PNAS
Proceedings of the National Academy of Sciences of the United States of America www.pnas.org

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PNAS 2007;104;6690-6695; originally published online Apr 10, 2007;
doi:10.1073/pnas.0611356104

This information is current as of May 2007.

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Notes:

β-Arrestin is a necessary component of Wnt/β-catenin signaling *in vitro* and *in vivo*

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Edited by Robert J. Lefkowitz, Duke University Medical Center, Durham, NC, and approved March 1, 2007 (received for review December 20, 2006)

The Wnt/β-catenin signaling pathway is crucial for proper embryonic development and tissue homeostasis. The phosphoprotein dishevelled (Dvl) is an integral part of Wnt signaling and has recently been shown to interact with the multifunctional scaffolding protein β-arrestin. Using Dvl deletion constructs, we found that β-arrestin binds a region N-terminal of the PDZ domain of Dvl, which contains casein kinase 1 (CK1) phosphorylation sites. Inhibition of Wnt signaling by CK1 inhibitors reduced the binding of β-arrestin to Dvl. Moreover, mouse embryonic fibroblasts lacking β-arrestins were able to phosphorylate LRP6 in response to Wnt-3a but decreased the activation of Dvl and blocked β-catenin signaling. In addition, we found that β-arrestin can bind axin and forms a trimeric complex with axin and Dvl. Furthermore, treatment of *Xenopus laevis* embryos with β-arrestin morpholinos reduced the activation of endogenous β-catenin, decreased the expression of the β-catenin target gene, Xnr3, and blocked axis duplication induced by X-Wnt-8, CK1ε, or DshΔDEP, but not by β-catenin. Thus, our results identify β-arrestin as a necessary component for Wnt/β-catenin signaling, linking Dvl and axin, and open a vast array of signaling avenues and possibilities for cross-talk with other β-arrestin-dependent signaling pathways.

canonical Wnt signaling | dishevelled | Frizzled | G protein-coupled receptor | *Xenopus*

The Wnt/Frizzled pathway is a crucial element in cellular communication (1), which is highly conserved through evolution (2) and is required for both embryonic development and tissue homeostasis. Dysfunction and deregulation of this pathway cause different diseases, including cancer and developmental defects (3).

Wnts are secreted lipoglycoproteins (4) that bind to their cognate receptors of the Frizzled family (FZD_{1-10}) as well as their coreceptors low-density lipoprotein receptor-related protein 5/6 (LRP5/6) (1). The phosphoprotein dishevelled (Dvl) is one of the most upstream modules in this pathway (5). Dvl associates with many different intracellular proteins and is phosphorylated by different kinases (for reviews, see e.g., refs. 5 and 6). In response to Wnt ligand, Dvl is phosphorylated and activated by kinases [as casein kinase 1 (CK1)δ/ε; ref. 7], resulting in a electrophoretic mobility shift (7, 8), hereafter referred to as PS-Dvl (phosphorylated and shifted Dvl). In the Wnt/β-catenin** signaling pathway, Dvl activation is followed by an inhibition of a destruction complex composed of axin, glycogen synthase kinase 3 and adenomatous polyposis coli, which results in a stabilization of β-catenin, which is now capable of regulating transcription by means of T cell factor (TCF)/lymphoid enhancer factor (Lef) transcription factors (1).

Recently, the multifunctional scaffolding protein β-arrestin was shown to interact with Dvl (9, 10). β-Arrestins are known to regulate G protein-coupled receptor desensitization and internalization as well as signaling (11). Initially, β-arrestin-mediated endocytosis was seen as a means of desensitizing a receptor system. However, recent findings point also to a role of β-arrestins as a signaling scaffold for diverse signaling modules, including kinases, phosphatases, small GTPases, phosphodiesterases, ubiquitin E3 ligases, and IκBα (for review, see refs. 12 and 13). With regard to Wnt signaling, β-arrestin1 was identified as a positive modulator of the Wnt/β-catenin pathway and β-arrestin2 as a mediator for the agonist-induced internalization of FZD_4 (9, 10). However, despite the information from previous overexpression studies, the mechanism of action and role of endogenous β-arrestin in Wnt signaling are still unknown.

Here, we aimed at characterizing the nature of the Dvl–β-arrestin interaction and its importance for Wnt/β-catenin signaling both *in vitro* and *in vivo*. In mouse embryonic fibroblasts (MEFs) genetically depleted of β-arrestin1 and/or 2, we show that endogenous β-arrestin is necessary for the Wnt-3a-induced activation of Dvl and for signaling to β-catenin. We identify axin as a β-arrestin-binding partner, and we suggest that β-arrestin forms a functional, trimeric Dvl–β-arrestin–axin complex. Moreover, loss-of-function experiments in *Xenopus laevis* embryos further indicated that β-arrestin is necessary during embryonic development for Wnt/β-catenin signaling *in vivo*.

Results

Based on previous findings on the interaction between β-arrestin and Dvl (9, 10), we aimed at characterizing that interaction in more detail. We first used as a model SN4741 cells (14), a dopaminergic cell line in which Wnt signaling has been characterized (7, 15, 16). We found that immunoprecipitation of either Myc-Dvl2 or HA-β-arrestin2, in cells ectopically expressing Myc-Dvl2 and FLAG-β-arrestin2 (Fig. 1*A*) or FLAG-Dvl3 and HA-β-arrestin2 (Fig. 1*B*), resulted in the pulldown of FLAG-β-arrestin2 and FLAG-Dvl3, respectively. Furthermore, expression of β-arrestin2-GFP and Myc-Dvl2 in SN4741 cells showed a strong colocalization of the two proteins in characteristic punctate Dvl aggregates (17, 18), as detected by immunocytochemistry and confocal laser scanning microscopy (Fig. 1*C*). Similar results have been obtained with Myc-Dvl2 and HA-β-arrestin2 or FLAG-β-arrestin2 (data not

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Author contributions: V.B., D.G., A.S., E.A., and G.S. designed research; V.B., D.G., A.S., and G.S. performed research; V.B., D.G., A.S., and G.S. analyzed data; and V.B., E.A., and G.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: CK1, casein kinase 1; D4476, 4-[4-(2,3-dihydro-benzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1*H*-imidazol-2-yl]benzamide; Δaxin, aa1–477 axin; Dsh, *Drosophila melanogaster* dishevelled; Dvl, mammalian dishevelled; FZD, Frizzled; KO, knockout; LEF, lymphoid enhancer factor; MEF, mouse embryonic fibroblast lacking β-arrestin1 (β-arr1KO), β-arrestin2 (β-arr2KO), or both (β-arr1/2dKO); MO, morpholino; PS-Dvl, phosphorylated and shifted Dvl; TCF, T cell factor; XDsh, *Xenopus* dishevelled.

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**Synonymous to the term ''canonical signaling,'' we rather use ''Wnt/β-catenin signaling.''

This article contains supporting information online at www.pnas.org/cgi/content/full/0611356104/DC1.

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Fig. 1. Interaction and colocalization of Dvl and β-arrestin2. Expression of Myc-Dvl2 and FLAG-β-arrestin2 (*A*) or HA-β-arrestin2 and FLAG-Dvl3 (*B*) in SN4741 cells allowed coimmunoprecipitation of Myc-Dvl2 together with FLAG-β-arrestin2 (*A*) or of HA-β-arrestin2 with FLAG-Dvl3 (*B*). Total cell lysates (TCL) analyzed for the presence of transfected FLAG-tagged proteins by immunoblotting. (*C*) SN4741 cells transfected with β-arrestin2-GFP (βarr2-GFP) and Myc-Dvl2 show substantial colocalization (indicated in yellow in merged picture) of those proteins in cytoplasmatic, nonvesicular punctae, which are the typical subcellular distribution of Dvl as analyzed by confocal microscopy. WB, Western blotting.

shown), to ensure that the association of β-arrestin with Dvl represents a true colocalization.

To narrow the interaction interface of β-arrestin2 with Dvl, we used a set of deletion mutants of FLAG-Dvl3 (19) (Fig. 2*A*) and FLAG-β-arrestin2 (Fig. 2*B*) expressed in COS-7 cells. Coimmunoprecipitation of HA-β-arrestin2 in cells overexpressing also FLAG-Dvl3 mutants revealed that HA-β-arrestin2 can interact with FLAG-Dvl3 mutants 1, 2, 5, 6, and 7 but not with 3 and 4 [supporting information (SI) Fig. 6*A*], which lack a region N-terminal of the PDZ domain of Dvl (Fig. 2*A*). Immunoprecipitation of Myc-Dvl2 resulted in the pulldown of full-length FLAG-β-arrestin2 (construct 1) as well as the deletion mutants 2, 3, 5 but not 4 (SI Fig. 6*B*). To confirm the weak interaction between β-arrestin2 construct 2 and Dvl2-Myc (SI Fig. 6*B*), we performed coimmunoprecipitation of construct 2 with XDsh-Myc (20) and found a strong interaction between them (SI Fig. 6*C*). Thus, our results identify the region aa163–300 as the Dvl-interacting domain on β-arrestin2.

The small portion of Dvl that was found to bind β-arrestin, N-terminal to the PDZ domain, was previously shown to contain Ser/Thr residues recognized by CK1ε (21) and PAR-1 (partitioning defective mutant in *C. elegans*) (20, 22). Moreover, endogenous CK1δ/ε is induced by Wnt ligands (16, 23), phosphorylates Dvl, and results in formation of active PS-Dvl (7, 16). To assess the importance of CK1-mediated phosphorylation in the Dvl–β-arrestin interaction, we treated cells with the CK1-specific inhibitor D4476 (24), which interferes with the activation of Dvl. In cells overexpressing Myc-Dvl2, treatment with D4476 reduced the formation of PS-Myc-Dvl2 (16) and reduced the interaction of Myc-Dvl2 with HA-β-arrestin2 (Fig. 2*C*), suggesting that phosphorylation of Dvl by CK1 increases the affinity for β-arrestin. We have shown previously (16) that expression of CK1ε in SN4741 cells promotes an even cytosolic distribution of Myc-Dvl2, whereas a kinase-dead mutant, CK1εK→R, promotes the typical punctate Dvl appearance in the cytosol. In triple-expression experiments using HA-β-arrestin2, Myc-Dvl2, and CK1ε or CK1εK→R (Fig. 2*D*), we found that HA-β-arrestin2 follows the localization of CK1ε–Dvl. In the presence of CK1ε, HA-β-arrestin2, Myc-Dvl2, and CK1ε were distributed evenly throughout the cytoplasm, whereas in cells expressing the kinase-dead mutant of CK1ε, the proteins adopted a punctate distribution. This finding demonstrated that β-arrestin overexpression does not affect the CK1ε-induced translocation of Myc-Dvl2 and suggests that βarrestin probably acts downstream from Dvl, phosphorylated by CK1. This finding also strengthens the biochemical data of β-arrestin interaction with both Dvl and PS-Dvl.

To examine whether endogenous β-arrestin is required for Wnt-3a signaling, we examined whether MEFs lacking (knockout, KO) β-arrestin1, β-arrestin2, or both (β-arr1KO, β-arr2KO, β-arr1/2dKO) respond to Wnt-3a stimulation (Fig. 3*A* and SI Figs. 7 and 8). The wild-type (WT) MEFs showed time courses and dose responses of Dvl activation similar to those reported previously for SN4741 cells (7). However, the ability of β-arr2KO or β-arr1/2dKO MEFs to induce the formation of PS-Dvl in response to Wnt-3a was delayed and severely reduced (Fig. 3 *A* and *B* and SI Fig. 7), suggesting an important role of β-arrestin in Dvl activation and PS-Dvl formation. Interestingly, complete ablation of β-arrestins resulted in a higher basal level of PS-Dvl compared with the WT, β-arr1KO, and β-arr2KO, suggesting up-regulation of yet unknown compensatory mechanisms. Moreover, endogenous β-arrestin2 seemed to be more important compared with β-arrestin1 because the defects on PS-Dvl formation were more pronounced in MEFs lacking β-arrestin2 compared with cells lacking β-arrestin1 (SI Fig. 8). Thus, our results suggest that β-arrestins are crucial components of the molecular machinery mediating Dvl activation and formation of PS-Dvl in response to Wnt-3a. Because the formation of PS-Dvl at the 2-h time point studied here is necessary for the substantial activation of β-catenin in response to Wnt-3a (7), we also investigated dephosphorylation and stabilization of β-catenin. Moreover, we also analyzed the activation and phosphorylation of Ser-1490 in LRP6, using a phosphospecific antibody (25). Notably, LRP6 phosphorylation was strongly induced by Wnt-3a treatment but was not affected by genetic deletion of arrestins (Fig. 3*C*). In contrast, agonist-induced β-catenin signaling in β-arr2KO and β-arr1/2dKO MEFs was almost completely abolished (Fig. 3*C*). β-arr1KO MEFs showed a pattern of β-catenin dephosphorylation similar to that of the Wnt-3a-stimulated WT MEFs (SI Fig. 8). Because dephosphorylated β-catenin is stabilized and leads to activation of transcription in a TCF/Lef-dependent manner, we also investigated the role of β-arrestin in Wnt-3a-induced activation of the TOPflash luciferase reporter. Although WT MEFs strongly responded to Wnt-3a stimulation with TCF/Lef reporter activity, the lack of both β-arrestins completely inhibited this response (Fig. 3*D*). MEFs lacking β-arrestin2 showed a partially reduced response to Wnt-3a stimulation, supporting the idea of redundancy between β-arrestin1 and β-arrestin2 in Wnt signaling. Again, depletion of β-arrestins 1 and 2 increased basal TCF/Lef signaling slightly, indicating the existence of compensatory mechanisms. Thus, our *in vitro* data argue for an important function of β-arrestin in Wnt signaling, in particular for the Wnt-induced formation of PS-Dvl and subsequent activation of the Wnt/β-catenin pathway.

In addition, we sought to characterize further the potential β-arrestin-dependent mechanisms upstream and downstream from Dvl. First, we investigated the role of β-arrestin in the FZD-induced redistribution of Dvl to the plasma membrane (26). Overexpression of FZD_4-GFP together with Myc-Dvl2 in both WT and β-arr1/2dKO MEFs, resulted in complete redistribution of Myc-Dvl2 from cytoplasmic punctae to the plasma membrane (SI Fig. 9). Thus, β-arrestin is not crucial for the FZD_4-induced Dvl2 translocation. Second, we analyzed the interaction of axin with β-arrestin by overexpression in COS-7 cells. As shown in Fig. 4*A*, a full-length axin or a deletion mutant 1–477 axin (Δaxin) lacking the DIX domain (27), which is necessary for the interaction with Dvl (28), coimmunoprecipitated with β-arrestin. Thus, β-arrestin can interact directly with axin, and such interaction is most likely not mediated by Dvl. These data suggest that a trimeric Dvl–β-arrestin–axin complex might be central for the recruitment of Dvl to axin and the subsequent dissociation of the destruction complex (27–29). To test this possibility, we transfected in Fig. 4*B* the indicated combinations of vectors encoding for Dvl (XDsh), β-arrestin, and full-

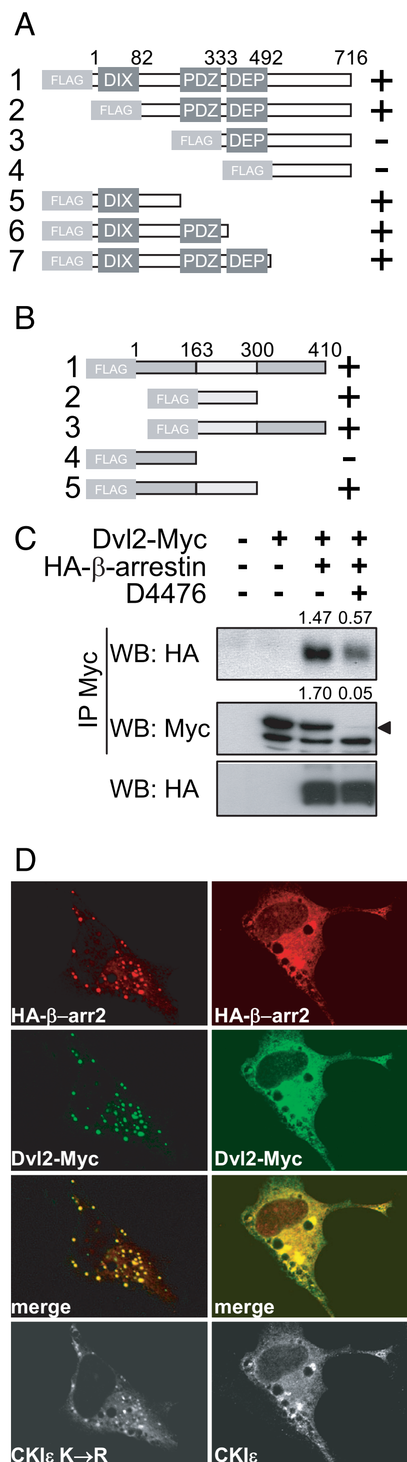

Fig. 2. Mapping of the interaction interfaces of β-arrestin2 and Dvl. Deletion mutants of FLAG-Dvl3 (*A*; constructs 1–7; ref. 19) and of FLAG-β-arrestin2 were used in combination with HA-β-arrestin2 and Myc-Dvl2, respectively, for coimmunoprecipitation. + and − indicate interaction with β-arrestin (*A*) or Dvl (*B*). For detailed presentation of coimmunoprecipitation data, see SI Fig. 6. (*C*) β-Arrestin2 preferentially binds phosphorylated Dvl2. SN4741 cells expressing Dvl2-Myc and HA-β-arrestin2 were grown with and without the CK1 inhibitor D4476. D4476 treatment prevents formation of PS-Dvl and decreases the amount of HA-β-arrestin2 that can be precipitated with Myc-Dvl2, suggesting a decreased affinity of β-arrestin to unphosphorylated Dvl. Numbers represent OD of β-arrestin signal and PS-Dvl/Dvl ratio. The arrowhead points to the localization of PS-Dvl band. (*D*) Overexpressed HA-β-arrestin2 (red; HA-β-arr2) and Dvl2-Myc (green) as well as CK1ε (b/w; kinase-dead mutant CK1ε K→R) are analyzed by confocal laser scanning microscopy. In the presence of WT CK1ε, β-arrestin2, Dvl2, and CK1 are distributed evenly throughout the cytoplasm. In the presence of CK1εK→R, β-arrestin2, Dvl2, and CK1ε are confined to nonvesicular punctae characteristic for Dvl aggregates. Overlap of distribution of β-arrestin and Dvl2-Myc is indicated in yellow in the merged pictures.

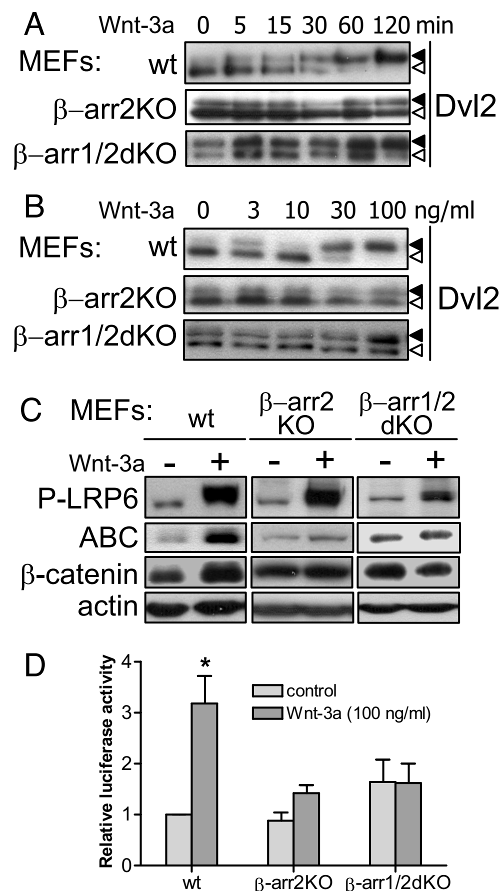

Fig. 3. β-Arrestin2 is a necessary component of canonical Wnt signaling in MEFs. (*A*) WT, β-arr2KO, or β-arr1/2dKO MEFs are treated with 100 ng/ml Wnt-3a for the indicated times. Dvl2 activation is assessed as the formation of PS-Dvl2 (Dvl, open; PS-Dvl, filled arrowheads) by immunoblotting. (*B*) Wnt-3a dose responses were performed at 2 h. (*C*) Quantification of data is available in SI Fig. 7. Phosphorylation of LRP6 at Ser-1490 (P-LRP6) and β-catenin signaling was analyzed in WT, β-arr2KO, and β-arr1/2dKO MEFs after 100 ng/ml Wnt-3a treatment for 2 h. Dephosphorylation of β-catenin (ABC) and β-catenin stabilization show that Wnt-3a-induced signaling to β-catenin is reduced in β-arr2KO MEFs and completely abolished in β-arr1/2dKO MEFs. For responses in β-arr1KO MEFs, see SI Fig. 8. (*D*) SuperTOPflash activity upon 100 ng/ml Wnt-3a stimulation is shown ($n = 3$). *, $P < 0.01$ as analyzed by ANOVA and Tukey's post hoc tests.

length/Δaxin for coimmunoprecipitation. We found that β-arrestin, XDsh, and axin are precipitated together and are, thus, likely to form a trimeric complex. Interestingly, Δaxin is also present in such complexes, suggesting that it might be recruited to XDsh by β-arrestin. However, β-arrestin is not necessary for the XDsh–axin interaction, at least not in an overexpression system, because overexpressed XDsh interacts to a similar extent with axin in both WT and β-arr1/2dKO MEFs (data not shown). In addition it appears that axin could act as a stabilizing component of XDsh/β-arrestin binding because the association of XDsh and β-arrestin is stronger when axin is coexpressed (compare lanes 3 and 4 with lane 5, WB: XDsh, Fig. 4*B*).

Fig. 4. β-Arrestin interaction with axin. (*A*) Immunoprecipitation (IP) experiments in COS-7 cells show the interaction of β-arrestin (*Right*) with full-length and aa1–477 axin (Δaxin). (*Left*) Total cell lysates (TCL). The arrowhead points to the IgG band in the immunoprecipitate. WB, Western blot. (*B*) Overexpression of HA-β-arrestin2, Myc-Dishevelled (*Xenopus* dishevelled, XDsh) and full length (FLAG-tagged) as well as Δaxin reveals the existence of trimeric Dvl-β–arrestin–axin complexes.

To examine the importance of β-arrestin for Wnt signaling *in vivo*, during embryonic development, we turned to one of the most classical and robust models to study Wnt signaling, the early *X. laevis* embryo. The axis duplication assay, as induced by injection of XWnt-8 or DshΔDEP mRNA in the ventral blastomeres, was used to examine the role of β-arrestin overexpression in canonical signaling. The lack of effect of β-arrestin RNA injection on axis duplication under basal conditions or in blastomeres injected with XWnt-8 or DshΔDEP suggested that β-arrestin *per se* is not sufficient to induce Wnt/β-catenin signaling. In contrast, down-regulation of *Xenopus* β-arrestins (for sequence and alignment, see SI Fig. 10) with β-arrestin morpholinos (β-arrMO) showed that β-arrestin is required for Wnt/β-catenin pathway (Fig. 5 *B* and *C*) because the XWnt-8-induced axis duplication was blocked (percent axis duplication: XWnt-8, 51.0; XWnt-8 + β-arrMO, 8.5). Control injections with

Fig. 5. β-Arrestin is not sufficient but necessary for Wnt/β-catenin signaling *in vivo*. (*A*) Percentage of secondary axis induction in *Xenopus* embryos injected with XWnt-8, DshΔDEP, and β-arrestin RNA. *n*, no. of analyzed embryos per condition. (*B*) Effect of β-arrestin morpholino (β-arrMO) treatment on XWnt-8-, DshΔDEP-, CK1ε-, and β-catenin-induced axis duplication; *n*, no. of analyzed embryos per condition. (*C*) Representative photographs of embryos. Secondary axis formation (arrowheads) is best seen by the appearance of a secondary neural tube in neurula (XWnt-8, β-catenin), tailbud (CK1ε), and tadpole stages (DshΔDEP). Arrows indicate the endogenous axes. (*D*) Immunoblotting of *Xenopus* lysates analyzed for β-catenin dephosphorylation (ABC) and β-arrestin levels (A1CT). Filled arrowheads indicate *Xenopus* β-arrestin signal. Open arrowhead indicates an unspecific band serving as loading control. Gene expression (Xnr3) was examined in *Xenopus* embryos by quantitative RT-PCR. (*E*) Error bars show mean ± SD.

β-arrMO alone did not result in axis duplication (data not shown; percent axis duplication: β-arrMO, 0, n = 261). In addition, β-arrMO (Fig. 5 *B* and *C*) completely blocked DshΔDEP − (percent axis duplication: DshΔDEP, 21.2; DshΔDEP + β-arrMO, 0) as well as CK1ε-induced secondary axis formation (percent axis duplication: CK1ε, 13.6; CK1ε + β-arrMO, 1.4). However, β-arrMO did not affect secondary axis induced by β-catenin (% axis duplication: β-catenin, 44.4; β-catenin + β-arrMO, 38.6), arguing for β-arrestin acting upstream from β-catenin and downstream from CK1ε and Dvl, which is in good agreement with our *in vitro* data. As control for the CK1ε injection we also injected a kinase-dead mutant of CK1εK→R, which alone or in combination with β-arrMO failed to induce axis duplication (data not shown; % axis duplication, CK1ε, 0, n = 87; CK1ε + β-arrMO, 0, n = 117). Detection of activated, dephosphorylated β-catenin (ABC, Fig. 5*D*) and expression of the β-catenin target gene Xnr3 (Fig. 5*E*) in control and β-arrestin depleted *Xenopus* embryos further showed that β-catenin activity is reduced in β-arrestin-depleted embryos. In summary, the *Xenopus* experiments demonstrate that endogenous β-arrestin is required for normal development and for Wnt/β-catenin signaling *in vivo*.

Discussion

Here, we identify the multipurpose scaffolding protein β-arrestin as a necessary component in the Wnt/β-catenin pathway *in vitro* and *in vivo*. We show that β-arrestin binds to and forms a trimeric complex with axin and Dvl. Moreover, endogenous β-arrestin does not affect phosphorylation of LRP6 but is necessary for proper activation of Dvl, signaling along the Wnt/β-catenin pathway, activation of endogenous Wnt target genes, and XWnt-8-, DshΔDEP-, or CK1ϵ-induced axis duplication in *Xenopus* embryos *in vivo*.

We mapped the domain of physical interaction between β-arrestin and Dvl (9, 10) to a region N-terminal of the PDZ domain of Dvl and aa163–300 of β-arrestin. This stretch of Dvl contains important CK1ε (21), PAR-1 (20, 22), and possibly also casein kinase 2 (CK2) (30) phosphorylation sites, which may work as potential regulators of β-arrestin–Dvl interaction. Importantly, CK1, CK2, and PAR-1 are all necessary for Wnt/β-catenin signaling (20, 31, 32), and at least CK1ε and CK2 kinase activity is induced directly by Wnts (16, 23, 33). It has been reported that the association of Dvl with β-arrestin is potentiated by CK2 phosphorylation (9), and we report herein that this interaction is reduced by CK1 inhibition, a finding that also supports this hypothesis. We also found that cells lacking β-arrestin resemble cells with CK1δ/ε knockdown (7, 16) in their inability to form PS-Dvl properly in response to Wnt. We thus suggest that the formation of the activated form of Dvl, PS-Dvl, is initiated by and depends on Dvl phosphorylation by Wnt-induced kinases, as we demonstrated previously for CK1ε (7, 16). In this model, β-arrestin recognizes phosphorylated (primed) Dvl and subsequently recruits other kinases to complete PS-Dvl formation and Dvl activation. A second function of β-arrestin that is likely to be crucial for Wnt signaling is its capacity to bind axin directly. This binding did not require the DIX domain of axin, which is necessary for the interaction of Dvl (7). This finding suggested the possibility of a trimeric complex consisting of Dvl, β-arrestin, and axin, which was confirmed by coimmunoprecipitation experiments. We hypothesize that such a trimeric complex could contribute to destabilize the degradation complex, resulting in the stabilization of β-catenin. Thus, our results suggest that the formation of a trimeric complex is an essential step in Wnt signaling that provides a molecular link between the LRP6/axin and FZD/Dvl branches of Wnt signaling after activation.

Experiments in β-arrestin-deficient MEFs suggest that β-arrestins 1 and 2 might be at least partially redundant with respect to mediating Wnt signaling and activation. Interestingly, unstimulated β-arr1/2KO MEFs contain a higher amount of PS-Dvl and higher background activity in the TOPflash reporter assay compared with WT and β-arr1KO or β-arr2KO MEFs, indicating that mechanisms exist to accomplish the formation of PS-Dvl in the absence of β-arrestin. These compensatory mechanisms become evident also regarding the obvious delay and decrease in efficacy of Wnt-3a-induced PS-Dvl formation rather than a complete block. It is possible that high concentrations of Wnt or long-term exposure may to some extent lead to PS-Dvl formation in MEFs deficient in β-arrestins, although not to the same extent as in WT MEFs. These findings show that β-arrestin is not indispensable for PS-Dvl formation and suggest that redundant mechanism(s) exist, which is also evident from the quantification of PS-Dvl/Dvl data (SI Fig. 7), indicating that absence of β-arrestin2 alone has a more pronounced effect on Dvl activation compared with cells depleted of both β-arrestins. However, β-arrestin seems to be uniquely required for proper dynamics of the PS-Dvl formation and for correct Wnt signaling *in vivo*, as demonstrated by the dramatic defects in β-arrestin-depleted *Xenopus* embryos. It should be noted, however, that although β-arrestin is a necessary component for Wnt signaling, it is not sufficient to induce PS-Dvl formation (Fig. 1*A*) or axis duplication in *Xenopus* embryos (Fig. 5). Thus, our data suggest that β-arrestin is required, but not sufficient, for Wnt signaling and that β-arrestin is recruited to Wnt signaling upon Wnt activation.

Our findings regarding the necessity of β-arrestin for Wnt signaling, together with the recognized function of β-arrestins as multifunctional adapter proteins, also open other interesting possibilities such as that β-arrestin might be necessary for other signaling pathways or that β-arrestin might mediate an interaction between Wnt and other signaling pathways (12). This assumption is further supported by structural data indicating that the domain of β-arrestin2 that binds Dvl (aa 163–300) lies in a region of the protein containing large parts of the C-terminal β-fold (β sheet X-XVI; see alignments of nonvisual vertebrate β-arrestins in SI Fig. 10). Thus, both the N and C termini of β-arrestin could remain open for interaction with other partners after Dvl binding. β-Arrestins are also known to play a very important role in clathrin-mediated endocytosis (34). In addition, blockade of endocytosis by, for example, hyperosmolar sucrose blocks Wnt-induced β-catenin signaling (35) and down-regulates Dvl (36). Combined, these findings together with the recently appreciated role of endocytosis in Wnt/Frizzled signaling (10, 35, 37–40) suggest the provocative possibility that β-arrestin-mediated endocytosis might be required for some aspects of Wnt signal transduction, an issue that remains to be explored.

In summary, we hereby identify β-arrestin as an integral component of the Wnt signaling pathway. This finding opens up the possibility that Wnts may signal through novel additional pathways, allowing extensive cross-talk with other, e.g., G protein-coupled receptor-dependent, pathways. Future studies will explore such interactions and will focus on further elucidating the role of β-arrestin in Dvl activation and the function of the trimeric complex formed by Dvl–β-arrestin–axin in Wnt signaling.

Methods

Cell Culture, Transfection, and Treatments. SN4741 cells were obtained from J. H. Son (14). WT MEFs and MEFs lacking β-arrestin1 (β-arr1KO), β-arrestin2 (β-arr2KO), or β-arrestins 1 and 2 (β-arr1/2dKO) were a gift from R. J. Lefkowitz (41). SN4741 cells were propagated in DMEM/10% FCS/2 mM L-glutamine/50 units/ml penicillin/50 units/ml streptomycin/0.6% glucose. MEFs and COS-7 cells were grown in identical medium without glucose. Cells (40,000–60.000 per well) were seeded in 24-well plates either directly (for biochemical analysis) or on sterile coverslips (for microscopy). The next day, cells were transfected (plasmids are listed in *SI Materials and Methods*) by

using Lipofectamine 2000 or polyethylenimine at 0.8 μg/ml in PBS (42). For confocal analysis, 0.2 μg per construct were used according to the manufacturer's instructions. Cells were harvested for immunoblotting or immunocytochemistry 24 h after transfection (for a detailed description of immunocytochemistry and confocal analysis, see *SI Materials and Methods*). Treatment with the D4476 (4-[4-(2,3-dihydro-benzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1*H*-imidazol-2-yl]benzamide), dissolved in DMSO, was done in 24-well plates in the presence of 1 μl per well FuGENE 6 reagent to increase cell penetration. For analysis of cellular signaling, the cells were stimulated with mouse Wnt-3a (from R&D Systems, Minneapolis, MN) for 2 h if not otherwise stated. Control stimulations were done with 0.1% BSA in PBS. For the TOPflash assay, see *SI Materials and Methods*.

Immunoprecipitation and Immunoblotting. Immunoprecipitation was done as described previously (16). For domain-mapping experiments, cells were lysed in 0.1% SDS instead of 0.5% Nonidet P-40. Immunoblotting and sample preparation were done as published previously (7). Protein extracts from *X. laevis* embryos were obtained by using 20 μl of lysis buffer (150 mM NaCl/1 mM EDTA/50 mM Tris·Cl, pH 7.4/0.5% Nonidet P-40) supplemented with protease inhibitors per embryo. After sonication, lysates were depleted of yolk and lipids by mixing with 30 μl of freon per embryo, vortexing, and spinning down (15 min at 12,000 × *g*). The upper phase was mixed with 5× Laemmli buffer (4:1), boiled, and analyzed further. A list of antibodies for immunoblotting and immunoprecipitation is available in the *SI Materials and Methods*.

Injection and Analysis of *X. laevis* Embryos. Capped mRNAs were transcribed from linearized DNA templates (pCS2-DshΔDEP, psp64T-XWnt-8, psp64T-β-catenin, pcDNA-HA-β-arrestin2) by using mMessage mMachine (Ambion, Austin, TX). For knock-down experiments in *Xenopus*, a morpholino antisense oligonucleotide targeted against *Xenopus* β-arrestin (β-arrMO: 5′-TCTCCCCCATCTTCCCAGCTCCGC-3′) was used. Eggs from human chorionic gonadotropin-treated females were fertilized by standard methods and staged according to (43). Morpholino and RNA were injected into the marginal zone of the ventral or dorsal blastomeres of four-cell stage embryos in a total volume of 4 nl. If not mentioned otherwise in the text, the following amounts were injected: XWnt-8, 20 pg; β-catenin, 250 pg; DshΔDEP, 500 pg; β-arrestin2, 500 pg; CK1ε, 500 pg; β-arrMO, 0.4 pg. Embryos were cultivated as described previously (44).

For real-time RT-PCR total RNA was extracted from stage 10.5 embryos (Nucleospin II; Macherey Nagel, Düren, Germany) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Real-time PCR was carried out using iQ-SybrGreen Supermix on an iCycler instrument (Bio-Rad, Hercules, CA). Primer sequences were: ODC-U, 5′-gcc att gtg aag act ctc tcc att c; ODC-D, 5′-ttc ggg tga ttc ctt gcc ac (45); Xnr-3 forward, 5′-aag aga tca aac ccg agt gc; and Xnr-3 reverse, 5′-ctg tgg aac tgc aca agt gg. Expression levels were calculated relative to ornithine decarboxylase and normalized to uninjected controls.

We thank Dr. R. J. Lefkowitz (Duke University Medical Center) for providing the β-arrestin KO MEFs, β-arrestin, and FZD_4-GFP constructs, and β-arrestin antibodies as well as for valuable discussions. Drs. S. Yanagawa (Kyoto University, Kyoto, Japan), R. T. Moon (University of Washington School of Medicine, Seattle, WA), O. Ossipova (Mt. Sinai School of Medicine, New York, NY), T. C. Dale (Cardiff School of Biosciences, Cardiff, U.K.), and J. M. Graff (University of Texas Southwestern Medical Center, Dallas, TX) provided additional plasmids, and we thank Dr. Son for SN4741 cells. This work was supported by the Swedish Foundation for Strategic Research (INGVAR and Center of Excellence in Developmental Biology), Swedish Research Council (VR), European Union (Eurostemcell) (to E.A.), Karolinska Institutet (to E.A. and G.S.), the Stiftelse Lars Hiertas Minne and Tore Nilsson Foundation (to G.S.), MoEYS CR Grant MSM0021622430 (to V.B.), Swedish Society for Medical Research and the Swedish Brain Foundation (Hjärnfonden) postdoctoral fellowships (to G.S.), and Deutsche Forschungsgemeinschaft Grants DFG GR 1802 (to D.G.) and DFG SCHA 965 (to A.S.).

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