Caprin-2 enhances canonical Wnt signaling through regulating LRP5/6 phosphorylation

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The low-density lipoprotein receptor–related proteins 5 and 6 (LRP5/6) are coreceptors for Frizzled and transmit signals from the plasma membrane to the cytosol. However, the mechanism for LRP5/6 signal transmission remains undefined. Here, we identify cytoplasmic activation/proliferation-associated protein 2 (Caprin-2) as a LRP5/6-binding protein. Our data show that Caprin-2 stabilizes cytosolic β-catenin and enhances lymphoid enhancer-binding factor 1/T cell factor–dependent reporter gene activity as well as the expression of Wnt target genes in mammalian cells. Morpholino-mediated knockdown of Caprin-2 in zebrafish embryos inhibits Wnt/β-catenin signaling and results in a dorsalized phenotype. Moreover, Caprin-2 facilitates LRP5/6 phosphorylation by glycogen synthase kinase 3, and thus enhances the interaction between Axin and LRP5/6. Therefore, Caprin-2 promotes activation of the canonical Wnt signaling pathway by regulating LRP5/6 phosphorylation.

Introduction

The Wnt signaling pathway plays pivotal roles during embryogenesis and is also linked to tumorigenesis (Logan and Nusse, 2004; Clevers, 2006). Two types of cell surface receptors, seven-transmembrane protein Frizzled (Fz), and single-pass membrane proteins low-density lipoprotein receptor–related protein 5 and 6 (LRP5/6), are required for transducing the Wnt signal. LRP5/6 is homo-oligomerized and hetero-oligomerized with Fz through binding to Wnt proteins (Tamai et al., 2000; Cong et al., 2004). After that, LRP5/6 is phosphorylated and activated (Davidson et al., 2005; Zeng et al., 2005). Activated LRP5/6 recruits Axin to the plasma membrane and promotes Axin degradation, which results in the activation of Wnt signaling (Mao et al., 2001b). Recently, caveolin-dependent internalization of the LRP6–Axin complex was also reported to be important for activation of Wnt signaling (Yamamoto et al., 2006).

Several mechanisms were raised to illustrate how the activity of LRP5/6 is regulated. For example, Dickkopf (DKK) binds LRP6, thus causing an inactivation of LRP6 (Bafico et al., 2001; Mao et al., 2001a), and R-Spondin 1 activates Wnt signaling through releasing LRP6 from the inhibition of DKK (Binnerts et al., 2007). Wise and SOST were also found to interact with LRP5/6 and compete with Wnt and Fz for binding to LRP5/6 (Itasaki et al., 2003; Semenov et al., 2005). ER-retained Wise also reduces LRP6 on the cell surface, and thereby inhibits Wnt signaling (Guidato and Itasaki 2007).

The activity of LRP5/6 is also regulated by phosphorylation. Phosphorylation results in the activation of LRP5/6 and is important for the interaction between LRP5/6 and Axin (Mao et al., 2001b, Davidson et al., 2005; Zeng et al., 2005). It has been previously shown that several PPP(S/T)P motifs within the intracellular domain of LRP5 are required for LRP5/6–Axin interaction (Mao et al., 2001b). Casein kinase I γ and glycogen synthase kinase 3 (GSK3) are responsible for the phosphorylation at the motif (Davidson et al., 2005; Zeng et al., 2005). Recent work suggested that formation of the LRP6 signalosome in response to Wnt stimulation is required for initiating LRP phosphorylation, and the process is believed to be mediated by Dishevelled (Dvl; Bilic et al., 2007). Axin was also reported to be involved in the regulation of LRP5/6 phosphorylation (Zeng et al., 2008). Nevertheless, the precise mechanism by which LRP5/6 phosphorylation is regulated remains elusive. In this work, we identified cytoplasmic activation/proliferation-associated protein 2 (Caprin-2) as a novel LRP5/6-binding protein. Our data show

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Abbreviations used in this paper: Caprin, cytoplasmic activation/proliferation-associated protein; Dvl, Dishevelled; Fz, Frizzled; hpf, hours postfertilization; LEF, lymphoid enhancer-binding factor; LRP, low-density lipoprotein receptor–related protein; MO, morpholino oligonucleotides; TCF, T cell factor.

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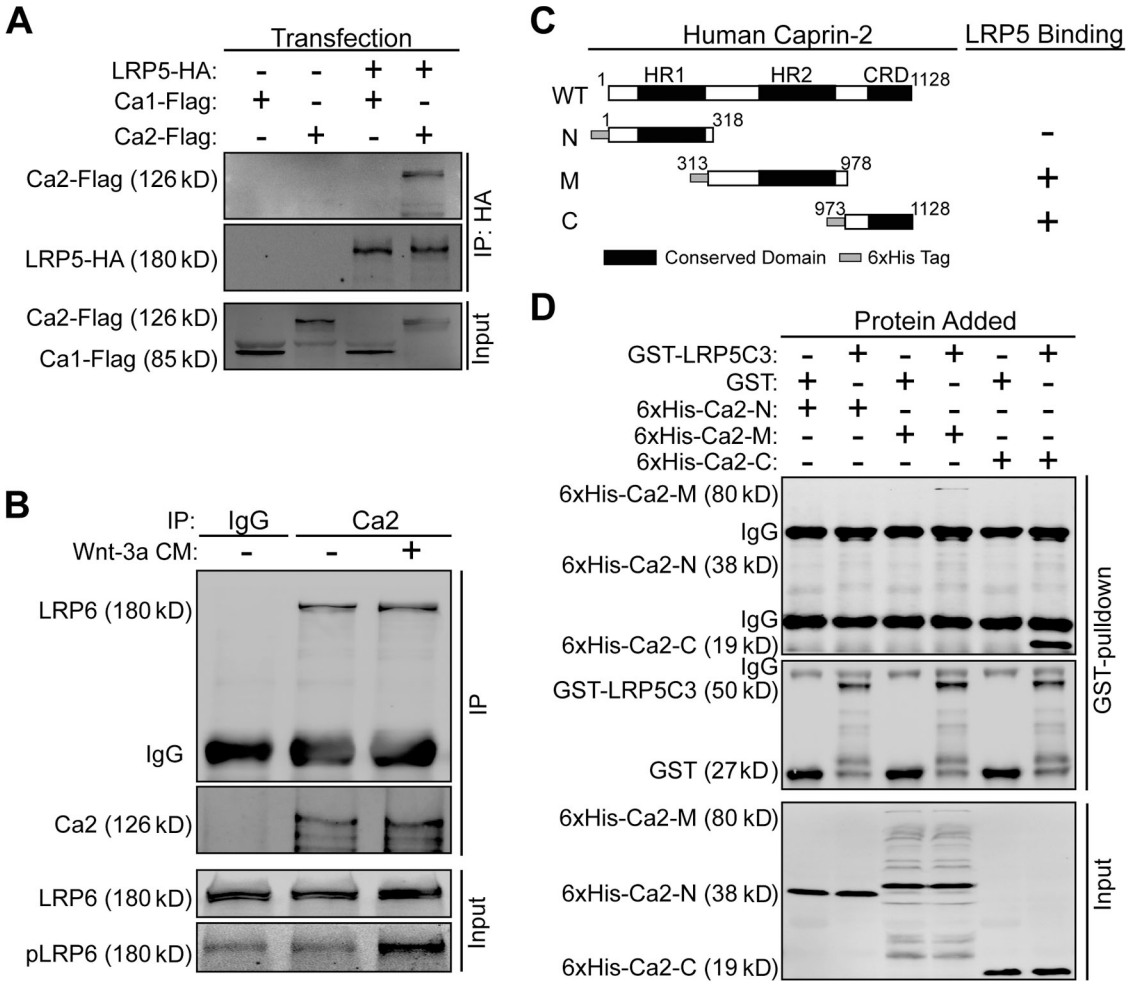

Figure 1. **Caprin-2 interacts with LRP5/6.** (A) HEK-293T cells were transiently transfected with HA-tagged LRP5 and Flag-tagged Caprin-1 (Ca1) or Caprin-2 (Ca2). Cell lysates were incubated with indicated antibodies and subsequently analyzed by Western blotting. (B) Coimmunoprecipitation of endogenous Caprin-2–LRP6 complexes in HEK-293T cells. Immunoprecipitation was performed with an anti–Caprin-2 polyclonal antibody. IgG was used as control. LRP6 was detected in the complex by an LRP6 antibody. (C) Schematic representation of Caprin-2's fragments. Numbers indicate amino acids. CRD, C1q region domain; HR, homologous region. (D) In vitro binding assay. GST fused LRP5C3 and 6× His-tagged Caprin-2 fragments were expressed in *E. coli*. Indicated proteins were mixed and then pulled down by an anti-GST antibody.

that Caprin-2 plays an important role in regulating GSK3-mediated phosphorylation of LRP5/6.

Results and discussion

Caprin-2 interacts with LRP5/6

LRP5/6 acts as a coreceptor of Fz to transduce signal from membrane to cytosol (Tamai et al., 2000; Cong et al., 2004). However, the precise mechanism by which LRP5/6 mediates Wnt signaling at the plasma membrane remains to be defined. To address this question, we sought to explore potential partners that interact with LRP5/6. HEK-293T cells were transiently transfected with a Flag-tagged truncated form of LRP5, LRP5C2-Flag, which lacks the extracellular domain and constitutively activates Wnt–β-catenin signaling (Mao et al., 2001b). Immunoprecipitation was performed with the anti-Flag antibody. Samples were then separated on SDS-PAGE and subsequently processed with mass spectrometry analysis followed by protein database searching. Among the proteins identified in the LRP5C2 complex, we found a novel LRP5-binding protein named Caprin-2, which was previously identified as a member of cytoplasmic activation/proliferation-associated proteins family (Aerbajinai et al., 2004; Grill et al., 2004). The Caprin family contains two members, which share two homologous regions that are highly conserved. The first discovered family member, Caprin-1, is highly expressed in brain and tissues capable of proliferation. It has been shown that the function of Caprin-1 is related to cell proliferation (Grill et al., 2004; Wang et al., 2005; Solomon et al., 2007). The function of Caprin-2 has remained unclear. A previous study showed that during blood cell differentiation, the expression level of Caprin-2 changes dramatically, which suggests that Caprin-2 may function in cell differentiation (Aerbajinai et al., 2004).

To confirm our mass spectrometry data, we performed coimmunoprecipitation assay in HEK-293T cells. LRP5 was cotransfected with either Caprin-1 or Caprin-2, and the result indicates that only Caprin-2 but not Caprin-1 (Fig. 1 A) interacts with LRP5. To substantiate this observation, we generated an antibody against Caprin-2. This antibody was shown to efficiently detect and immunoprecipitate both endogenously and exogenously expressed Caprin-2 protein. We then pulled down endogenous Caprin-2

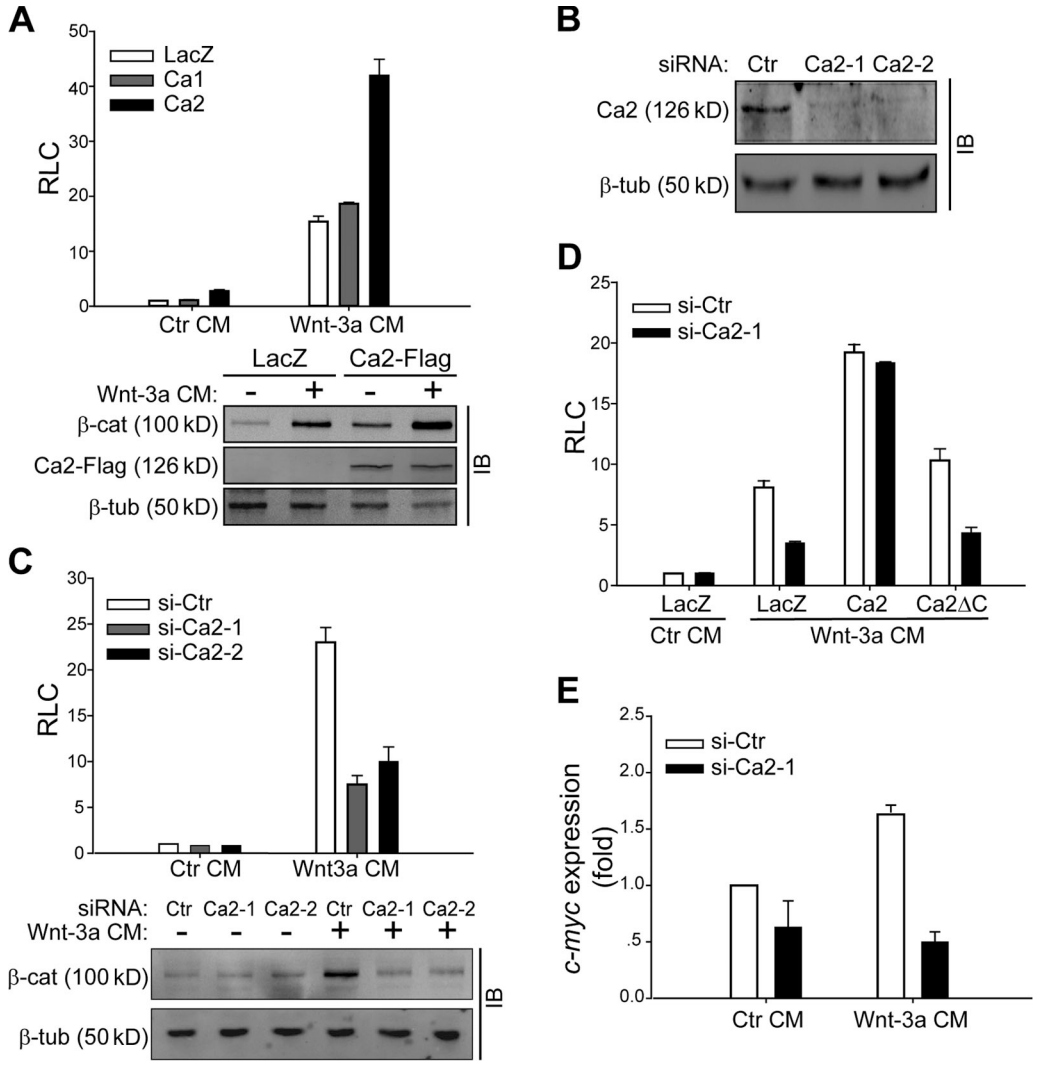

Figure 2. **Caprin-2 activates Wnt-induced LEF-1/TCF transcriptional activity.** (A) Caprin-1 or Caprin-2 was overexpressed in HEK-293 cells with LEF-1 and LEF-luciferase reporter. 14 h after transfection, cells were treated with control or Wnt-3a conditioned medium (CM) for 6 h, then luciferase activity was determined (top). To detect the β-catenin level in cytosol, cells were stimulated with Wnt-3a CM for 3 h and examined by anti–β-catenin antibody, and β-tubulin was used as a cytoplasmic marker (bottom). (B) The efficiency of Caprin-2 siRNAs was examined. Two independent siRNAs against Caprin-2 were used. 72 h after transfection, cells were lysed and detected by Caprin-2 antibody. (C) Control and Caprin-2 siRNAs were transfected with a LEF-1–dependent reporter plasmid. After 72 h, cells were treated with control or Wnt-3a CM, and luciferase activity (top) and cytoplasmic β-catenin (bottom) were examined. (D) A rescue experiment was performed using either the full-length or C-terminal C1q region truncated form of Caprin-2 plasmids (amino acids 1–935, Ca2ΔC) with Caprin-2 siRNA. (E) Real-time PCR analysis of *c-myc* RNA expression in HEK-293 cells. Expression levels of GAPDH mRNA were used as internal control. Error bars indicate SD of duplicated assays in one experiment. Each experiment was repeated at least three times.

with this antibody, and the result showed that endogenous LRP6 was coimmunoprecipitated with Caprin-2 (Fig. 1 B). We also found that Wnt-3a stimulation has little effect on the binding of Caprin-2 with LRP6.

To determine whether Caprin-2 interacts with LRP5/6 directly, we performed an in vitro binding experiment using recombinant proteins produced from *Escherichia coli.* Because full-length Caprin-2 was hardly expressed in *E. coli.*, we divided it into three fragments according to a previous study (Fig. 1 C; Grill et al., 2004). These fragments include an N-terminal homologous region-1 (HR-1) domain (Ca2-N), the middle HR-2 domain (Ca2-M), and a C1q region domain (CRD) in the C terminus (Ca2-C). All of these fragments were fused with a 6× His tag, and GST was fused to the intracellular domain of LRP5 (LRP5C3; Mao et al., 2001b). As shown in Fig. 1 D, both Ca2-M and Ca2-C interacted with LRP5C3, which suggests that there might be multiple LRP5/6-binding sites within Caprin-2. Together, our results establish that Caprin-2 is a binding partner of LRP5/6.

Caprin-2 activates Wnt-induced lymphoid enhancer-binding factor 1 (LEF-1)/T cell factor (TCF) transcriptional activity

To determine whether Caprin-2 is involved in the Wnt–β-catenin pathway, we overexpressed Caprin-2 in HEK-293 cells and evaluated its effect using the LEF-1 reporter system. As shown in Fig. 2 A, overexpression of Caprin-2 enhanced LEF-1/TCFs–dependent reporter activity. We also examined the potential role of Caprin-1, and our results showed that Caprin-1 did not affect Wnt-induced LEF-1/TCFs-dependent reporter activity. In Caprin-2 overexpressing cells, cytosolic β-catenin was stabilized, similar

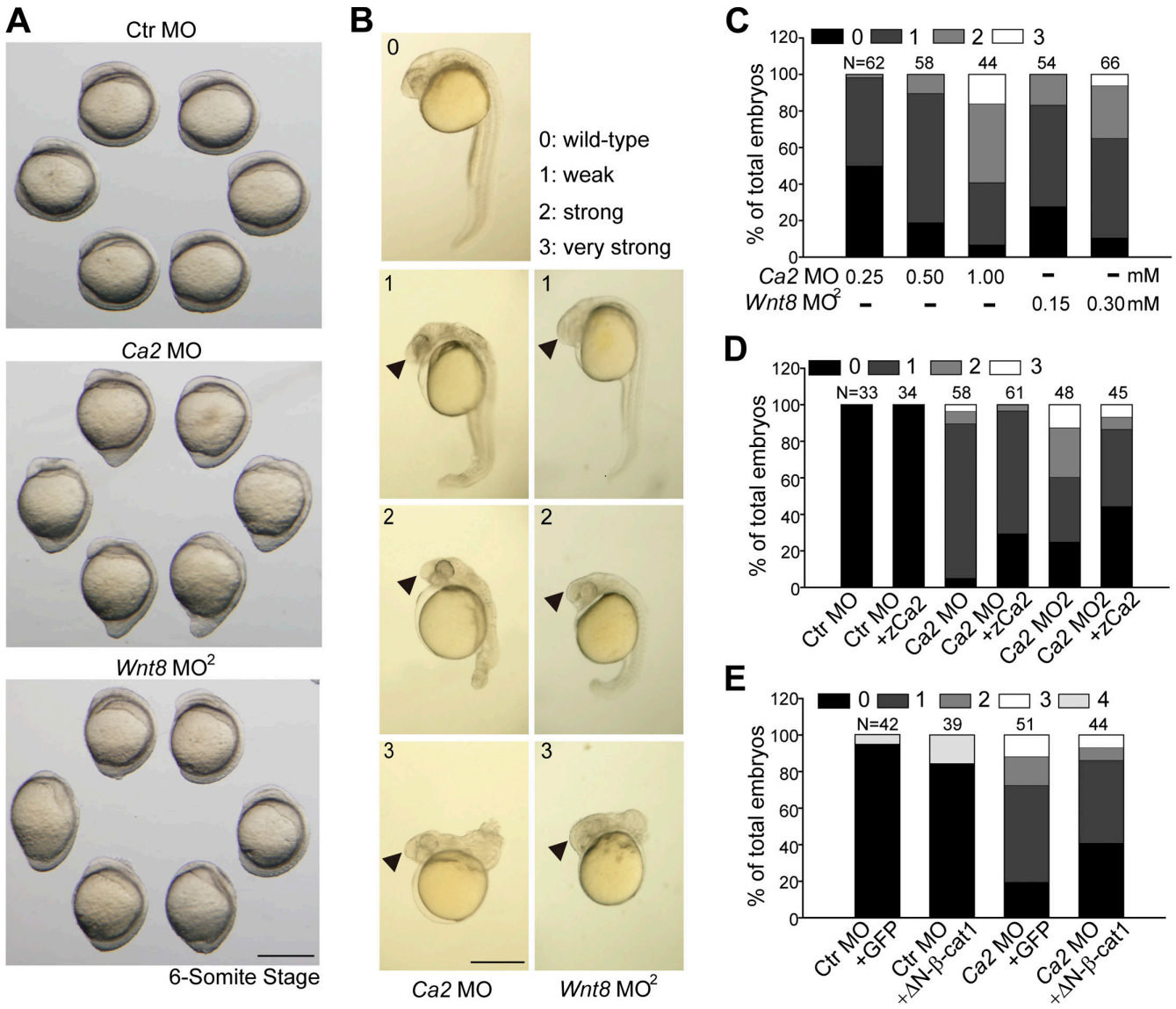

Figure 3. ***Caprin-2* morphants display dorsalized phenotypes.** (A) Morphology of the *Caprin-2* MO– and *Wnt8* MO^2–injected embryos at the 6-somite stage. The concentrantion for *Caprin-2* MO was 0.5 mM, and for each of *Wnt8* MO^2, 0.3 mM. (B) Phenotype of the *Caprin-2* MO– and *Wnt8* MO^2–injected pharyngula stage (24 hpf) embryos. The dorsalized phenotypes of the injected embryos were classified into four categories. 0, wild type; 1, modest enlargement of the telencephalon (arrowhead in panel 1); 2, strong anteriorization phenotype with enlargement of the head (arrowhead in panel 2) and reduction of the tail; and 3, very strong anteriorization phenotype with super enlargement of the telencephalon (arrowhead in panel 3) and major loss of trunk and tail. Bars, 500 µm. (C) Percentages of embryos were evaluated. Embryos were injected with *Caprin-2* MO or *Wnt8* MO^2 as indicated. The number of embryos scored (*n*) is shown on top of each bar. (D) Phenotype penetrance is shown. Embryos were coinjected with 0.5 mM *Caprin-2* MO or 0.2 mM *Caprin-2* MO2 with 20 pg/nl of *zCaprin-2*. (E) *Caprin-2* morphants could be partially restored by 12 pg/nl of ΔN–β-catenin coinjection. Injection of the plasmids generates abnormal embryos with a reduced tail and small head (indicated as 4).

to what is seen in cells treated with Wnt-3a (Fig. 2 A, bottom). These data indicate that overexpression of Caprin-2 facilitates the activation of Wnt signaling by increasing the accumulation of cytoplasmic β-catenin.

To confirm the function of Caprin-2 in vivo, we performed knockdown experiments by using Caprin-2 siRNAs. The efficiency of RNAi was examined by Western blotting using the Caprin-2 antibody (Fig. 2 B). We then examined the effects of Caprin-2 knockdown on canonical Wnt signaling. As shown in Fig. 2 C, knockdown of Caprin-2 significantly decreased Wnt-3a–stimulated LEF-1–dependent reporter activity and diminished Wnt-induced accumulation of cytoplasmic β-catenin.

We then performed a rescue experiment. We cotransfected full-length Caprin-2 with its siRNA and found that the knockdown effect could be rescued by Caprin-2 itself. One major difference between Caprin-1 and Caprin-2 is that Caprin-2 has an extra C1q region. Thus, we asked whether the C1q region is critical for Caprin-2's activity. To test this, we analyzed a C-terminal truncated form of Caprin-2, and found that this truncated form failed to rescue the effect of Caprin-2 knockdown (Fig. 2 D).

We also investigated the effect of Caprin-2 knockdown on the expression of native Wnt target genes. We found that in cells transfected with Caprin-2 siRNA, the Wnt-3a–induced expression

of *c-myc* (Fig. 2 E) and *axin-2* (not depicted) were reduced. This observation further confirmed that Caprin-2 is involved in the Wnt signaling pathway in mammalian cells. Our findings that Caprin-2 but not Caprin-1 interacts with LRP5/6 and activates Wnt signaling (Figs. 1 and 2) suggest that Caprin-2 and Caprin-1 have distinct functions.

Caprin-2 is involved in the Wnt signaling pathway in zebrafish development

To further confirm the role of Caprin-2 as a component or modulator of canonical Wnt signaling, we extended our analyses to the organism level using the zebrafish model system. During zebrafish embryonic development Wnt–β-catenin signaling is essential for the establishment of ventral and posterior fates (Erter et al., 2001; Lekven et al., 2001; Thorpe et al., 2005). We first cloned the zebrafish *Caprin-2* homologue and performed in situ hybridization analysis, which showed that *zCaprin-2* was broadly expressed within 24 h postfertilization (hpf; unpublished data). Next, we used antisense morpholino oligonucleotides (MO) targeting the translation initiation region of *zCaprin-2* to knock down its expression.

Embryos injected with *Caprin-2* MO exhibited dorsalized phenotypes: an oval shape at early somite stage, with the tailbud premature protrusion from the yolk instead of tight attachment around the yolk (Fig. 3 A), and enlargement of the telencephalon and reduction of the tail at 24 hpf (Fig. 3 B), which are similar to *Wnt8* morphants (Lekven et al., 2001; Waxman et al., 2004), whereas embryos injected with control MO developed normally. The severity of *Caprin-2* morphant phenotypes could be enhanced by increasing the amount of MO injected, indicating a dose-dependent effect (Fig. 3 C).

To verify the specificity of morpholino knockdown, we performed rescue experiments with MO-resistant mRNA. The phenotypes induced by a suboptimal dose (0.5 mM) of *Caprin-2* MO were partially reversed by coinjection of 20 pg/nl *zCaprin-2* mRNA (Fig. 3 D), which suggests that *Caprin-2* MO in our experiments specifically targeted *Caprin-2*. These results were confirmed by using a second *Caprin-2* MO (*Caprin-2* MO2; Fig. 3 D).

If the phenotype of Caprin-2 morphants was indeed attributable to specific inhibition of the Wnt–β-catenin pathway, activation of the Wnt pathway by ΔN–β-catenin (the constitutively activated form of β-catenin) should suppress the phenotype. To test this, we injected the plasmid expressing zebrafish ΔN–β-catenin1 into the animal cap of one-cell-stage embryos, and found that Caprin-2 MO-induced phenotypes could be rescued by coinjection of ΔN–β-catenin (Fig. 3 E).

In agreement with their phenotypes, *Caprin-2* morphants showed a decreased expression level of the ventral markers *eve1* and *tbx6* and expanded expression of the dorsal marker *goosecoid* (*gsc*; Fig. 4 A). In contrast, the expression of *no tail* (*ntl*), the general marker of nascent mesoderm, was unaffected, which suggests that the function of Caprin-2 in zebrafish development is to promote the specification of ventral cell fates (Fig. 4 A). We also observed the posterior expansion of *opl* (telencephalon) and *pax2.1* (midbrain/hindbrain boundary), coupled with lateral extent of *myoD* at the early somite stage, which resembles the neuroectoderm posteriorization phenotypes of *Wnt8* morphants (Fig. 4 B).

To further support that the phenotypes of *Caprin-2* morphants indeed resulted from the disruption of Wnt signaling, we examined the expression of Wnt target genes, the ventrolateral mesoderm marker *tbx6* (Szeto and Kimelman, 2004) at the early gastrula stage, and *cdx4* (Shimizu et al., 2005) at the 75% epiboly stage. The expression of both genes was reduced by *Caprin-2* MO, and the reduction could be reversed by zCaprin-2 mRNA injection (Fig. 4 C), which suggests a specific effect of *Caprin-2* MO on Wnt target gene expression. Collectively, we conclude that endogenous Caprin-2 activates canonical Wnt signaling in zebrafish embryos.

Caprin-2 enhances GSK3-mediated LRP5/6 phosphorylation

We next asked how Caprin-2 functions in Wnt signaling. Because we have identified that Caprin-2 is an LRP5/6 binding partner, we investigated whether Caprin-2 modulates LRP5/6's activity. We first cotransfected Caprin-2 siRNA with LRP5C2. The result showed that Caprin-2 knockdown blocked the activity of LRP5C2 (Fig. 5 A), which indicates that Caprin-2 may regulate the activity of LRP5/6. Previously, we have demonstrated that LRP5/6–Axin interaction is critical for the activity of LRP5/6 (Mao et al., 2001b). We thus explored whether Caprin-2 affects LRP5/6–Axin interaction. HEK-293T cells were cotransfected with LRP5 and Axin with or without Caprin-2. According to our previous finding that the interaction between LRP5 and Axin requires GSK3, we included GSK3β as a positive control in this experiment. Consistent with our previous study (Mao et al., 2001b), the interaction between Axin and LRP5 was intensified in the presence of GSK3β (Fig. 5 B). Interestingly, Caprin-2 also enhanced the interaction between Axin and LRP5, and the interaction was notably elevated when Caprin-2 was cotransfected with GSK3β (Fig. 5 B). The knockdown experiment using Caprin-2 siRNA confirmed the idea that Caprin-2 is required for optimum LRP5/6–Axin interaction (Fig. 5 C).

Several previous studies have indicated that Wnt-3a–induced LRP5/6 phosphorylation is required for the initiation of signal transduction (Davidson et al., 2005; Zeng et al., 2005; Zeng et al., 2008). Upon canonical Wnt stimulation, LRP5/6 is phosphorylated at multiple sites, including Thr-1479, Ser-1490, and Thr-1493, which is mediated by kinases such as GSK3 and Cdk inhibitor; and the phosphorylation of LRP5/6 is required for its binding with Axin (Mao et al., 2001b; Davidson et al., 2005; Zeng et al., 2005). Therefore, we further asked whether overexpression or knockdown of Caprin-2 might affect Wnt-stimulated LRP5/6 phosphorylation. We overexpressed Caprin-2 in HEK-293T cells and examined the level of phosphorylated LRP6 using a phospho-LRP6 antibody, which specifically detects Ser-1490 phosphorylation caused by GSK3. Result showed that although Caprin-2 alone could increase LRP6 phosphorylation, Caprin-2 overexpression and the stimulation of Wnt-3a led to a synergistic increase of LRP6 phosphorylation (Fig. 5 D). The knockdown experiment using siRNAs confirmed that Caprin-2 is a bona fide regulator of LRP6 phosphorylation. As shown in Fig. 5 E, when endogenous Caprin-2 was knocked down, the level of phospho-LRP6 upon Wnt-3a stimulation was remarkably decreased. Putting these results together, we propose that Caprin-2 functions through regulating LRP5/6 phosphorylation.

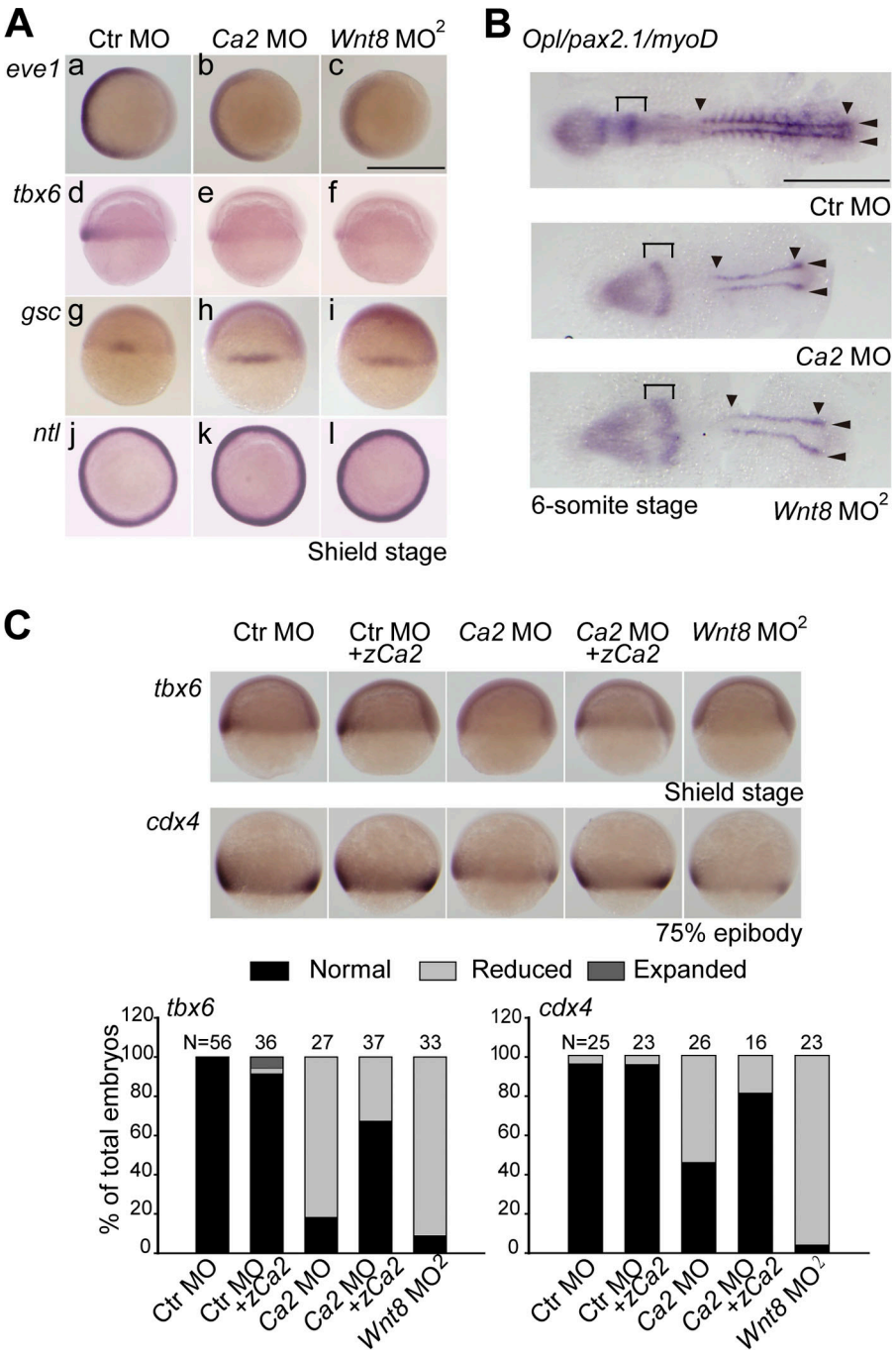

Figure 4. **Morpholino-mediated silencing of zebrafish Caprin-2 represses Wnt8–β-catenin signaling.** (A) Knockdown of Caprin-2 results in expansion of dosal fates. Embryos injected with the indicated morpholinos were fixed at the shield stage and stained for *eve1* (a–c), *tbx6* (d–f), *goosecoid* (*gsc*; g–i), and *no tail* (*ntl*; j–l). Embryos are shown in animal pole views with the dorsal toward the right (a and j–k), lateral views with dorsal toward the right (d–f), and dorsal views with the animal pole toward the top (g–i). (B) Knockdown of Caprin-2 leads to a modest expansion of dorsoanterior tissues and a severe loss of ventroposterior structures. Embryos were fixed at 6-somite stage and stained for *opl*, *pax2.1*, and *myoD*. Arrowheads indicate the length and width of the notochord. Brackets indicate the ventral and posterior expansion of *opl* and *pax2.1*. Embryos were flat-mounted, with the anterior is toward the left. Bars, 500 µm. (C) The expression of *tbx6* and *cdx4* were examined at the shield stage and 75% epiboly, respectively (top). Graph shows the statistical data, respectively (bottom).

A recent study has discovered that LRP6 was clustered with Fz, Dvl, Axin, and GSK3 in an LRP6 signalosome in response to Wnt stimulation (Bilic et al., 2007). Formation of this signalosome not only requires oligomerization of LRP6 but also aggregation of Dvl. The C-terminal region of Caprin-2 contains a C1q domain, which also mediates protein oligomerization (Tom Tang et al., 2005). We thus hypothesize that Caprin-2 might participate in the process of LRP signalosome formation. We suggest the following possibilities to interpret our observations in light of the current framework of canonical Wnt signaling. (1) Caprin-2 may directly regulate LRP5/6 aggregation. In normal naive cells, Caprin-2 is maintained in a monomer form and binds to LRP5/6. When cells are stimulated by Wnt ligand, Caprin-2 is oligomerized through its C1q domain and promotes LRP5/6 aggregation, which triggers the phosphorylation of LRP5/6. (2) Caprin-2 regulates the association of LRP5/6 and GSK3. In the absence of Wnt, Caprin-2 may separately associate with LRP5/6 and GSK3 (unpublished data), respectively. In the presence of Wnt, Caprin-2 undergoes oligomerization and thereby bridges the interaction between LRP5/6 and GSK3, thus promoting the phosphorylation of LRP5/6 and leading to the activation of Wnt signaling. (3) The fact that knockdown of Caprin-2 inhibited LRP5C2-induced LEF-1 reporter activity (Fig. 5 A) suggests that Caprin-2 may also function downstream of LRP5/6 activation. Actually, we found that Caprin-2 also interacts with Axin (unpublished data). It is reasonable to hypothesize

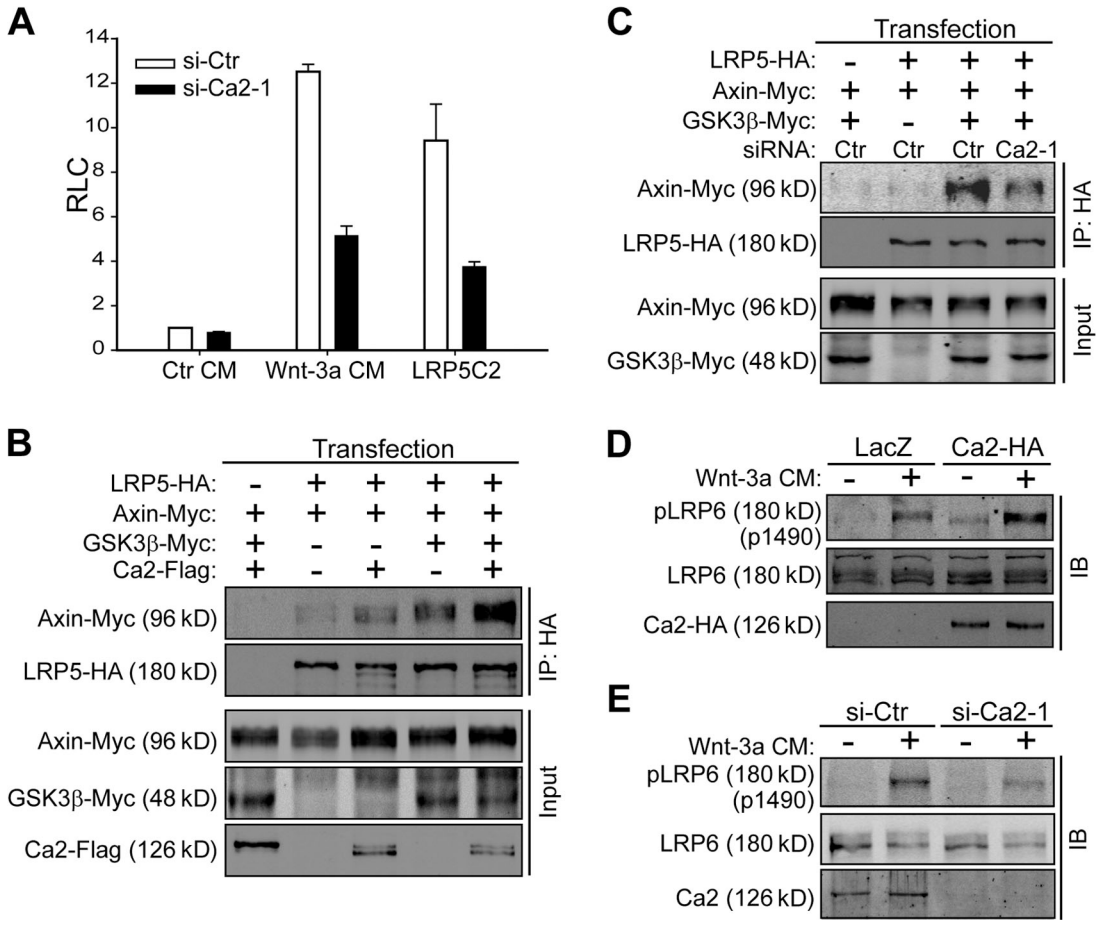

Figure 5. **Caprin-2 enhances GSK3-mediated LRP5/6–Axin interaction and LRP5/6 phosphorylation.** (A) LRP5C2 was cotransfected with Caprin-2 siRNA. LEF-luciferase activity was examined. Error bars indicate SD of duplicated assays in one experiment. Each experiment was repeated at least three times. (B) Lysates of HEK-293T cells expressing LRP5-HA, Axin-Myc, GSK3β-Myc, and Caprin-2-Flag as indicated were immunoprecipitated with anti-HA antibody, and the immunoprecipitates were probed with the indicated antibodies. (C) Caprin-2 siRNA was transfected into HEK-293T cells along with LRP5-HA, Axin-Myc, and GSK3β-Myc as indicated. Lysates were then treated and detected with the indicated antibodies. (D) HEK-293T cells were transfected with Caprin-2-HA. Cells were treated with control or Wnt-3a conditioned medium (CM) for 30 min, the membrane fraction was isolated, and phosphorylation of endogenous LRP6 was then detected using an anti-pLRP6 (p1490) antibody. The anti-LRP6 antibody was used as internal control. (E) Caprin-2 siRNA was transfected in HEK-293T cells for 72 h. Cells were treated with control or Wnt-3a CM for 30 min. The membrane fraction was isolated, and phosphorylation of LRP6 was then detected.

that Caprin-2 may also play a role in modulating Axin binding to LRP5/6 directly via its interaction with both of LRP5/6 and Axin. Work is in progress to examine these hypotheses.

Materials and methods

cDNA constructions

cDNA encoding human Caprin-2 (available from GenBank/EMBL/DDBJ under accession no. NM_001002259) was amplified from total RNA of HEK-293T cells by RT-PCR. PCR product was cloned into mammalian expression vectors that were tagged with HA and Flag, respectively. Deletion constructs of Caprin-2 encoding amino acids 1–318, 313–978, and 973–1128 were generated by PCR and then subcloned to pET28c. Other plasmids have been used previously (Mao et al., 2001b).

Cell culture and transfection

HEK-293 and HEK-293T cells were propagated in DME (Invitrogen) plus 10% FBS (Invitrogen). Cells were seeded in plates 24 h before transfection. Plasmids were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. For the siRNA assay, Lipofectamine 2000 (Invitrogen) was used.

Antibodies

A polycolonal antibody of human Caprin-2 that was raised against *E. coli* expressed a recombinant N terminal of human Caprin-2 (amino acids 1–318). Anti-HA (Covance), anti-Myc (Covance), anti-Flag (Sigma-Aldrich), anti–β-catenin (BD Biosciences), and anti–β-tubulin (Sigma-Aldrich) antibodies were used in this work. Anti-LRP6 and anti–phospho-LRP6 antibodies were obtained from Cell Signaling Technology.

RNAi

Two pairs of independent siRNA against human Caprin-2 for knocking down endogenous Caprin-2 were designed. Target sequences were: si-1, 5′-GAACUUGACUACCUCAUUAAGUUUU-3′; and si-2, 5′-GGCUAUCUUCUUUAUCAAGAUUGAA-3′.

Reporter gene assay

HEK-293 cells in a 24-well plate were transfected with 250 ng of plasmids in total, including 20 ng of reporter plasmid LEF-1–dependent reporter gene and 5 ng of LEF-1 plasmid. 50 ng of GFP plasmid was cotransfected as the transfection control. After 18 h of transfection, cells were treated with Wnt-3a conditioned medium or control medium for additional 6 h. Cells were then lysed and luciferase assays were performed. The luciferase activities presented were normalized against the levels of GFP expression as described previously (Li et al., 1999)

Immunoprecipitation and Western blot analysis

After transfection, cells were harvested and lysed in protein lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% [vol/vol] Triton X-100, 5 mM EDTA, and proteinase inhibitors) and centrifuged at 16,000 *g* for 15 min at 4°C. The lysates were incubated with primary antibody for 1 h at 4°C. Protein A/G PLUS agarose (Santa Cruz Biotechnology, Inc.) was added

and incubated at 4°C for 3 h. Samples were washed three times, eluted by SDS loading buffer, separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies. Results were visualized using Odyssey Infrared Imaging System 9120 (LI-COR).

In vitro binding

Recombinant proteins (GST or 6× His tagged) were expressed in *E. coli*. Proteins were mixed with antibodies for 1 h at 4°C, and the protein A/G PLUS agarose was added for an additional 3 h. The beads were washed three times and resuspended in SDS loading buffer.

Membrane and cytoplasmic fractionations

HEK-293 and HEK-293T cells were plated into 6-well plates. Membrane and cytosolic fractions were isolated using ProteoExtract native membrane protein extraction kit (EMD).

RT-PCR and quantitative real-time PCR

Total RNAs were extracted from cultured cells with Trizol, and reverse transcription of purified RNA was performed using Superscript III reverse transcription kit according to the manufacturer's instructions (Invitrogen). The quantification of all gene transcripts was done by quantitative PCR using a Quantitect SYBR green PCR kit (QIAGEN) and a Rotor-Gene RG-3000A apparatus (Corbett). The primer pairs used for human *c-myc* gene were 5′-TGCTCCATGAGGAGACA-3′ and 5′-CCTCCAGCAGAAGGTGA-3′. For the human *GAPDH* gene, the sequences were 5′-GCACCACCAACTGCTTA-3′ and 5′-AGTAGAGGCAGGGATGAT-3′.

Zebrafish experiments

Zebrafish were raised under standard conditions. The wild-type embryos were derived from the Tüebingen strain. Antisense MO and a standard control MO were obtained from Gene Tools, LLC. The MO sequences are *Caprin-2* MO1 (signed as *Caprin-2 MO*), 5′-TTCTCATGCGTCTCTGCTGGAGTGT-3′; and *Caprin-2* MO2, 5′-GTGTGTTTGTTTGCTGCGTTTCAGA-3′. The *Wnt8* MO[2] (*Wnt8*-ORF1 MO+ *Wnt8*-ORF2 MO) has been described previously (Lekven et al., 2001). For sense RNA injections, capped mRNA was synthesized using the mMessage mMachine kit (Ambion). In MO and plasmid coinjection experiments, a volume of ∼2–3 nl was injected into the animal cap of one-cell stage embryos. However, in all the other microinjection experiments, a volume of ∼4–5 nl was injected into the yolk of one-cell stage embryos. Whole-mount in situ hybridizations using digoxigenin-labeled mRNA probes were performed using standard methods (Oxtoby and Jowett, 1993) with minor modifications. All images were captured at room temperature using a camera (DP71; Olympus) on a microscope (SZX16, 1×; Olympus). The acquiring software was DP Controller and DP Manager (DP71; Olympus).

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