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IQGAP1 Protein Regulates Nuclear Localization of β -Catenin via Importin- β 5 Protein in Wnt Signaling*

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Background: The molecular mechanisms underlying the β -catenin nuclear localization remain unclear.

Results: IQGAP1 is a regulator of β -catenin nuclear localization.

Conclusion: Importin- β 5 and Ran contribute to the nuclear localization of β -catenin.

Significance: Novel molecular mechanisms were found in Wnt signaling.

In the canonical Wnt signaling pathway, the translocation of β -catenin is important for the activation of target genes in the nucleus. However, the molecular mechanisms underlying its nuclear localization remain unclear. In the present study, we found IQGAP1 to be a regulator of β -catenin function via importin- β 5. In *Xenopus* embryos, depletion of IQGAP1 reduced Wnt-induced nuclear accumulation of β -catenin and expression of Wnt target genes during early embryogenesis. Depletion of endogenous importin- β 5 associated with IQGAP1 also reduced expression of Wnt target genes and the nuclear localization of IQGAP1 and β -catenin. Moreover, a small GTPase, Ran1, contributes to the nuclear translocation of β -catenin and the activation of Wnt target genes. These results suggest that IQGAP1 functions as a regulator of translocation of β -catenin in the canonical Wnt signaling pathway.

β -Catenin acts as an indispensable component in canonical Wnt signaling (1, 2). In the Wnt off-state, β -catenin levels are kept low through the interaction with Axin-APC-glycogen synthase kinase-3 β complex in the cytoplasm. After phosphorylation by glycogen synthase kinase-3 β and CK1, β -catenin is degraded through the ubiquitin pathway. In the Wnt on-state, DVL inactivates the APC² complex for the degradation of β -catenin, and β -catenin is accumulated in the cytoplasm (3, 4). The stabilized β -catenin then translocates to the nucleus and associates with TCF/LEF transcription factors, which activate the Wnt target genes (5, 6). Thus, nuclear translocation of β -catenin is a key step in canonical Wnt signaling. In *Xenopus*, nuclear accumulation of β -catenin at the dorsal side is important for axis formation and expression of Wnt target genes during early embryogenesis (7). Overexpression of *Xwnt-8*, β -catenin, and *DVL2* induces a secondary axis and expression

of Wnt target genes, such as *Siamois*, *Xnr3*, and *Xtwn*, at the ventral side (8–13).

IQGAP1 is a scaffolding protein and is highly conserved throughout the evolution from yeast to human (14). IQGAP1 is a 190-kDa and contains multiple protein-interacting domains; the CHD (calponin homology) domain binds to actin, the WW domain binds to ERK2, the IQ repeat motifs bind to calmodulin and myosin light chain, the RasGAP-like domain binds to Cdc42 and Rac1, and the RasGAP_c domain at the C terminus binds to β -catenin and E-cadherin (15–21). IQGAP1 is involved in various cellular processes including cytoskeletal reorganization, cell adhesion, and cell cycle (22). It is also reported that IQGAP1 stimulates β -catenin-mediated transcriptional activation (20). Despite the presence of the RasGAP-like domain, IQGAP1 demonstrates no GAP activity (23). However, IQGAP1 is known as a potential effector of Cdc42 and Rac (23). The subcellular localization of IQGAP1 occurs in a variety of cultured cells, and IQGAP1 is localized in the cytoplasm, cell membrane, and nucleus (24). These subcellular localizations are presumably linked to its cellular functions. Our recent study showed that IQGAP1 binds to DVL and translocates DVL into the nucleus in canonical Wnt signaling (25).

The importin- β family includes more than 20 genes and mediates the translocation of proteins that have the nuclear localizing signal (NLS) by making a complex with importin- α in the classical nuclear transport of proteins (26–28). Importin- β genes also mediate the translocation of non-NLS proteins by directly binding to them (29). The transport direction of importin- β -cargo complex is determined by the gradient of different nucleotide-bound states of a small GTPase, Ran. In the nucleus, the Ran guanine exchange factor RCC1 (RanGEF) converts the inactive form of Ran (RanGDP) to the active form (RanGTP). In the cytoplasm, most of the Ran is in the GDP-bound form because of the GTPase-activating protein RanGAP (27). At the nucleoplasmic side of the nuclear pore, RanGTP binds to importin- β , and a target protein is dissociated from the importin- β -cargo complex (27).

In the present study, we found that IQGAP1 complexed with DVL and β -catenin. Complex of these proteins mutually contributed to their nuclear localization. The depletion of endogenous IQGAP1 in *Xenopus* embryos suppressed secondary axis

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² The abbreviations used are: APC, adenomatous polyposis coli; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; NLS, nuclear localizing signal; MO, morpholino oligonucleotide; xpo- β 5, *Xenopus* importin- β 5; x, *Xenopus*; IQGAP1, IQ motif containing GTPase-activating protein 1; Importin- β 5, karyopherin β family protein; DVL, Dishevelled.

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induction and expression of Wnt target genes. We also found that IQGAP1 associated with importin- β 5 and that the depletion of importin- β 5 reduced the nuclear localization of β -catenin and expression of Wnt target genes. Moreover, it was suggested that Ran plays important roles in the canonical Wnt signaling pathway. These results reveal a novel role for IQGAP1 in modulating the subcellular localization and transcriptional activation of components of the Wnt signaling pathway.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The *Xenopus xIpo- β 5*, *xRan1*, *xRanGAP*, and *xRanGEF* were amplified by RT-PCR from cDNA templates prepared from *Xenopus* embryos and subcloned into the pCS2+ vectors. *xIQGAP1- Δ RGD* was constructed by PCR and contained the following amino acid sequences: *xIQGAP1*, 1–1013 and 1372–1657 amino acids. We made a GFP construct of β -catenin by conjugating it to the GFP sequence at the C terminus. The nuclear localization signal, PKKKRKV (30), is conjugated to the N terminus of β -catenin to construct the NLS- β -catenin. The active (GTP-bound) form of Ran proteins has double amino acid substitutions (G19V and Q69L). The inactive (GDP-bound) form of Ran proteins has single amino acid substitutions (T24N). Other constructs were previously reported (25).

Embryo Handling and Morpholino Oligonucleotides—Capped mRNAs were synthesized from linearized vectors using the mMESSAGE mMACHINE kit (Ambion). The morpholino oligonucleotides (MOs) (Gene Tools, LLC) used here were previously reported (25), and the sequences used were: 5'-TTTC-AACCGTTTCCAAAGAACCAGG-3' (β -catenin-MO), 5'-CCGCCATTGCTGCTGCTCCAGAAAC-3' (*xIpo- β 5*-MO), and 5'-AGTTTGTCAGCCTTCCAGAAGCGTC-3' (*xRan1*-MO). The specificity of each MO was confirmed by its ability to inhibit the translation of FLAG-tagged mRNAs containing the targeted site with or without 5'-mismatched sequences. MO- (10 ng) and FLAG-tagged mRNAs (100 pg) were co-injected with β -globin-FLAG mRNA (100 pg) as loading control into the animal poles of four-cell stage embryos, and the injected animal caps were dissected at stage 10. Lysates from the animal caps were subjected to Western blotting with anti-FLAG antibody (M2, Sigma) (data not shown).

MOs and mRNAs were injected into four animal blastomeres at the eight-cell stage for dissection of animal caps or into two dorsal or ventral blastomeres at the four-cell-stage for quantitative RT-PCR analysis and observation of embryo phenotypes. Animal cap explants of the injected (10 pg of mRNA of each GFP-fused construct) embryos were dissected at the early gastrula stage (stage 10) and fixed for DAPI staining as reported previously (31). We counted the number of cells that have fluorescence signals as in our previous study (25). Dorsal or ventral sectors of the injected embryos were dissected at stage 10, and total RNA was extracted for RT-PCR analysis. The cytoplasmic and nuclear fractions were prepared as described with modifications (32).

RT-PCR Analysis—Total RNA was prepared using TRIzol (Invitrogen). cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative PCR was performed with an Applied Biosystems

7300 real-time PCR cycler (ABI) using THUNDERBIRD SYBR quantitative PCR mix (TOYOBO). The sequences of the primer pairs were previously reported (25). *Xenopus* embryonic ornithine decarboxylase (*ODC*) was used for normalization of cDNA samples.

Chromatin Immunoprecipitation (ChIP) Assay—MYC-tagged mRNAs were injected into two dorsal blastomeres of four-cell embryos. Injected dorsal sectors were dissected at stage 10 and cross-linked in 1% formaldehyde for 1 h. Sonication, immunoprecipitation, and DNA purification were performed as described previously (33). The nested PCR was performed using specific primers as follows: Siamois: outer/forward, 5'-GAAGTCTTGCCAACTTCTCTCA-3'; outer/reverse, 5'-GTCCTTTGATGATTCTGATGAC-3'; inner/forward, 5'-CCAACTTCTCTCACTCAGTC-3'; and inner/reverse, 5'-TTTCCCTTGATCTTGCCC-3'; *Xnr3*: outer/forward, 5'-ATAGCTTTAATGTGCCACAATCTAC-3'; outer/reverse, 5'-GTACAGTCTTGGGAGTTCCTG-3'; inner/forward, 5'-CATAAAGGCAAATGGTTTCTGC-3'; and inner/reverse, 5'-TTATAC-TGGGATGGACAGAGGC-3'.

Antibodies, Cell Lines, and siRNA Transfection—The following antibodies were used for immunoprecipitation and/or Western blotting analysis: horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare); horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare); anti-FLAG (M2 and F7425, Sigma); anti-MYC (9B11, Cell Signaling); anti-DVL1 (3F12 and Q-25, Santa Cruz Biotechnology); anti-IQGAP1 (H-109, Santa Cruz Biotechnology); anti- β -catenin (C2206, Sigma); anti-importin- β 5 (sc-11369, Santa Cruz Biotechnology); anti-Ran (sc-20802, Santa Cruz Biotechnology); anti-active-Ran (26915, NewEast Biosciences); anti- β -tubulin (sc-58884, Santa Cruz Biotechnology); and anti-histone-H3 (sc-10809, Santa Cruz Biotechnology). We used the following cell lines: HEK 293 cells, HEK 293T cells, SW480 cells, L cells, and L Wnt3A cells (25). Recombinant human Wnt3A (R&D Systems; 20 ng/ml) or 3 day Wnt-3A conditioned medium from L-Wnt-3A cells was used for Wnt stimulation of cultured cells. The FLAG peptide (F3290, Sigma; 100 μ g/ml) was used for elution of FLAG tag proteins before the second immunoprecipitation. The growth medium for each cell type was as recommended by the American Type Culture Collection.

Protein Identification by LC-MS/MS Analysis—FLAG-human IQGAP1 was expressed in HEK 293 cells, and associated proteins were recovered from cell extracts by immunoprecipitation with anti-FLAG antibody. The IQGAP1-associated complexes were digested with *Achromobacter* protease I, and the resulting peptides were analyzed using a nanoscale LC-MS/MS system, as described previously (34).

GST Pulldown, GAP, and GEF Assay—GST-*xRan1*-FLAG was expressed in *Escherichia coli* BL21 (DE3). Bacteria were lysed with GST lysis buffer (PBS with 1% Triton X-100, 1 mM DTT, and Complete protease inhibitor cocktail (Roche Applied Science)). GST fusion proteins were purified by affinity chromatography with glutathione-Sepharose 4B (GE Healthcare) and eluted with 50 mM Tris-HCl (pH 8.0) and 20 mM glutathione. Purified proteins were mixed in TNE buffer (10 mM Tris-HCl (pH 7.8), 0.1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and Complete protease inhibitor cocktail (Roche Applied Science)). The protein solutions were

added to glutathione-Sepharose 4B (GE Healthcare) and incubated for 2 h at 4 °C. The GAP or GEF activity toward xRan1 was examined by the Western blotting of immunoprecipitates with the anti-active-Ran antibody. Each reaction performed in 50 μ l of buffer A (50 mM HEPES, pH 7.4, 1.5 mM magnesium chloride, 5 mM EGTA, 1 mM DTT, 1 mM ATP, and Complete protease inhibitor cocktail (Roche Applied Science)) with the addition of 1 μ l of cell lysate. Cell lysates were prepared from HEK 293T cells transfected with xIQGAP1-MYC, xRanGAP-MYC, xRanGEF-MYC, or pCS2+, using buffer B (buffer A + 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 60 mM magnesium chloride). Reactions were incubated at room temperature for 5 min and terminated by adding 250 μ l of buffer B. We performed immunoprecipitation with the same tubes for 30 min. For GAP assays, GST-xRan1-FLAG was preloaded with GTP (1 mM) for 10 min at room temperature. For GEF assays, we added 0.1 mM GTP and 0.1 mM GDP in reactions.

RESULTS

IQGAP1 Forms a Complex with β -Catenin and DVL—In canonical Wnt signaling, the nuclear translocation of β -catenin is very important for transactivation of Wnt target genes (4). Our recent study has shown that xIQGAP1 plays a role in the translocation of xDVL2 into the nucleus in the canonical Wnt signaling pathway (25). Because both IQGAP1 and DVL bind to β -catenin (14, 25, 35), we examined whether IQGAP1 also contributes to the nuclear localization of β -catenin. The interaction of ectopically expressed *Xenopus* β -catenin with xDVL2 and xIQGAP1 was confirmed in HEK 293T cells (Fig. 1, A and B). The endogenous interaction of human β -catenin to both human DVL1 and human IQGAP1 was also confirmed in SW480 cells, which possess a mutation in the APC gene that interferes with β -catenin turnover (Fig. 1C). To investigate whether these proteins could form a complex, we performed a stepwise immunoprecipitation of β -catenin with xDVL2 and xIQGAP1. We detected an association of xDVL2 and β -catenin with xIQGAP1 in the first immunoprecipitation (Fig. 1D). In the second immunoprecipitation, we detected the binding of β -catenin to xDVL2 (Fig. 1D). The Wnt stimulation increased the binding of β -catenin in the second immunoprecipitates (Fig. 1, D and E). We also conducted this stepwise immunoprecipitation in a different order and obtained the same results; xIQGAP1 and β -catenin were found associated with xDVL2 in the first immunoprecipitation, and the β -catenin was associated with xIQGAP1 in the second immunoprecipitation (data not shown). These results suggest that xIQGAP1, xDVL2, and β -catenin can form a complex and that their association was increased by Wnt stimulation.

IQGAP1 Is Required for Nuclear Localization of β -Catenin—We then asked whether IQGAP1 and xDVL2 also contribute to the nuclear localization of β -catenin induced by Wnt signaling. We examined the distribution of β -catenin fused to GFP (β -catenin-GFP) in *Xenopus* embryonic (animal cap) cells injected with a morpholino oligonucleotide of xIQGAP1-MO or xDVL2-MO. We also examined the effect of depleting β -catenin on the nuclear localization of xDVL2-GFP and xIQGAP1-GFP. We detected nuclear localization of β -catenin when *Xwnt-8* was co-expressed in animal cap cells (Fig. 1F).

Depletion of xIQGAP1 reduced the proportion of β -catenin-GFP localized in the nucleus in cells co-expressing *Xwnt-8* (Fig. 1, F and G, lane 5), whereas depletion of xDVL2 did not affect the nuclear localization of β -catenin (Fig. 1G, lane 6). We therefore examined whether the other xDVL family members, *i.e.* xDVL1 and xDVL3, may be involved in the nuclear localization of β -catenin. We found that depletion of all three xDVLs (xDVL1, xDVL2, and xDVL3) reduced the nuclear localization of β -catenin in cells co-expressing *Xwnt-8* (Fig. 1, F and H, lanes 2 and 4). We confirmed that depletion of xIQGAP1 or xDVLs reduced the amounts of β -catenin in the nuclear fractions of animal cap cells and did not alter the amounts of cytoplasmic β -catenin (Fig. 2, upper panels). Conversely, depletion of β -catenin by a morpholino oligonucleotide (β -catenin-MO) reduced the nuclear localization of both xDVL2-GFP and xIQGAP1-GFP in cells co-expressing *Xwnt-8* (Fig. 1, I and J, lane 4). Western blotting analysis also showed that the amounts of xDVL2 and xIQGAP1 in the nuclear fractions of animal cap cells were reduced by depletion of β -catenin (Fig. 2, bottom panels). To elucidate whether the interaction between xIQGAP1 and β -catenin is necessary for the nuclear localization of β -catenin, we made a C-terminal truncated construct of xIQGAP1, xIQGAP1- Δ C, which hardly bound to β -catenin (21) (Fig. 3A). Expression of xIQGAP1- Δ C reduced the nuclear localization of β -catenin-GFP in cells co-expressing *Xwnt-8* (Fig. 3B, lane 4) and did not rescue expression of Wnt target genes that were reduced by xIQGAP1-MO (Fig. 3C). These results indicate that Wnt-induced nuclear localization of β -catenin, xIQGAP1, and xDVL2 requires the mutual presence of all three components.

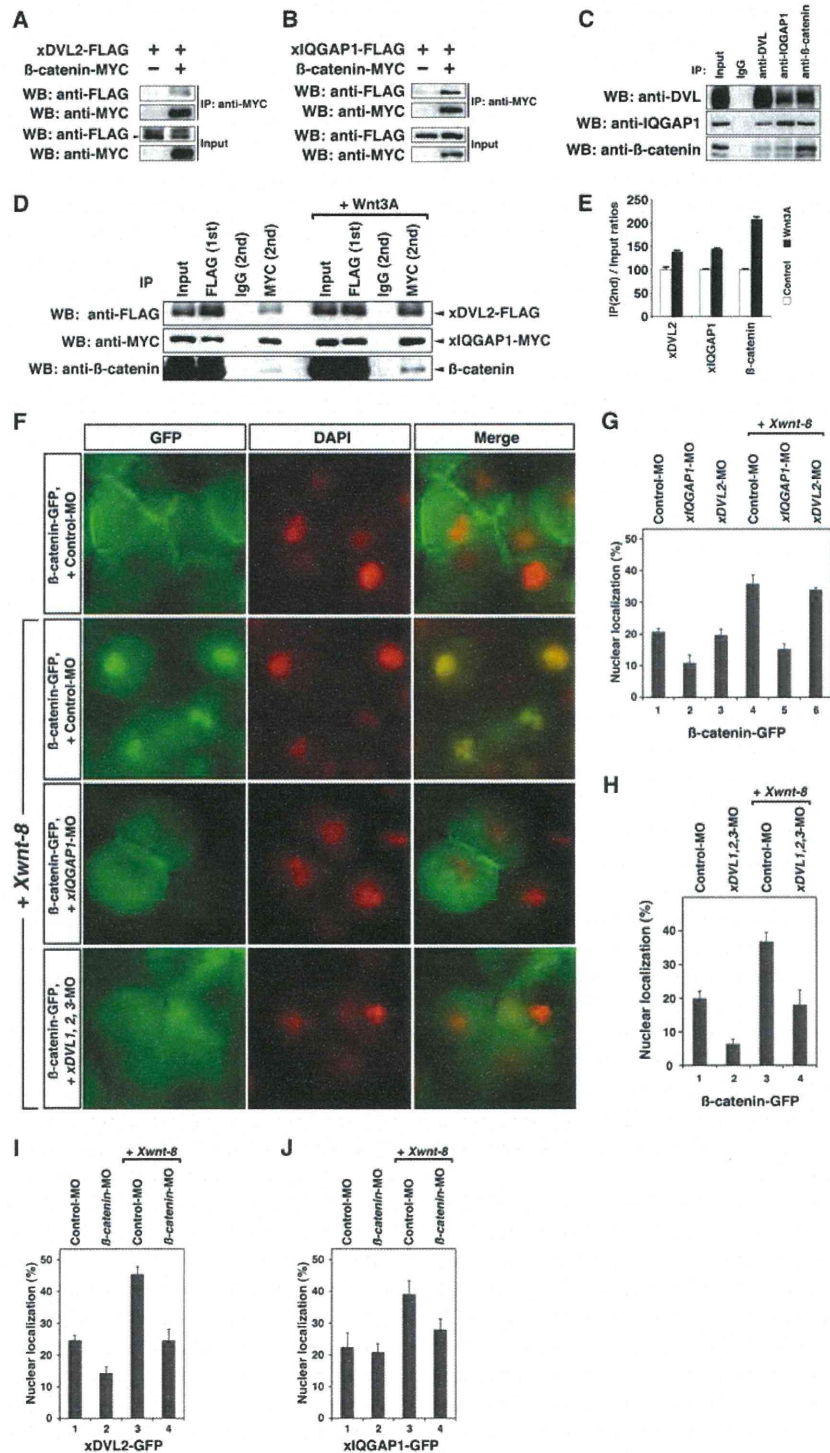
IQGAP1 Is Necessary for the Canonical Wnt Pathway—Our recent study showed that the depletion of endogenous IQGAP1 in *Xenopus* embryos suppressed secondary axis induction and expression of Wnt target genes (25). We confirmed that the induction of the Wnt target genes and partial secondary axis induced by β -catenin was suppressed by the depletion of xIQGAP1 (Fig. 3, D and E, lane 2). On the other hand, depletion of xIQGAP1 did not affect both of the Wnt target genes and the secondary axis formation induced by NLS- β -catenin, fusing with a peptide encoding NLS at the N terminus, which is predominantly localized in the nucleus without Wnt signaling stimulation (Fig. 3, D and E, lane 4). Moreover, we examined whether xIQGAP1 affects the localization of NLS- β -catenin. We observed that the nuclear localization of NLS- β -catenin-GFP was not suppressed by xIQGAP1-MO or xDVLs-MO, (Fig. 3, F and G). To confirm whether xIQGAP1 affects the stabilization of β -catenin in cytoplasm, we investigated the amounts of cytoplasmic β -catenin. We observed that xIQGAP1-MO injection had no effect on the β -catenin stability in the cytoplasm of the dorsal marginal cells (Fig. 3H). Interestingly, the ChIP assay revealed that xIQGAP1 was not recruited to the promoter regions of the Wnt target genes, whereas both xDVL2 and β -catenin were recruited (Fig. 3I). These results suggest that xIQGAP1 functions as an intermediate molecule in the canonical Wnt signaling pathway in early development, promoting the nuclear localization of β -catenin.

Importin- β 5 Regulates the Nuclear Localization of IQGAP1—To elucidate the mechanisms by which IQGAP1 is translocated

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into the nucleus, we performed a high throughput analysis of proteins that co-immunoprecipitated with human IQGAP1 in HEK 293 cells. We identified human importin- β as a candidate protein that may interact with IQGAP1. Importin- β family proteins mediate the nuclear import of several proteins that

bind directly to them (36). An interaction between ectopically expressed xIQGAP1 and ximportin- β 5 (xIpo- β 5) was confirmed in HEK 293T cells, but xDVL2 and β -catenin did not associate with xIpo- β 5 (Fig. 4A). We also confirmed endogenous interaction between human IQGAP1 and human impor-



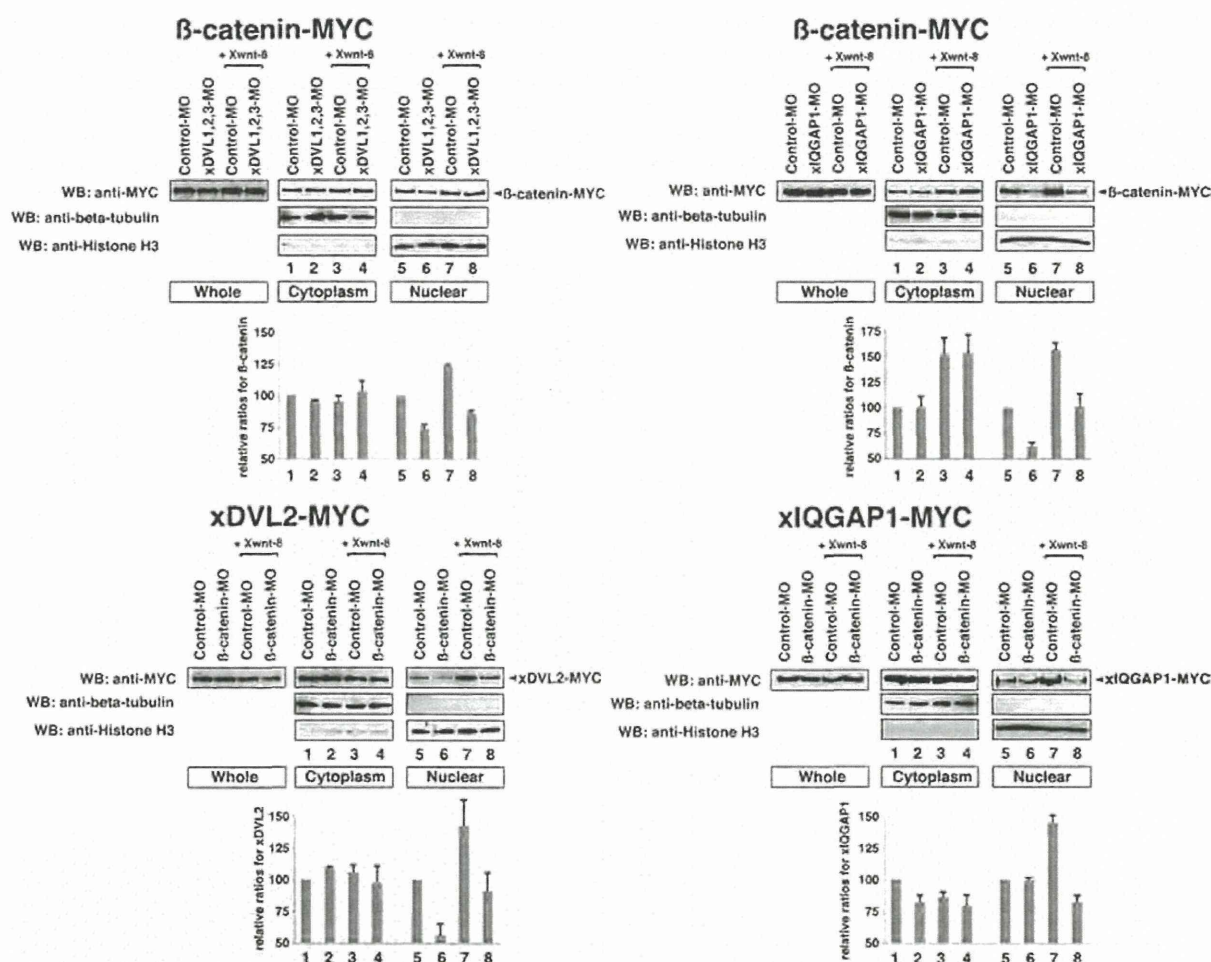


FIGURE 2. **Cytoplasmic and nuclear distribution of xDVL2, xIQGAP1, and β -catenin in animal cap cells.** Each MYC-tagged mRNA (100 pg) was co-injected into the animal poles of four-cell stage embryos with the indicated morpholino oligonucleotides, and the injected animal caps were dissected at stage 10. Lysates from the animal caps were fractionated and subjected to Western blotting (WB) with the indicated antibodies. *Upper panels*, β -catenin-MYC. *Lower panels, left*, xDVL2-MYC. *Lower panels, right*, xIQGAP1-MYC. In the case of β -catenin-MYC, cytoplasmic fraction was refracted to cytosolic and membrane fractions.

tin- β 5 in HEK 293T cells (Fig. 4B). The Wnt stimulation and the co-expression of xDVL2 and β -catenin increased the interaction between xIpo- β 5 and xIQGAP1 (Fig. 4, C and D). Dorsal injection of xIpo- β 5-MO reduced the endogenous expression of Wnt signal target genes (Fig. 4E). Expression of the Wnt target genes induced ectopically by Xwnt-8 was also reduced by xIpo- β 5-MO and induced by xIpo- β 5 (Fig. 4F). Depletion of xIpo- β 5 reduced the nuclear localization of xIQGAP1-, xDVL2-, and β -catenin-GFP fusion proteins by co-expressing

Xwnt-8 in *Xenopus* animal cap cells (Fig. 4, G–J). On the other hand, overexpression of xIpo- β 5 mRNA increased the nuclear localization of β -catenin-GFP nuclear localization (Fig. 4J). These results suggest that the interaction between xIQGAP1 and xIpo- β 5 mediates the nuclear import of the xDVL2-xIQGAP1- β -catenin complex in Wnt signaling pathway.

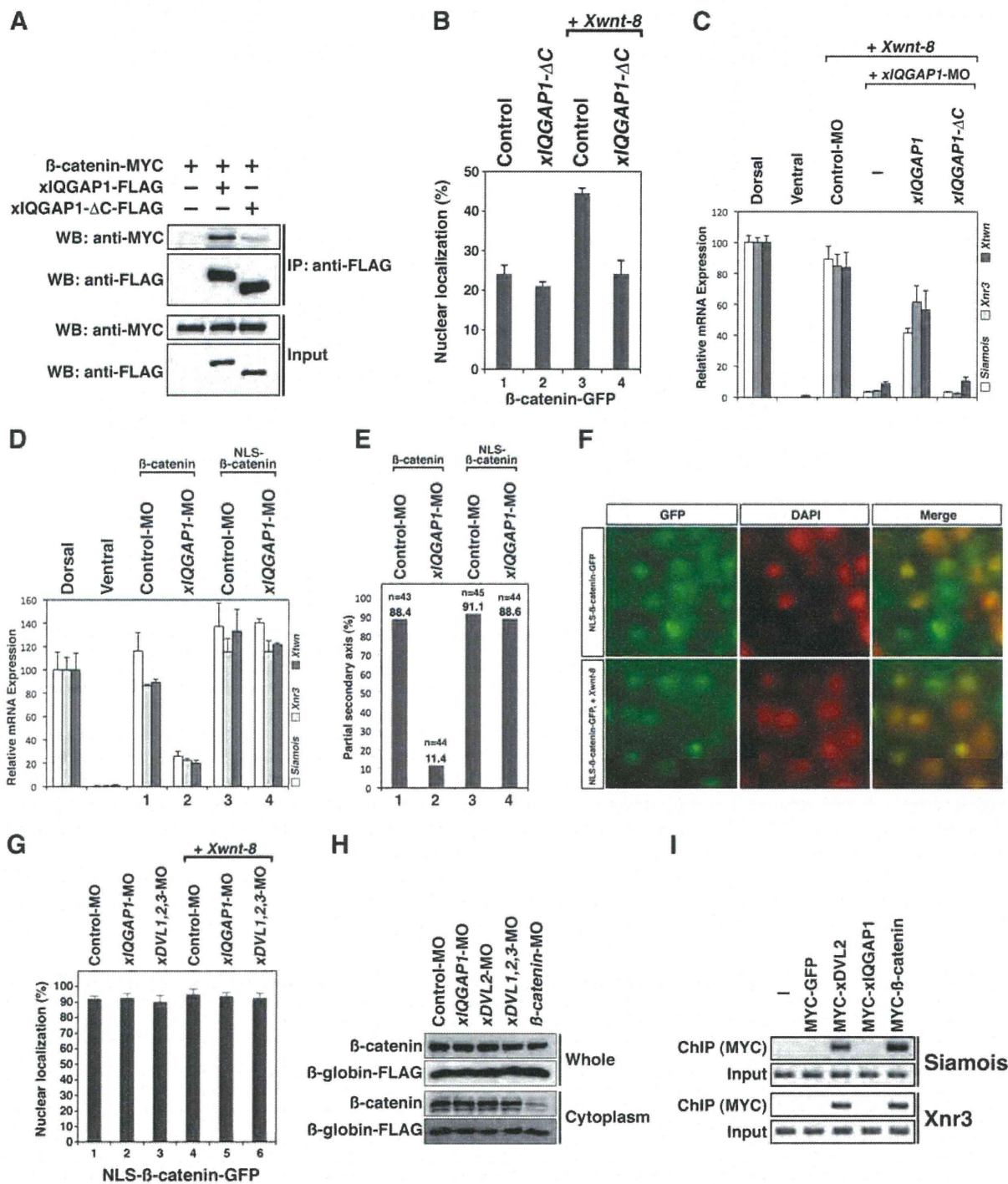
Ran1 Plays a Role of Nuclear Localization of IQGAP1 in Canonical Wnt Signaling—The transport cycle of importin- β is regulated by a small GTPase, Ran (27, 37). To confirm whether

FIGURE 1. **Interaction between β -catenin, xDVL2, and xIQGAP1.** A, interaction between ectopically expressed xDVL2 and β -catenin in HEK 293T cells. WB, Western blotting; IP, immunoprecipitation. B, interaction between ectopically expressed xIQGAP1 and β -catenin in HEK 293T cells. C, interaction among endogenous β -catenin, hDVL1, and hIQGAP1 in SW480 cells. D, interaction among ectopically expressed β -catenin, xDVL2, and xIQGAP1 in HEK 293T cells. Anti-FLAG antibody was used for the first immunoprecipitation, and the precipitate was subjected to a second immunoprecipitation with IgG or MYC antibodies. E, the second immunoprecipitation/input ratios for xDVL2, xIQGAP1, and β -catenin in panel D. F, nuclear localization of β -catenin-GFP in stage 10 *Xenopus* animal cap cells overexpressing Xwnt-8. Control-MO (15 ng), xIQGAP1-MO (15 ng), or xDVL1-MO, xDVL2-MO, and xDVL3-MO (each 5 ng) was co-injected with β -catenin-GFP mRNA (10 pg). *Left*, GFP signals. *Center*, DAPI staining of animal cap cells. *Right*, merge. G–J, the ratio of cells that had nuclear fluorescence signals of GFP. The average of ratio was taken with six explants in three independent experiments. Error bars represent S.D. with six explants. G, the ratio of nucleus-localized β -catenin-GFP in cells injected with xIQGAP1-MO and xDVL2-MO. Lane 1, $n = 1770$, 20.6%; lane 2, $n = 1437$, 10.9%; lane 3, $n = 1662$, 19.6%; lane 4, $n = 1655$, 35.8%; lane 5, $n = 1763$, 15.2%; lane 6, $n = 1874$, 33.9%. H, the ratio of nucleus-localized β -catenin-GFP in cells injected with xDVL1-MO, xDVL2-MO, and xDVL3-MO. Lane 1, $n = 1858$, 19.9%; lane 2, $n = 4689$, 6.4%; lane 3, $n = 5382$, 36.8%; lane 4, $n = 3650$, 18.0%. I, the ratio of nucleus-localized xDVL2-GFP in cells injected with β -catenin-MO. Lane 1, $n = 971$, 24.6%; lane 2, $n = 989$, 14.2%; lane 3, $n = 347$, 45.5%; lane 4, $n = 326$, 24.5%. J, the ratio of nucleus-localized xIQGAP1-GFP in cells injected with β -catenin-MO. Lane 1, $n = 784$, 21.9%; lane 2, $n = 616$, 20.9%; lane 3, $n = 544$, 39.0%; lane 4, $n = 885$, 27.9%.

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Ran is related to the nuclear import of IQGAP1 in the canonical Wnt signaling pathway, we investigated the effects of Ran in cultured cells and *Xenopus* embryos. We confirmed that both ectopically expressed *xIQGAP1* and ectopically expressed *xIpo-β5* bound to *xRan1* (Fig. 5, A and B). The endogenous interaction of human Ran to both human IQGAP1 and human importin-β5 was also confirmed (Fig. 5, C and D). Dorsal injection

of *xRan1*-MO reduced the endogenous expression of Wnt signal target genes at the gastrula stage (Fig. 5E). We also found that the expression of the Wnt target genes induced by ventral injection of *Xwnt-8* was reduced by depletion of *xRAN1* and that its reduction was rescued by *xRan1* mRNA (Fig. 5F). The injection of both *xIpo-β5*-MO and *xRan1*-MO synergistically reduced Wnt target gene expression induced by ventral injection



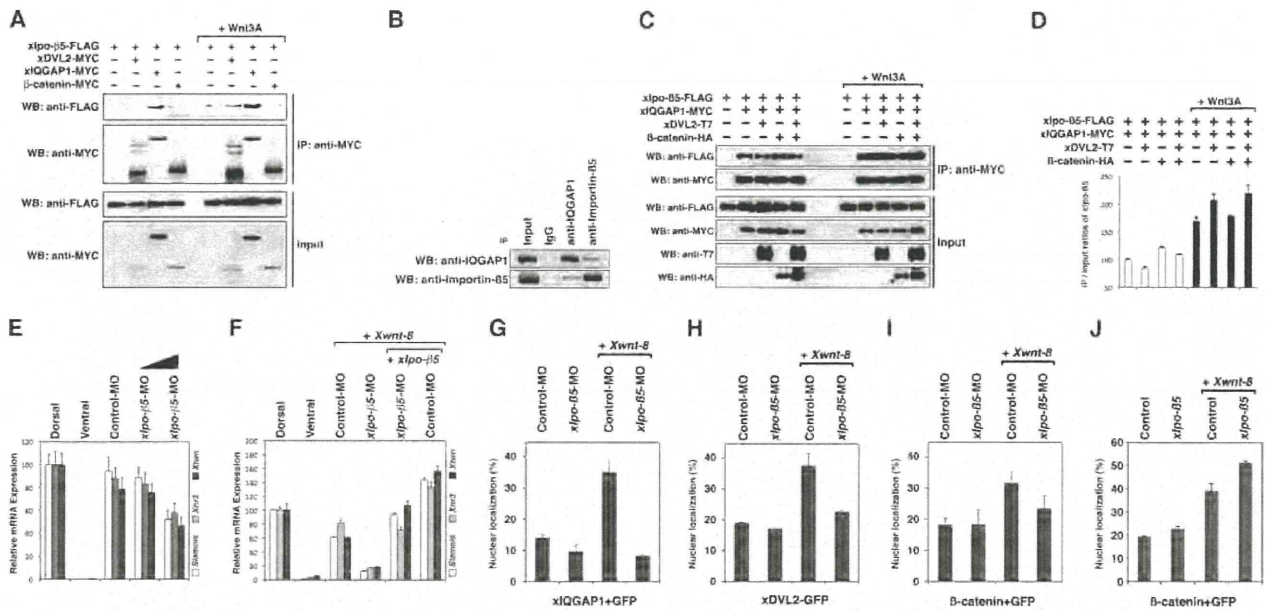


FIGURE 4. Interaction between xIQGAP1 and xIpo-β5 in canonical Wnt signaling. *A*, interaction between ectopically expressed xIpo-β5 and xDVL2, xIQGAP1, and β-catenin in HEK 293T cells. *WB*, Western blotting; *IP*, immunoprecipitation. *B*, interaction between endogenous human IQGAP1 and human importin-β5 in HEK 293T cells. *C*, interaction between xIQGAP1 and xIpo-β5 with ectopically expressed xDVL2 and β-catenin in HEK 293T cells. Immunoprecipitates obtained using anti-MYC antibody were subjected to Western blotting with the indicated antibodies. +, present; -, absent. *D*, the immunoprecipitates/input ratios for xIpo-β5 in panel *C*. *Error bars* represent S.D. in three experiments. *E*, quantitative RT-PCR analysis of early dorsal Wnt target genes (*n* = 3). Control-MO (80 ng) or xIpo-β5-MO (low dose: 40 ng, high dose: 80 ng) was injected into two dorsal blastomeres of four-cell embryos. The following procedure was described in Fig. 3C. *F*, quantitative RT-PCR analysis of early dorsal Wnt target genes (*n* = 3). Control-MO (40 ng), xIpo-β5-MO (40 ng), and Xwnt-8 (0.5 pg) mRNA were ventrally co-injected with xIpo-β5 mRNA (500 pg). *G–J*, the ratio of GFP-fused constructs localized in the nucleus in cells injected with xIpo-β5-MO or xIpo-β5 mRNA. *G*, xIQGAP1-GFP. Lane 1, *n* = 1859, 14.0%; lane 2, *n* = 1216, 9.7%; lane 3, *n* = 909, 35.1%; lane 4, *n* = 887, 8.3%. *H*, xDVL2-GFP. Lane 1, *n* = 932, 18.9%; lane 2, *n* = 540, 16.9%; lane 3, *n* = 669, 37.2%; lane 4, *n* = 402, 22.4%. *I*, β-catenin-GFP. Lane 1, *n* = 1125, 18.3%; lane 2, *n* = 636, 18.5%; lane 3, *n* = 1317, 40.0%; lane 4, *n* = 752, 23.7%. *J*, β-catenin-GFP. Lane 1, *n* = 495, 19.4%; lane 2, *n* = 966, 22.8%; lane 3, *n* = 796, 39.2%; lane 4, *n* = 399, 51.4%. *Error bars* represent S.D. with six explants.

tion of Xwnt-8 (Fig. 5G). In addition, depletion of xRan1 reduced the nuclear localization of β-catenin-GFP by co-expressing Xwnt-8 in animal cap cells (Fig. 5H). On the other hand, overexpression of xRan1 mRNA increased the nuclear localization of β-catenin-GFP nuclear localization (Fig. 5I). We confirmed that both depletions of xIpo-β5 and xRan1 did not affect the stability of cytoplasmic β-catenin (Fig. 5J). Interestingly, the Ras-GAP domain-deleted xIQGAP1 construct, xIQGAP1-ΔRGD, did not bind to xRan1 (Fig. 6A) and xIpo-β5 (Fig. 6B). The expression of xIQGAP1-ΔRGD did not rescue expression of Wnt target genes that were reduced by xIQGAP1-MO (Fig. 6C) and reduced the nuclear localization of

β-catenin-GFP in the Xwnt-8-expressing cells (Fig. 6D). These results suggest that the interaction between xIQGAP1 and xIpo-β5, or xIQGAP1 and xRan1, is indispensable for the function of xIQGAP1 in Wnt signaling. Interestingly, the expression of xIQGAP1 in HEK 293T cells increased the GTP-bound activated form of xRan1 in the same way as the effect of xRanGEF (Fig. 6E), but the interaction between xIQGAP1 and either xRanGAP or xRanGEF was not observed (Fig. 6F). The hydrolysis of xRAN1 by xRanGAP was reduced by xIQGAP1 (Fig. 6G), and the expression of xIQGAP1 inhibited the interaction between the active form of xRAN1 and xRanGAP (Fig. 6H). These results suggest that xIQGAP1 has no GAP activity but

FIGURE 3. The role of xIQGAP1 in canonical Wnt signaling. *A*, interaction between ectopically expressed β-catenin and xIQGAP1 or xIQGAP1-ΔC in HEK 293T cells. *B*, the ratio of β-catenin-GFP localized in the nucleus in cells injected with xIQGAP1-ΔC mRNA. Lane 1, *n* = 635, 24.3%; lane 2, *n* = 1517, 21.2%; lane 3, *n* = 459, 44.7%; lane 4, *n* = 1271, 24.3%. *C*, quantitative RT-PCR analysis of early dorsal Wnt target genes (*n* = 3). Control-MO (15 ng) or xIQGAP1-MO (15 ng) was co-injected with xIQGAP1 (400 pg) or xIQGAP1-ΔC (400 pg) mRNA into two ventral blastomeres of four-cell embryos. RNAs from dissected ventral sectors of injected embryos were extracted at stage 10. RNAs from dissected dorsal and ventral sectors of uninjected embryos were used as controls. The value obtained for each gene was normalized to the level of ODC (ornithine decarboxylase). The value of dorsal sectors was set to 100, and other values were computed. *Error bars* represent S.D. in three experiments. *D*, quantitative RT-PCR analysis of early dorsal Wnt target genes (*n* = 3). Control-MO (15 ng) or xIQGAP1-MO (15 ng) was co-injected with β-catenin (20 pg) or NLS-β-catenin (20 pg) mRNA into two ventral blastomeres of four-cell embryos. The following procedure is indicated in panel *C*. *E*, the ratio of injected embryos exhibiting a partial secondary axis. The numbered lanes indicate the injected mRNAs and MOs consistent with the numbering in panel *D*. *F*, nuclear localization of NLS-β-catenin-GFP in stage 10 *Xenopus* animal cap cells overexpressing Xwnt-8. *Left panels*, GFP signals. *Center panels*, DAPI staining. *Right panels*, merge. *G*, the ratio of nucleus-localized NLS-β-catenin-GFP in cells injected with xIQGAP1-MO and xDVL1-, 2-, 3-MO in *Xenopus* animal cap cells at stage 10. Lane 1, *n* = 352, 92.0%; lane 2, *n* = 347, 92.5%; lane 3, *n* = 396, 89.9%; lane 4, *n* = 907, 94.7%; lane 5, *n* = 512, 93.6%; lane 6, *n* = 359, 92.4%. *H*, Western blotting analysis using β-catenin antibody. Control-MO (15 ng), xIQGAP1-MO (15 ng), xDVL2-MO (15 ng), xDVL3-MO (15 ng), xDVL1-MO (5 ng), xDVL2-MO (5 ng), xDVL3-MO (5 ng), or β-catenin-MO (15 ng) was co-injected with β-globin-FLAG mRNA (100 pg) into two dorsal blastomeres of four-cell embryos. Lysates from whole embryos or cytoplasmic fractions were obtained dissected dorsal sectors at stage 10 and were subjected to Western blotting with the β-catenin and FLAG antibodies. *I*, CHIP assay for Siamesis and Xnr3 promoter regions. Each indicated mRNA (200 pg) was injected into two dorsal blastomeres of four-cell embryos. Injected dorsal sectors were dissected and cross-linked at stage 10. Immunoprecipitates using anti-MYC antibody were examined by PCR using specific primers of Siamesis and Xnr3 promoter regions.

A Role of IQGAP1 in Wnt Signaling

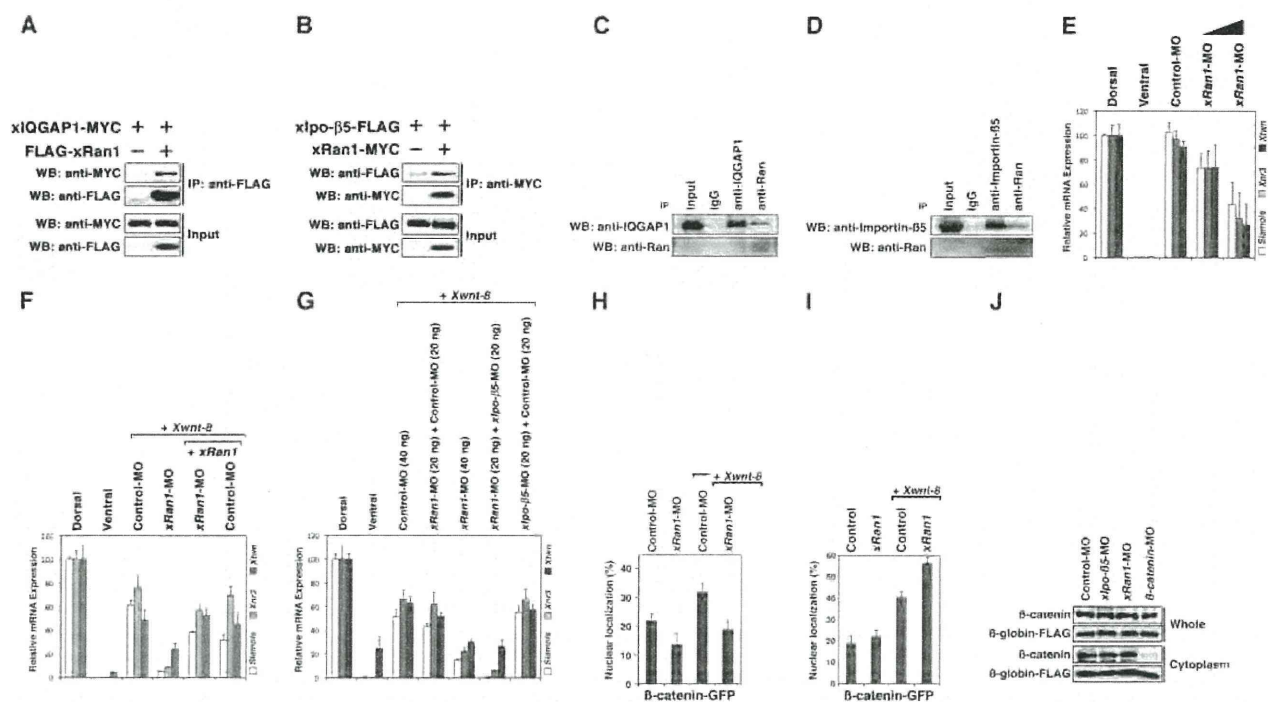


FIGURE 5. The role of xRan1 in canonical Wnt signaling. *A*, interaction between ectopically expressed xIQGAP1 and xRan1 in HEK 293T cells. *B*, interaction between ectopically expressed xIpo- β 5 and xRan1 in HEK 293T cells. *C*, interaction between endogenous human IQGAP1 and human Ran in HEK 293T cells. *D*, interaction between endogenous human importin- β 5 and human Ran in HEK 293T cells. *E–G*, quantitative RT-PCR analysis of early dorsal Wnt target genes ($n = 3$). *E*, control-MO (80 ng) or xRan1-MO (low dose: 40 ng, high dose: 80 ng) was injected into two dorsal blastomeres of four-cell embryos. RNAs from dissected dorsal sectors of injected embryos were extracted at stage 10. The following procedure was described in Fig. 3C. *F*, control-MO (40 ng), xRan1-MO (40 ng), and *Xwnt-8* (0.5 pg) mRNA were ventrally co-injected with xRan1 mRNA (500 pg). *G*, the indicated amounts of control-MO, xIpo- β 5-MO, and xRan1-MO were ventrally co-injected with *Xwnt-8* mRNA (0.5 pg). *H*, the ratio of β -catenin-GFP localized in the nucleus in cells injected with xRan1-MO. Lane 1, $n = 914$, 21.8%; lane 2, $n = 1006$, 13.6%; lane 3, $n = 2155$, 31.8%; lane 4, $n = 2245$, 18.8%. *I*, the ratio of β -catenin-GFP localized in the nucleus in cells injected with xRan1 mRNA. Lane 1, $n = 466$, 19.1%; lane 2, $n = 718$, 22.3%; lane 3, $n = 1582$, 40.8%; lane 4, $n = 448$, 56.7%. *J*, Western blotting analysis using β -catenin antibody. Control-MO (40 ng), xIpo- β 5-MO (40 ng), xRan1-MO (15 ng), or β -catenin-MO (15 ng) was co-injected into two dorsal blastomeres of four-cell embryos. The following procedure was described in Fig. 3H.

indirectly maintains the active status of Ran. On the other hand, the GTP-bound form of xRan1 was promoted by xRanGEF, but not by xIQGAP1 *in vitro* (Fig. 6I), and the interaction between the inactive form of xRan1 and xRanGEF was not inhibited by xIQGAP1 (Fig. 6J). These data suggest that xIQGAP1 has no GEF activity and does not inhibit RanGEF function via Ran. Moreover, xIQGAP1 bound preferably to the GDP-bound form of xRan1 rather than the GTP-bound form (Fig. 6K). Taken together, these results suggest that Ran regulates the nuclear import of IQGAP1 and β -catenin in Wnt signaling pathway through the alteration of Ran activation status by IQGAP1.

DISCUSSION

In the present studies, we show that IQGAP1 is necessary for the nuclear localization of β -catenin in the canonical Wnt signaling pathway. We have also shown that β -catenin physically interacts with IQGAP1. Two previous studies have also reported a relationship between IQGAP1 and β -catenin (14, 15). One showed that IQGAP1 inhibits β -catenin function in cell-cell adhesion and plays a role in the dissociation of α -catenin from the cadherin-catenin complex (15). Another study showed that IQGAP1 stimulates β -catenin-mediated transcription (14). Thus, it appears that IQGAP1 promotes the

translocation of β -catenin from the cell membrane to the cytoplasm, resulting in an increase in cytoplasmic β -catenin, which facilitates its own import into the nucleus to activate Wnt target genes. However, our data clearly showed that the depletion of xIQGAP1 suppressed the nuclear localization of β -catenin, formation of secondary axis, and induction of Wnt target genes without any effects on the β -catenin stability in the cytoplasm. We also observed that xIQGAP1-MO injection had no effect on the predominant nuclear localization of NLS- β -catenin. Thus, our results provide the first indication that IQGAP1 is critical in determining the nuclear translocation of β -catenin in the Wnt signaling pathway.

Our results show that xIQGAP1, xDVL2, and β -catenin form a complex and that Wnt stimulation increases the interaction of these proteins. Depletion of xIQGAP1, β -catenin, or xDVL2 reduced the Wnt-stimulated nuclear localization of other proteins. Moreover, formation of the secondary axis and induction of Wnt target genes by xDvl2, *Xwnt-8*, or β -catenin were also suppressed by the depletion of xIQGAP1 (our results and see Ref. 25). Taken together, these results clearly indicate that xIQGAP1, xDVL2, and β -catenin form a complex and are mutually required for nuclear localization and transactivation of Wnt target genes.

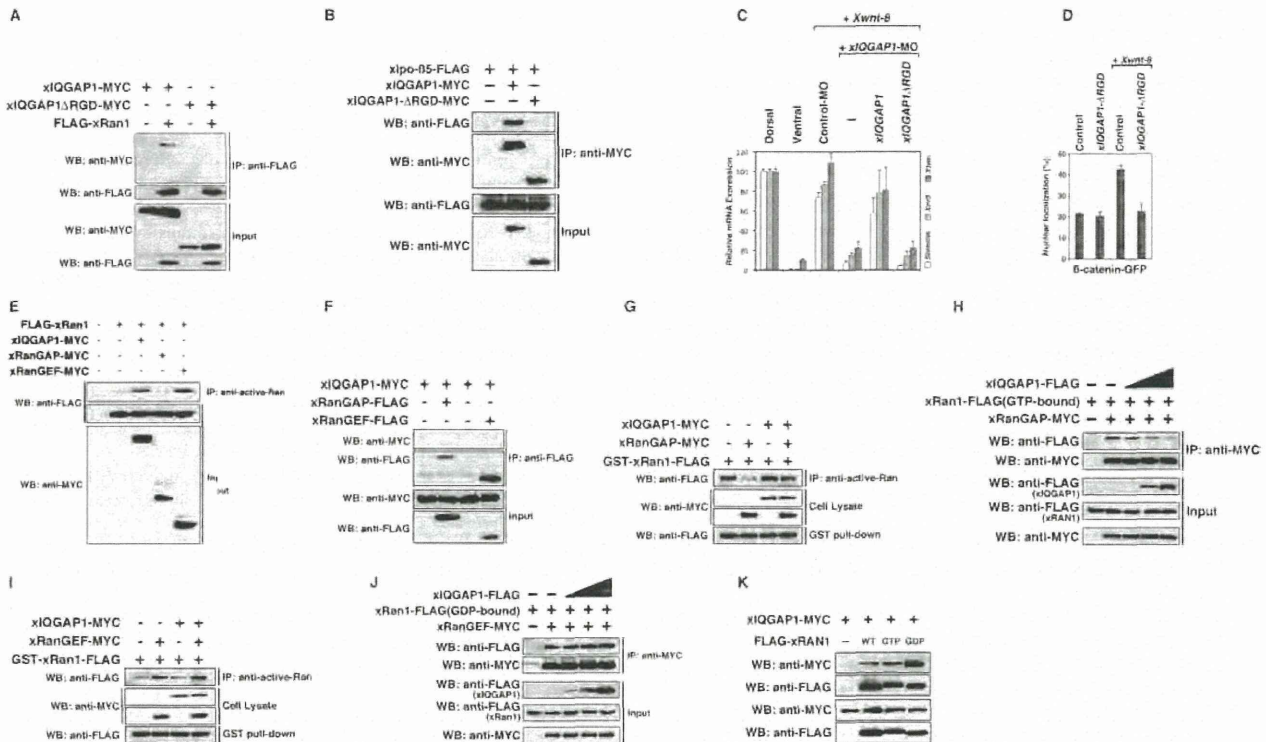


FIGURE 6. Interaction between xIQGAP1 and xRan1 in canonical Wnt signaling. *A*, interaction between ectopically expressed xRan1 and xIQGAP1 or xIQGAP1-ΔRGD in HEK 293T cells. *WB*, Western blotting; *IP*, immunoprecipitation. *B*, interaction between ectopically expressed xIpo-β5 and xIQGAP1 or xIQGAP1-ΔRGD in HEK 293T cells. *C*, quantitative RT-PCR analysis of early dorsal Wnt target genes ($n = 3$). Control-MO (15 ng), xIQGAP1-MO (15 ng), and Xwnt-8 (0.5 pg) mRNA were ventrally co-injected with xIQGAP1 (400 pg) or xIQGAP1-ΔRGD mRNA (400 pg). *D*, the ratio of β-catenin-GFP localized in the nucleus in cells injected with xIQGAP1 constructs mRNA. Lane 1, $n = 771$, 21.4%; lane 2, $n = 2165$, 20.5%; lane 3, $n = 856$, 42.5%; lane 4, $n = 549$, 22.6%. *E*, Ran activation assay. xRan1 and xIQGAP1, xRanGAP, or xRanGEF were ectopically expressed in HEK 293T cells. Immunoprecipitates obtained using anti-active-Ran antibody were subjected to Western blotting with the indicated antibodies. *F*, interaction between ectopically expressed xIQGAP1 and xRanGAP or xRanGEF in HEK 293T cells. No interaction was observed. *G*, GAP assay. GTP-loaded GST-xRan1-FLAG was incubated with cell lysates from HEK 293T cells transfected with pCS2+ (control), xRanGAP-MYC, or xIQGAP1-MYC and immunoprecipitated with anti-active Ran antibody. *H*, interaction between ectopically expressed xRanGAP and active form of xRan1 (G19V, Q69L) with xIQGAP1 in HEK 293T cells. Their interactions were inhibited by expression of xIQGAP1 in a dose-dependent manner. *I*, GEF assay. GST-xRan1-FLAG was incubated with cell lysates from HEK 293T cells transfected with pCS2+ (control), xRanGEF-MYC, or xIQGAP1-MYC and immunoprecipitated with anti-active Ran antibody. *J*, interaction between ectopically expressed xRanGEF and the inactive form of xRan1 (T24N) with xIQGAP1 in HEK 293T cells. *K*, interaction between ectopically expressed xIQGAP1 and active or inactive form of xRan1 in HEK 293T cells. *WT*, wild type; *GTP*, active form of xRan1; *GDP*, inactive form of xRan1.

It is known that the nuclear import of β-catenin does not involve importin or an importin-mediated mechanism and that β-catenin itself functions as a nuclear import receptor (38–40). Our data showed that xIpo-β5 is associated with xIQGAP1, but not with either xDVL2 or β-catenin, and that xIpo-β5 is necessary for the nuclear localization of β-catenin and the induction of Wnt target genes. Because previous works did not detect the presence of IQGAP1, they might not be able to find the importin-dependent nuclear localization of β-catenin. Although our findings suggest a new molecular mechanism mediating xIQGAP1-importin-dependent nuclear localization of β-catenin, we cannot exclude the importin-independent nuclear localization of β-catenin. Further studies might need to clarify this discrepancy.

Our results clearly indicate that the nuclear import of xDVL2-xIQGAP1-β-catenin complex is mediated by the interaction between xIpo-β5 and xIQGAP1. In the meantime, a target protein is released from importin-β-cargo complex by the binding of the active form of Ran, RanGTP, in the nucleus (27), and RanGEF generates enrichment of the RanGTP form in the

nucleus (37). We found that xIQGAP1 had no GAP or GEF activity and indicated different affinities between RanGAP and RanGEF. Interestingly, the expression of xIQGAP1 increased the RanGTP form through the reduction of interaction between RanGAP and Ran. In addition, xDVL2 and β-catenin were recruited to the promoter regions of the Wnt target genes, whereas IQGAP1 was not recruited. Thus, these results suggest that xIQGAP1 promotes the release of xDVL2 and β-catenin from importin-β-cargo complex through the active Ran in the nucleus. Altogether, our results demonstrate the existence of a new molecular mechanism regulating the nuclear import of β-catenin and modulating canonical Wnt signaling.

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