β -Arrestin and casein kinase 1/2 define distinct branches of non-canonical WNT signalling pathways

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Recent advances in understanding β -catenin-independent WNT (non-canonical) signalling suggest an increasing complexity, raising the question of how individual non-canonical pathways are induced and regulated. Here, we examine whether intracellular signalling components such as β -arrestin (β -arr) and casein kinases 1 and 2 (CK1 and CK2) can contribute to determining signalling specificity in β -catenin-independent WNT signalling to the small GTPase RAC-1. Our findings indicate that β -arr is sufficient and required for WNT/RAC-1 signalling, and that casein kinases act as a switch that prevents the activation of RAC-1 and promotes other non-canonical WNT pathways through the phosphorylation of dishevelled (DVL, xDSH in *Xenopus*). Thus, our results indicate that the balance between β -arr and CK1/2 determines whether WNT/RAC-1 or other non-canonical WNT pathways are activated.

Keywords: convergent extension movements; RAC-1; RHO-like GTPases; *Xenopus laevis*; WNT-5A

EMBO reports (2008) 9, 1244–1250. doi:10.1038/embor.2008.193

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Received 11 August 2008; accepted 8 September 2008;
published online 24 October 2008

INTRODUCTION

WNTs are secreted glycolipoproteins that activate β -catenin-dependent and -independent (non-canonical) pathways. Several recent reports have provided evidence for the complexity of the non-canonical WNT pathways (Semenov *et al*, 2007). The individual β -catenin-independent WNT branches, which mediate planar cell polarity (for a list of abbreviations, see supplementary information online) or convergent extension movements, are so diverse that it has become necessary to name them by the signalling components involved. Here, we refer to the following pathways as defined in the original studies: WNT/RHO (Habas *et al*, 2003), WNT/RAC-1 (Habas *et al*, 2003), WNT/ Ca^{2+} (Kühl *et al*, 2001), WNT/ROR2/CDC42 (Schambony & Wedlich, 2007) and WNT/CK1/RAP1 (Tsai *et al*, 2007). β -Arrestin (β -arr) was recently found to regulate WNT/ β -catenin signalling (Bryja *et al*, 2007b) and convergent extension movements in *Xenopus laevis* through RHO-A (Kim & Han, 2007). The frequent involvement of β -arr in WNT signalling prompted us to examine whether it mediates the effects of WNTs on other branches of non-canonical signalling.

We found that β -arr was required for WNT/RAC-1 signalling, but was not essential for the WNT/ROR2/CDC42 pathway. Furthermore, *in vitro* and *in vivo* data support that CK1/2 prevent the activation of RAC-1, but promote the formation of phosphorylated and shifted dishevelled (PS-DVL; dishevelled, xDSH in *Xenopus*). This indicates that the recruitment of β -arr to DVL selects and is required for WNT/RAC-1 signalling, and that casein kinases switch signalling from WNT/RAC-1 towards non-canonical pathways mediated by PS-DVL.

RESULTS AND DISCUSSION

WNT-induced activation of RAC-1 requires β -arr

First, we examined the activation of small GTPases by WNT-5A in mouse embryonic fibroblasts (MEFs) using GTPase pull-down assays. Although WNT-5A, which does not affect β -catenin signalling (supplementary Fig S1A online), activated RAC-1 (Fig 1A; supplementary Fig S1B online; 4.1-fold $\pm$ 0.8 standard

Fig 1 | β-Arrestin is necessary for WNT-induced activation of RAC-1. (A) WNT-5A induced the activation of RAC-1 (GTP-RAC-1) in wild-type MEFs but not in MEFs lacking β-arrestin (β-arr1/2 double knock out (dKO)). One representative experiment is shown; in total, three are summarized in supplementary Fig S1B online. (B) WNT-5A-induced formation of phosphorylated and shifted (DVL2, open arrowhead; PS-DVL2, filled arrowhead) in a dose- and time-dependent manner in wild-type and β-arr1/2dKO MEFs is shown. (C) The activation and expression of RAC-1 were monitored in MEFs overexpressing β-arr2-FLAG. (D) Downregulation of β-arr is compensated by constitutively active RAC-1 and RHO-A. Keller open-face explants were scored for elongation on injection with Xβ-arr MO and indicated RNAs. Asterisks indicate values that are significantly different ($P > 0.95$, t -test) from xβ-arr MO. (E) Downregulation of β-arr1/2 expression by short interfering RNA (siRNA) in HEK293 cells does not affect the DVL2-MYC-induced activation of RAC-1 (GTP-RAC-1). Levels of β-arr are detected by the A1CT antibody. ca, constitutively active; DVL/DSH, dishevelled; HEK, human embryonic kidney; MEF, mouse embryonic fibroblast; MO, morpholino; WT, wild type.

error of the mean, s.e.m.), it activated neither RHO-A nor CDC42 (supplementary Fig S1C online). Interestingly, the activation of RAC-1 by WNT-5A was blocked in MEFs lacking β-arr1 and β-arr2 (β-arr1/2 double knock out (dKO)), arguing that β-arr is required for WNT/RAC-1 signalling. In the same cells, WNT-5A-induced formation of PS-DVL (González-Sancho *et al*, 2004; Bryja *et al*, 2007c) was less efficient and delayed compared with wild-type MEFs (Fig 1B). It is important to note that although the lack of β-arr interferes with WNT-induced dynamics of PS-DVL, it increases the basal PS-DVL (Fig 1B). This suggests that β-arr is not necessary for PS-DVL but modulates its formation. Re-transfection of β-arr1 in dKO MEFs reduces the basal PS-DVL and restores the WNT-5A-induced activation of RAC-1 (supplementary Fig S1D online). To analyse whether β-arr is sufficient for the activation of RAC-1, we overexpressed β-arr2-FLAG in MEFs and observed increased activation of RAC-1 (Fig 1C; supplementary Fig S1E online). To confirm these results, we overexpressed β-arr2-FLAG and

downregulated xβ-arr by using antisense morpholinos (Xβ-arr MO; Bryja *et al*, 2007b) in *X. laevis* embryos. We found that overexpression of β-arr increased basal RAC-1 activity and that it was decreased on downregulation (supplementary Fig S1F online). Furthermore, the effect of the xβ-arr MO could be reversed by overexpression of MO-insensitive, murine β-arr, emphasizing the specificity of the xβ-arr MO. These findings identify β-arr as a signalling component required for the regulation of RAC-1 *in vitro* and *in vivo*. For functional analysis, we performed Keller open-face explant elongation assays in *X. laevis* embryos, an established measure of convergent extension (Keller *et al*, 2003; Schambony & Wedlich, 2007). Both overexpression and downregulation of β-arr modulated convergent extension, and the latter was reversed by co-injection of the MO-insensitive haemagglutinin (HA)-β-arr (supplementary Fig S1F online), confirming previous results (Kim & Han, 2007). Kim & Han (2007) showed that β-arr2 regulates convergent extension movements in

X. laevis through RHO-A. Thus, our results, combined with those reported by Kim & Han, suggest that various subsets of RHO family GTPases are regulated by β -arr.

β -Arr acts upstream from RAC-1 in convergent extension

To examine the regulation of RAC-1 by β -arr, we analysed the effect of the following RNAs encoding for WNT signalling components on the convergent extension phenotype in β -arr knockdowns: xDSH Δ DIX (a mutant lacking the DIX domain), a constitutively active WNT/planar cell polarity DVL mutant, constitutively active caRHO-A, caRAC-1 or caCDC42, and xNR3, a WNT/ β -catenin transcriptional target (Fig 1D; Kühl *et al*, 2001; Bryja *et al*, 2007b). Both RAC-1 and RHO-A compensated for the lack of β -arr, and xDSH Δ DIX partly restored explant elongation, whereas CDC42 and xNR3 had no effect (Fig 1D). Complementary results from β -arr1/2 short interfering RNA (siRNA) experiments indicate that β -arr is not required for the DVL-induced activation of RAC-1 (Fig 1E). Other epistatic experiments showed that RHO-A and RAC-1 act downstream from xDSH in the regulation of convergent extension (supplementary Fig S1H online). When discussing the WNT-induced β -arr-dependent formation of PS-DVL, it is worthwhile mentioning that WNT-3A does not induce the activation of RAC-1 in MEFs (supplementary Fig S1I online), despite the fact that it induces β -arr-dependent PS-DVL (Bryja *et al*, 2007b) with a similar time course (Bryja *et al*, 2007c).

Taken together, these results indicate that: β -arr acts upstream or on the level of DVL; RHO-A and RAC-1 but not CDC42 are mediators downstream from β -arr and DVL; and the WNT/ β -catenin pathway is not involved in the observed phenotype.

β -Arr is not required for xWNT-5A/ROR signalling

It has been shown previously that WNT-regulated convergent extension in *X. laevis* Keller explants is mediated by several distinct molecular mechanisms (Tada *et al*, 2002). Although xWNT-11 exerts its effects on convergent extension by activation of the WNT/RAC-1, WNT/RHO and WNT/FZD $_7$ /Ca $^{2+}$ pathways (Winklbauer *et al*, 2001; Habas *et al*, 2003), xWNT-5A is not required for Keller explant elongation but instead for explant constriction, and activates the WNT/ROR2/CDC42 pathway (Unterseher *et al*, 2004; Schambony & Wedlich, 2007). To examine the function of β -arr for xWNT-11 or xWNT-5A signals, we examined whether β -arr mediated their effects in Keller explants. Strikingly, only xWNT-11 (Fig 2A), but not xWNT-5A (Fig 2B), was reversed by β -arr MO co-injection. Consistently, the negative effects of xWNT-11 MO on explant elongation were partly reversed by the overexpression of β -arr (Fig 2A); however, β -arr did not restore constriction in xWNT-5A-depleted explants (Fig 2B), arguing that β -arr is not involved in the xWNT-5A/ROR2 pathway.

To relate the xWNT-11-induced pathway to the small GTPases, we examined whether caRHO-A, caRAC-1 and caCDC42 or dnRHO-A and dnRAC-1 (supplementary Fig S2A online) rescued the effects of xWNT-11 MO or xWNT-11, respectively. Reduced explant elongation in xWNT-11-depleted explants was partly rescued by coexpression of caRHO-A and caRAC-1 (but not caCDC42), and xWNT-11 overexpression was compensated by dnRHO-A, dnRAC-1 and xDSH Δ DEP, a mutant lacking the RAC-1-activating DEP domain (supplementary Fig S2B online;

Fig 2 | β -Arrestin mediates WNT-11- but not WNT-5A-induced gastrulation movements in *Xenopus laevis* embryos. Keller open-face explant elongation (A,B, upper panels) and constriction (B, lower panel) were scored in *X. laevis* embryos injected with indicated RNAs and morpholinos (MOs). Asterisks indicate values that are significantly different ($P > 0.05$, t -test) from xWNT-11 and xWNT-11 MO.

Fig 3 | Casein kinases 1 and 2 differentially regulate the formation of PS-DVL and the activation of RAC-1. (A) The activation of RAC-1 (GTP-RAC-1), and the expression levels of RAC-1 and DVL2/PS-DVL2 (DVL2, open arrowhead; PS-DVL2, filled arrowhead) in MEFs were monitored in the absence (–) or presence (+) of the CK1 (100 μM) or CK2 inhibitor (100 μM). (B) HEK293 cells were transfected with RAC-1-MYC in the presence of DVL3-FLAG and/or CK1ε. RAC-1-MYC activity (GTP-RAC-1) was determined by pull down, and RAC-1-MYC, DVL3-FLAG, CK1ε and actin levels were determined in cell lysates. Note the CK1ε-induced mobility shift of DVL3-FLAG. (C,D) Explant elongation after downregulation of β-arrestin (C; xβ-arr MO) or xWNT-11 (D; xWNT-11 MO), in combination with CK1ε, CK2α, CK2β and CK2α/β, or CK1 and CK2 inhibitors, was evaluated. Asterisks indicate values that are significantly different ($P > 0.95$, t -test) from (C) xβ-arr MO and (D) xWNT-11 MO. (E) Effect of xWNT-11 MO on the activation of RAC-1 in the absence (–) and presence (+) of CK1 or CK2 inhibitors was measured by active GTP-RAC-1 pull down from *Xenopus* embryo lysates. Levels of RAC-1 in the lysates are shown in the lower panel. CK, casein kinase; HEK, human embryonic kidney; MEF, mouse embryonic fibroblast; MO, morpholino; PS-DVL, phosphorylated and shifted dishevelled.

Habas *et al*, 2003). These experiments confirmed the crucial function of RAC-1 and RHO-A downstream from xWNT-11 in convergent extension movements and indicated that xWNT-11 signalling to RHO/RAC also includes xDSH.

CK1/2 as switches in non-canonical WNT signalling

It is known that WNTs directly activate at least two kinases: casein kinase (CK)1 and CK2 (Willert *et al*, 1997; Swiatek *et al*, 2004; Gao & Wang, 2006; Bryja *et al*, 2007d), which, in turn, phosphorylate DVL to form PS-DVL. In line with these findings, it has been shown previously that CK1δ/ε are crucial components of the β-catenin-independent WNT signalling machinery (McKay *et al*, 2001; Klein *et al*, 2006; Strutt *et al*, 2006; Bryja *et al*, 2007d). Furthermore, it was recently shown that CK1ε acts through the small GTPases RAP1 (Tsai *et al*, 2007). Interestingly, CK1 and CK2 can physically interact with β-arr (Xiao *et al*, 2007), and we have already shown that CK1 mediates communication between β-arr and DVL in the WNT/β-catenin pathway (Bryja *et al*, 2007b). To test whether PS-DVL and CK1 have a similar function in β-arr-dependent WNT/RAC-1 signalling, we inhibited CK1 pharmacologically with D4476 (4-[4-(2,3-dihydro-benzo[1,4]-dioxin-6-yl)-5-pyridin-2-yl-1*H*-imidazol-2-yl]benzamide). As expected,

D4476 led to a reduction of basal PS-DVL2 levels but, surprisingly, it increased the activation of RAC-1 (Fig 3A). Similar results were obtained with the inhibition of CK2 by TBBt (4,5,6,7-tetrabromo-2-azabenzotriazole; Fig 3A). The presence of the DVL protein *per se* seems to be required for both the activation of RAC-1 and the maintenance of RAC-1 expression levels (supplementary Fig S3A online). Thus, DVL as a protein, but not PS-DVL, which is dependent on CK1 and CK2, is required for the activation of RAC-1. In summary, our data suggest that CK1 and CK2, and possibly also PS-DVL, negatively regulate WNT/RAC-1 signalling *in vitro*.

Importantly, WNTs seem to concomitantly evoke the activation of RAC-1/RHO-A (Habas *et al*, 2003; Fig 1A) and the formation of PS-DVL (González-Sancho *et al*, 2004; Schulte *et al*, 2005; Bryja *et al*, 2007d; Fig 1B), which depend on CK1 (Bryja *et al*, 2007d) and CK2 (Gao & Wang, 2006; Fig 3A). Interestingly, PS-DVL is usually formed 30–45 min after stimulation with WNT-5A (Bryja *et al*, 2007d; Fig 1B), whereas RAC-1 was shown to be already activated 5 min after stimulation (Kurayoshi *et al*, 2006), raising the possibility that the activation of RAC-1 precedes the formation of PS-DVL and activation of other branches of non-canonical signalling. Can CK1/CK2 thus act as a switch between individual

Fig 4 | DVL-dependent and -independent function of casein kinases. (A) Convergent extension was quantified in embryos treated with both xDSH MO and xWNT-11 MO in combination with overexpression of CK2 α , CK2 β or CK1, CK2 inhibition. Elongation of Keller explants was quantified. Asterisks indicate values that are significantly different ($P > 0.05$, t -test) from xWNT-11 MO/xDSH MO. (B) Immunocytochemical localization of xDSH-MYC (nuclear counterstain DAPI) in *Xenopus* mesoderm in control (ctrl), WNT- or CK1 ϵ -overexpressing or D4476-treated embryos. Arrowheads point at membranous xDSH-MYC. (C) Corresponding biochemical data. xDSH-MYC was detected in embryo lysates and analysed for the expression of xDSH-MYC (xDSH, open arrowhead; PS-xDSH, filled arrowhead). The antigen B62A12 was used as a loading control. (D) Schematic view of the role of casein kinases and β -arrestin in the regulation of formation of PS-DVL and WNT-induced signalling to RAC-1; for details, see text. CE, convergent extension; CK, casein kinase; DAPI, 4,6-diamidino-2-phenylindole; DVL/DSH, dishevelled; MO, morpholino; PS-DVL, phosphorylated and shifted DVL.

β -catenin-independent WNT pathways at the level of DVL? To test this possibility, we induced the activation of RAC-1 directly by DVL3 and studied the effects of CK1 on this process (Fig 3B). Although overexpression of DVL3 increased the activation of RAC-1, this positive effect on RAC-1 was abolished when the formation of PS-DVL3 was induced by simultaneous CK1 ϵ co-expression. Furthermore, we found, by using the AP-1 reporter assay, that basal and DVL3—but not phorbol ester—or ca-RAC-1-induced AP-1 activity was enhanced by treatment with D4476 (supplementary Fig S3B online). These data suggest that: PS-DVL (mediated by CK1/CK2 activity) does not participate in the WNT/ β -arr/RAC-1/AP-1 pathway; and that the activation of CK1/CK2 and formation of PS-DVL leads to a change in the signalling function of DVL, displacing it from the RAC-1 pathway.

CK 1 and 2 in the regulation of convergent extension

In vivo, we confirmed that both CK1 and CK2 are involved in and required for the regulation of convergent extension movements in

Keller explants (supplementary Fig S3C online). However, we observed differences in the ability of CK1 and CK2 to restore elongation in β -arr or xWNT-11-depleted explants. β -arr MO was not rescued by CK1 ϵ , CK2 α or CK2 holoenzyme and only partly by CK2 β (Fig 3C). By contrast, CK2 α , CK2 β and CK2 holoenzyme, but not CK1 ϵ rescued xWNT-11 depletion (Fig 3D). Consistently, xWNT-11 overexpression was rescued by dominant negative (dn) CK2 α , but not by dnCK1 ϵ (supplementary Fig S3D online). Furthermore, in agreement with our biochemical data, chemical inhibition of CK1 or CK2 restored explant elongation (Fig 3D) and RAC-1 activity (Fig 3E) in xWNT-11 MO-injected explants. Simultaneous downregulation of xWNT-11 and xDSH (for optimizing xDSH MOs, see supplementary Fig S4A online) resulted in an additive phenotype that was rescued by co-injection of CK2 α , but not CK2 β (Fig 4A). Overall, these results indicate that *in vivo* CK1 ϵ and CK2 α do not positively regulate β -arr-dependent branches of non-canonical signalling, and that xWNT-11 activates not only β -arr/xDSH/RAC-1 signalling but also

other pathways that involve CK2, most likely WNT/Ca²⁺ signalling. This assumption is supported by the observation that CK2α rescues convergent extension in PTX-treated explants (unpublished observation).

Interestingly, the positive effect of inhibition of CK1 on convergent extension was strictly xDSH dependent, whereas inhibition of CK2 led to explant elongation (Fig 4A) and the activation of RAC-1 (supplementary Fig S4B online) even after knockdown of xWNT-11/xDSH. To address the requirement of DVL for the CK-modulated activation of RAC-1 in mammalian cells, we used pretreatment with hyperosmolar sucrose to deplete cells of DVL (Bryja et al, 2007a). Under these conditions, only inhibition of CK2 increased the activation of RAC-1, further supporting the idea that CK2 but not CK1 mediates the activation of RAC-1 at least partly independently of DVL (supplementary Fig S4C online).

When we analysed xDSH subcellular localization (Fig 4B) in response to D4476, we observed that inhibition of CK1 activity resulted in translocation of xDSH-MYC to the cell membrane, similar to the overexpression of xWNT-11, but not of xWNT-5A or xWNT-8, whereas xDSH-MYC was found predominantly in the cytoplasm in CK1ε-overexpressing cells. The effect of the treatment was also monitored by the decrease or increase of PS-xDSH, respectively (Fig 4C). This indicates that submembraneous DVL could represent a pool of DVL that is active in the RAC-1 pathway and that is not phosphorylated by CK1/2, but does not form PS-DVL.

In summary, our data first suggest that β-arr is not essential for the WNT/ROR2/CDC42 and non-canonical pathways involving CK1/2 and PS-DVL. However, we found that β-arr is selectively required for the WNT/RAC-1 pathway. This finding selectively links WNT/RAC-1 signalling with other β-arr-regulated pathways and functions, such as receptor endocytosis, internalization and desensitization.

Second, our *in vitro* and *in vivo* data support that the formation of PS-DVL (which requires CK1 and/or CK2) and the activation of RAC-1 (which requires inhibition of CK1/2) represent independent events, and potential ways to measure two β-catenin-independent pathways activated by a common ligand (WNT5A or xWNT-11). We propose that CK1 and CK2 can act as a central relay to select the pathways and signalling components being positively and negatively regulated (PS-DVL and RAC-1, respectively, schematized in Fig 4D). Thus, CK1 and CK2 can be considered as switches between individual branches of β-catenin-independent WNT signalling.

METHODS

Cell culture, transfection and treatments. Wild-type MEFs and MEFs lacking β-arr 1 and 2 (β-arr1/2dKO) were a gift from Dr Lefkowitz (Kohout et al, 2001). MEFs and human embryonic kidney (HEK) 293 cells were grown in DMEM, 10% FCS, L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 U/ml). Transfection and treatment with D4476, casein kinase 2 inhibitor I (Calbiochem, Nottingham, UK)-TBBT or recombinant mouse WNT-5A (from R&D Systems, Abingdon, UK) were carried out as described previously (Bryja et al, 2007c). For a list of expression vectors, see the supplementary information online.

GTPase pull-down assay and immunoblotting. GST-PAK-CRIB (for glutathione-S-transferase-p21-activated kinase-CDC42/RAC

interactive binding domain), GST-WASP-CRIB (WASP for Wiskott–Aldrich syndrome protein) and GST-RHOTekin recombinant proteins were coupled to glutathione sepharose beads for the detection of activated RAC-1, CDC42 and RHO-A, respectively, and the assay performed as described in the supplementary information online. Immunoblotting and sample preparation were carried out as described previously (Bryja et al, 2007c).

Xenopus oocytes and Keller explants. For RNA transcription and oocyte injection, see the supplementary information online. Embryos were cultivated until Nieuwkoop and Faber stage 10.5, and Keller explants were prepared, cultivated and scored as described previously (Unterscher et al, 2004). For inhibitor treatment, D4476 and CK2 inhibitors were added to the medium at 1 μM at the blastula stage (stage 8). xDSH-MYC translocation was examined after injection of 100 pg xDSH-MYC RNA. At onset of gastrulation (stage 10.5), the dorsal marginal zone and the blastocoel roof were prepared and fixed in 4% formalin. Tissue was stained with 9E10 anti-MYC, mounted and examined with a laser scanning confocal microscope.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

The study was supported by the Swedish Foundation for Strategic Research (INGVAR and Center of Excellence for Developmental Biology), Swedish Research Council, European Union (Eurostemcell), Karolinska Institutet, Åke Wiberg, Signe & Olof Wallenius, Jeansson, Tore Nilsson Foundations, German Research Foundation (DFG), Ministry of Education and Grant Agency of the Academy of Sciences of the Czech Republic and the European Molecular Biology Organization (for more details, see supplementary information online).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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