. 3. TNIK acts downstream of Rap2 in regulation of LRP6 stability. HEK293T cells were transfected with Co siRNA (10 nM) or *Rap2* siRNA (10 nM) and 48 h later, re-transfected as indicated with Myc-*LRP6*, *XRap2B*, *XTNIK* or *XMINK*. After an additional 24 h, cells were harvested and subjected to Western blotting analysis. The graph shows quantification of the results from three independent analyses. Error bars denote the standard error (SE).

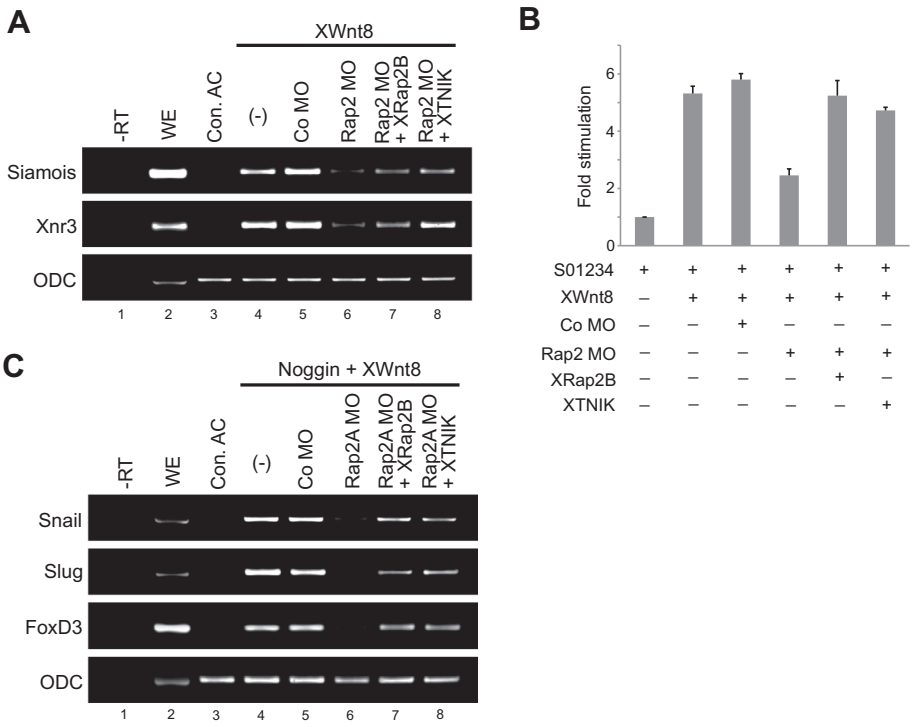

Fig. 4. TNIK mediates the activity of Rap2 in the Wnt/β-catenin pathway. (A, C) Four-cell stage embryos were injected in the animal pole region with the indicated combination of *XWnt8* (400 pg for A, 200 pg for C), *noggin* (100 pg), *XRap2B* (200 pg), *XTNIK* (400 pg), Co MO (40 ng), *Rap2A* MO (40 ng) and *Rap2* MO (40 ng, a combination of *Rap2A* MO and *Rap2B* MO). Animal caps were excised at stage 9 and cultured to stage 10.5 (A) or 16 (C) prior to harvesting for RT-PCR analysis. ODC serves as a loading control. WE, stage 10.5 whole embryo. –RT, a control in the absence of reverse transcriptase. Con.AC, uninjected control animal caps. (B) Luciferase reporter assay in *Xenopus* embryos injected as indicated with β-catenin/Tcf factor-dependent reporter (S01234, 40 pg), *XWnt8* (400 pg), *XRap2B* (200 pg), *XTNIK* (400 pg), Co MO (40 ng) and *Rap2* MO (40 ng). Error bars denote the standard deviation (SD).

cell surface a few hours after stimulation [6]. A recent study shows that Wnt receptor complex containing GSK3 is translocated to the late endosomes or MVBs for efficient signaling [9]. Notably, these endocytic compartments serve as precursors for lysosomal degradation or protein recycling to the cytosol or plasma membrane [32]. It has been shown that Rap2 is localized to the recycling endosomes [26]. This small GTPase regulates the recycling back to the cell surface of integrin receptor to control T cell migration [33]. Furthermore, Rap2 facilitates the trafficking of internalized Activin/Nodal receptors into a recycling pathway, thereby preventing their degradation [20]. Given these findings, it is tempting to speculate that Rap2 functions to direct the sorting of LRP6 receptor into recycling or degradation pathway or to regulate the rate of its trafficking into a degradation route. Frizzled receptor also displays similar endocytic trafficking to that of LRP6 [6]. An ubiquitinylation/deubiquitinylation cycle regulates constitutively the proportion of Frizzled destined for lysosomal degradation versus recycling back to the cell surface, thereby modulating cell responsiveness to Wnt ligands [12]. It will be necessary to further investigate whether and how Rap2 could control the trafficking and cellular levels of Frizzled as well as LRP6 receptors for Wnt signaling. Besides, we have shown that TNIK, but not MINK, acts as an effector of Rap2 in control of LRP6 stability and the β-catenin/Tcf complex-dependent gene expression. In contrast, MINK acts downstream of Rap2 to regulate cell movements in the non-canonical Wnt pathway [28]. In addition, both of these kinases have been shown to function as effectors of Rap2 differentially or similarly to regulate neuronal processes [25,27]. These findings suggest that Rap2 employs as its effector TNIK and/or MINK in a tissue or context-dependent manner. It has been reported that TNIK binds to the promoter of β-catenin/Tcf target genes in a β-catenin-dependent manner to activate Wnt transcriptional programme during colorectal cancer growth and *Xenopus* embryonic axis formation [29,30]. This nuclear function of TNIK appears not to be compatible with its role in control of LRP6 stability. However, TNIK is localized in the cytosol as well as in the nucleus, and regulates as an effector of Rap2 actin cytoskeleton [16,30,34]. Furthermore, this kinase also colocalizes with Rap2 to the recycling endosome to control cell spreading [26]. Thus, the role of TNIK in the stabilization of LRP6 seems likely to be correlated with its localization to the cytoplasmic area, presumably recycling endosomes. Future experiments are warranted to elucidate the mechanisms by which TNIK controls as an effector of Rap2 the trafficking of Wnt receptors in this intracellular compartment.

Acknowledgments

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References

[1] H. Clevers, Wnt/beta-catenin signaling in development and disease, Cell 127 (2006) 469–480.
[2] B.T. MacDonald, K. Tamai, X. He, Wnt/beta-catenin signaling: components, mechanisms, and diseases, Dev. Cell 17 (2009) 9–26.
[3] T. Buechling, M. Boutros, Wnt signaling signaling at and above the receptor level, Curr. Top. Dev. Biol. 97 (2011) 21–53.
[4] K.M. Cadigan, M. Peifer, Wnt signaling from development to disease: insights from model systems, Cold Spring Harb. Perspect. Biol. 1 (2009) a002881.
[5] C. Niehrs, J. Shen, Regulation of Lrp6 phosphorylation, Cell Mol. Life Sci. 67 (2010) 2551–2562.

[6] H. Yamamoto, H. Komekado, A. Kikuchi, Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin, Dev. Cell 11 (2006) 213–223.
[7] J. Bilic, Y.L. Huang, G. Davidson, T. Zimmermann, C.M. Cruciat, M. Bienz, C. Niehrs, Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation, Science 316 (2007) 1619–1622.
[8] C.M. Cruciat, B. Ohkawara, S.P. Acebron, E. Karaulanov, C. Reinhard, D. Ingelfinger, M. Boutros, C. Niehrs, Requirement of prorenin receptor and vacuolar H+–ATPase-mediated acidification for Wnt signaling, Science 327 (2010) 459–463.
[9] V.F. Taelman, R. Dobrowolski, J.L. Plouhinec, L.C. Fuentealba, P.P. Vorwald, I. Gumper, D.D. Sabatini, E.M. De Robertis, Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes, Cell 143 (2010) 1136–1148.
[10] A. Yamamoto, T. Nagano, S. Takehara, M. Hibi, S. Aizawa, Shisa promotes head formation through the inhibition of receptor protein maturation for the caudalizing factors, Wnt and FGF, Cell 120 (2005) 223–235.
[11] H.X. Hao, Y. Xie, Y. Zhang, O. Charlat, E. Oster, M. Avello, H. Lei, C. Mickanin, D. Liu, H. Ruffner, X. Mao, Q. Ma, R. Zamponi, T. Bouwmeester, P.M. Finan, M.W. Kirschner, J.A. Porter, F.C. Serluca, F. Cong, ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner, Nature 485 (2012) 195–200.
[12] A. Mukai, M. Yamamoto-Hino, W. Awano, W. Watanabe, M. Komada, S. Goto, Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt, EMBO J. 29 (2010) 2114–2125.
[13] J.L. Bos, Ras-like GTPases, Biochim. Biophys. Acta. 1333 (1997) M19–M31.
[14] S.C. Choi, J.K. Han, Rap2 is required for Wnt/beta-catenin signaling pathway in *Xenopus* early development, EMBO J. 24 (2005) 985–996.
[15] C.A. Fu, M. Shen, B.C. Huang, J. Lasaga, D.G. Payan, Y. Luo, TNIK, a novel member of the germinal center kinase family that activates the c-Jun N-terminal kinase pathway and regulates the cytoskeleton, J. Biol. Chem. 274 (1999) 30729–30737.
[16] K. Taira, M. Umikawa, K. Takei, B.E. Myagmar, M. Shinzato, N. Machida, H. Uezato, S. Nonaka, K. Kariya, The Traf2- and Nck-interacting kinase as a putative effector of Rap2 to regulate actin cytoskeleton, J. Biol. Chem. 279 (2004) 49488–49496.
[17] P.D. Nieuwkoop, J. Faber, Normal table of *Xenopus laevis* (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis, Garland Pub, New York, 1994.
[18] G. Davidson, W. Wu, J. Shen, J. Bilic, U. Fenger, P. Stannek, A. Glinka, C. Niehrs, Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction, Nature 438 (2005) 867–872.
[19] L.L. McGrew, S. Hoppler, R.T. Moon, Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in *Xenopus*, Mech. Dev. 69 (1997) 105–114.
[20] S.C. Choi, G.H. Kim, S.J. Lee, E. Park, C.Y. Yeo, J.K. Han, Regulation of activin/nodal signaling by Rap2-directed receptor trafficking, Dev. Cell 15 (2008) 49–61.
[21] A.H. Monsoro-Burq, R.B. Fletcher, R.M. Harland, Neural crest induction by paraxial mesoderm in *Xenopus* embryos requires FGF signals, Development 130 (2003) 3111–3124.
[22] K. Mizuseki, M. Kishi, M. Matsui, S. Nakanishi, Y. Sasai, *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction, Development 125 (1998) 579–587.
[23] X. Zeng, K. Tamai, B. Doble, S. Li, H. Huang, R. Habas, H. Okamura, J. Woodgett, X. He, A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation, Nature 438 (2005) 873–877.
[24] K. Tamai, X. Zeng, C. Liu, X. Zhang, Y. Harada, Z. Chang, X. He, A mechanism for Wnt coreceptor activation, Mol. Cell 13 (2004) 149–156.
[25] H. Nonaka, K. Takei, M. Umikawa, M. Oshiro, K. Kuninaka, M. Bayarjargal, T. Asato, Y. Yamashiro, Y. Uechi, S. Endo, T. Suzuki, K. Kariya, MINK is a Rap2 effector for phosphorylation of the postsynaptic scaffold protein TANC1, Biochem. Biophys. Res. Commun. 377 (2008) 573–578.
[26] Y. Uechi, M. Bayarjargal, M. Umikawa, M. Oshiro, K. Takei, Y. Yamashiro, T. Asato, S. Endo, R. Misaki, T. Taguchi, K. Kariya, Rap2 function requires palmitoylation and recycling endosome localization, Biochem. Biophys. Res. Commun. 378 (2009) 732–737.
[27] N.K. Hussain, H. Hsin, R.L. Huganir, M. Sheng, MINK and TNIK differentially act on Rap2-mediated signal transduction to regulate neuronal structure and AMPA receptor function, J. Neurosci. 30 (2010) 14786–14794.
[28] R.H. Lee, H. Iioka, M. Ohashi, S. Iemura, T. Natsume, N. Kinoshita, XRab40 and XCullin5 form a ubiquitin ligase complex essential for the noncanonical Wnt pathway, EMBO J. 26 (2007) 3592–3606.
[29] R. Satow, M. Shitashige, T. Jigami, K. Honda, M. Ono, S. Hirohashi, T. Yamada, Traf2- and Nck-interacting kinase is essential for canonical Wnt signaling in *Xenopus* axis formation, J. Biol. Chem. 285 (2010) 26289–26294.
[30] T. Mahmoudi, V.S. Li, S.S. Ng, N. Taouatas, R.G. Vries, S. Mohammed, A.J. Heck, H. Clevers, The kinase TNIK is an essential activator of Wnt target genes, EMBO J. 28 (2009) 3329–3340.
[31] A.H. Monsoro-Burq, E. Wang, R. Harland, Msx1 and Pax3 cooperate to mediate FGF8 and WNT signals during *Xenopus* neural crest induction, Dev. Cell 8 (2005) 167–178.
[32] J. Gruenberg, H. Stenmark, The biogenesis of multivesicular endosomes, Nat. Rev. Mol. Cell Biol. 5 (2004) 317–323.
[33] P. Stanley, S. Tooze, N. Hogg, A role for Rap2 in recycling the extended conformation of LFA-1 during T cell migration, Biol. Open 1 (2012) 1161–1168.
[34] A. Mikryukov, T. Moss, Agonistic and antagonistic roles for TNIK and MINK in non-canonical and canonical Wnt signalling, PLoS One 7 (2012) e43330.