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The planar cell polarity (PCP) protein Diversin translocates to the nucleus to interact with the transcription factor AF9

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ABSTRACT

The planar cell polarity (PCP) pathway, a β-catenin-independent branch of the Wnt signaling pathway, orients cells and their appendages with respect to the body axes. Diversin, the mammalian homolog of the *Drosophila* PCP protein Diego, acts as a molecular switch that blocks β-catenin-dependent and promotes β-catenin-independent Wnt signaling. We report now that Diversin, containing several nuclear localization signals, translocates to the nucleus, where it interacts with the transcription factor AF9. Both Diversin and AF9 block canonical Wnt signaling; however, this occurs independently of each other, and does not require nuclear Diversin. In contrast, AF9 strongly augments the Diversin-driven activation of c-Jun N-terminal kinase (JNK)-dependent gene expression in the nucleus, and this augmentation largely depends on the presence of nuclear Diversin. Thus, our findings reveal that components of the PCP cascade translocate to the nucleus to participate in transcriptional regulation and PCP signaling.

Introduction

Wnt signaling cascades activate cellular programs that range from migration and proliferation to cell fate determination and stem cell renewal. These pathways enable cells to translate environmental cues into complex morphogenetic programs. In canonical Wnt signaling, binding of secreted Wnt molecules to Frizzled receptors and low-density-lipoprotein-related protein (LPR) induces phosphorylation of Dishevelled, inhibiting the β-catenin degradation complex. As a consequence, β-catenin escapes proteasomal degradation and translocates to the nucleus, where it interacts with transcription factors of the LEF/TCF family to activate Wnt-specific gene transcription. In *Drosophila*, a β-catenin-independent branch of the Wnt signaling pathway, the planar cell polarity (PCP) pathway, orients epithelial cells in the plane of a tissue (reviewed in [1,2]). By an unknown mechanism, upstream PCP components such as Fat and Dachsous trigger the asymmetric subcellular distribution of the PCP core proteins Frizzled (Fz), Dishevelled (Dvl), Flamingo/Starry Night (Fmi, Stan), Strabismus/Van Gogh (Stbm/Vang), Prickle (Pk), and Diego (Dgo). In the *Drosophila* wing, Fz, Dvl, and Dgo move to the distal side of the cell, while Pk and Stbm accumulate at the proximal plasma membrane. PCP effectors like Inturned (In), Fuzzy (Fy), and RhoA then reorganize the cytoskeleton to ensure proper cell morphogenesis (reviewed in [1,3]). However, in some tissues such as the *Drosophila* eye, normal PCP signaling requires JNK-dependent gene transcription [4,5].

Dgo, a six ankyrin repeat protein, was originally identified to localize Fmi in response to Frizzled signaling to the proximal/distal boundaries of the *Drosophila* wing [6]. Dgo is recruited to the plasma membrane by Frizzled receptors, where it interacts with Pk and Stbm/Vang through its N-terminal ankyrin repeats to maintain the apical localization of Fmi [7]. The mammalian homolog of Dgo, Diversin, interacts with Dvl, and recruits Casein kinase Iε and members of the Axin family to target β-catenin for degradation, thereby inhibiting canonical Wnt signaling [8]. Diversin is also involved in JNK activation, and plays a crucial role in zebrafish gastrulation and heart formation [9]. In the present study we describe that Diversin translocates to the nucleus, where it interacts with the putative transcriptional modulator AF9 to promote JNK-dependent gene transcription.

Materials and methods

Reagents and plasmids. Full-length human AF9 and mouse Diversin were generous gifts from C. Hemenway and W. Birchmeyer, respectively. Full-length and truncated version were created by PCR and standard cloning techniques, and fused to YFP- (eYFP-C1, Clontech), FLAG- (pcDNA6, Invitrogen), or V5-containing expression vectors (pcDNA6, Invitrogen). Antibodies used in this study included mouse M2 antibody to FLAG (Sigma), mouse antibody to V5 (Serotec), mouse antibody to GFP (MBL), rabbit

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antibody to MLLT3 (Atlas), and mouse antibody to Diversin (Nanotools). The position of amino acids are based on NM_001012450 (Diversin) or NM_004529 (AF9).

Yeast two-hybrid cDNA screening. Yeast two-hybrid screening was performed by the Genomics & Proteomics Core Facilities of the DKFZ (Heidelberg, Germany; Dr. M. Kögl) using Diversin without the ankyrin repeat domain (amino acid 270–712) as bait in combination with a human fetal brain cDNA library (Pretransformed Human Fetal Brain Matchmaker cDNA Library, Clonetech). The pGBKT7-Diversin was transformed into *Saccharomyces cerevisiae* strain AH109 (MATα). Yeast two-hybrid library screening was carried out by the yeast-mating procedure using *S. cerevisiae* Y187 (MATα) pretransformed with pACT2. After mating, clones were selected on minimal synthetic dropout medium (-Trp-Leu-His-Ade) containing 0.4 mM 3-amino-1, 2, 4-triazole (3AT). Protein–protein interactions were independently confirmed by yeast-two-hybrid analysis and determination of α-galactosidase activity.

Co-immunoprecipitation. Co-immunoprecipitation experiments were carried out as described, using transiently transfected HEK 293T cells [10]. For endogenous immunoprecipitation, three 10 cm dished of IMCD cells were pooled. For compartment-specific immunoprecipitation, soluble cytosolic and nuclear fractions were separated using the method described by Dignam [11].

Luciferase assay. HEK293T cells were seeded in 12-well plates and transiently transfected with a TOPflash luciferase reporter

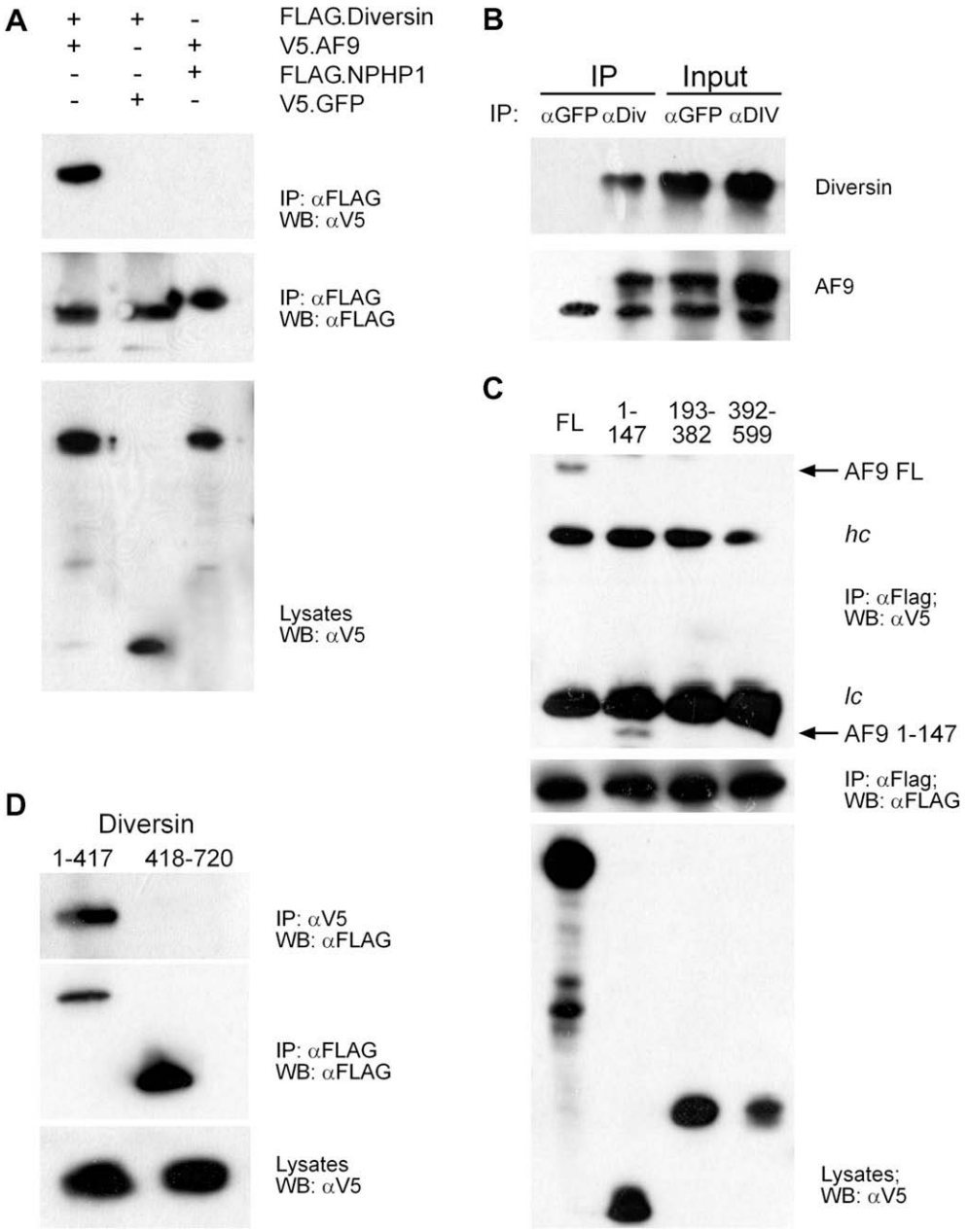

Fig. 1. The amino-terminus of Diversin interacts with AF9. (A) HEK 293T cells were transiently transfected with plasmids as indicated. Precipitated FLAG.Diversin, but not FLAG.NPHP1 immobilized V5.AF9. The control protein V5.GFP was not detectable in either precipitate. (B) Endogenous AF9 was precipitated from IMCD cells by an antibody directed against endogenous Diversin, but not by a control antibody directed against GFP. (C) FLAG9.Diversin was coexpressed with full-length and truncated forms of V5.AF9. Only full-length AF9 and the N-terminal fragment spanning amino acid 1–147 were found in immuncomplexes with Diversin. (D) Flag-tagged amino-terminal or carboxy-terminal parts of Diversin (1–417 or 418–712, respectively) were coexpressed with full-length V5-tagged AF9. Only the N-terminal fragment (spanning amino acid 1–417) interacted with full-length AF9.

construct, a β-galactosidase expression vector, and vectors directing the expression of the desired proteins. The JNK-dependent reporter assay was performed using the PathDetect Kit (Stratagene) according to manufacturer's instructions. Cells were collected after incubation for 12 h, collected in cold PBS and lysed in reporter lysis buffer (Applied Biosystems) for 10 min at room temperature. Debris was removed by short centrifugation (13,500 rpm, 5 min). Luciferase activity was determined using a commercial assay system (Applied Biosystem), and normalized to β-galactosidase activity to correct for transfection efficiency.

Results

Diversin interacts with the putative transcription factor AF9

We noticed that Diversin has five putative nuclear localization sequences (NLS), prompting us to investigate whether Diversin is present in the nucleus. Despite a predominant cytosolic accumulation, wild-type Diversin was detectable in the nucleus, while deletion of the five NLS prevented access of Diversin to the nucleus (Supplementary Fig. 1). To identify potential nuclear binding part-

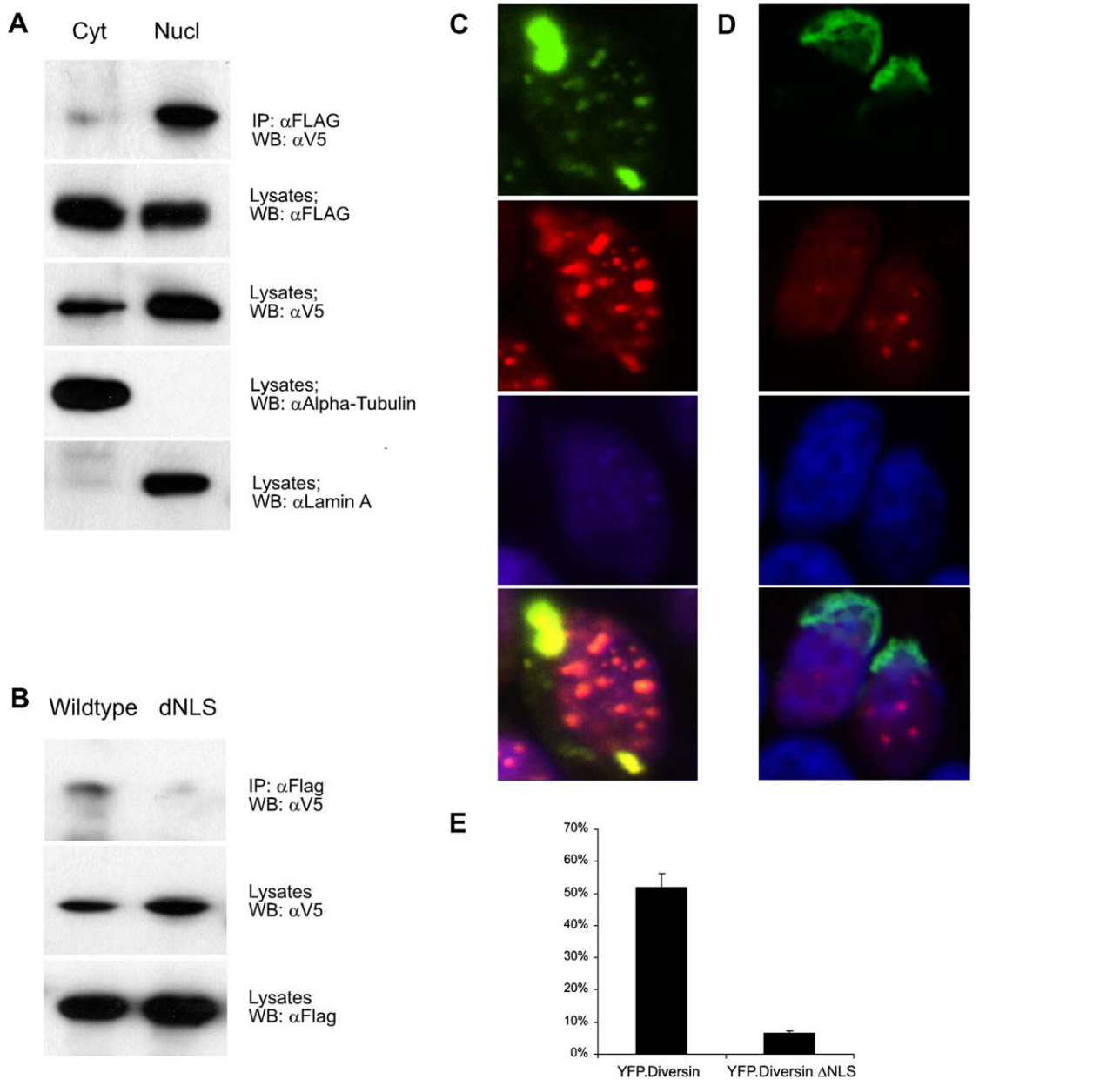

Fig. 2. The interaction between Diversin and AF9 was restricted to the nuclear compartment of the cell. (A) The interaction between Diversin and AF9 was restricted to the nuclear compartment of the cell. HEK 293T cells expressing V5.AF9 and FLAG9.Diversin were lysed and separated into nuclear and cytosolic fractions. Protein levels were equalized, and Diversin was precipitated using anti-FLAG beads. V5.AF9 was only detected in the nuclear precipitate. The purity of the fractions was controlled by anti-Lamin A (nucleus) and anti-α-tubulin (cytosol) staining. (B) V5-tagged wild-type Diversin or a mutant lacking the NLS sequences were coexpressed with FLAG9.AF9. Wild-type (WT), but only small amounts of mutant Diversin ΔNLS) interacted with AF9 precipitated by an antibody directed against the V5 epitope from whole cell lysates. (C) YFP.Diversin (green) and CFP.AF9 (false-colored, red) were coexpressed in HEK 293T cells. Overlapping signals were detected in the nucleus (Hoechst, blue). (D) The YFP.Diversin ΔNLS mutant (green) was retained in a perinuclear compartment, and not present in the nucleus (Hoechst, blue). YFP.Diversin ΔNLS did not co-localize with CFP.AF9 (false-colored, red). (E) 200 HEK293T cells transfected with both Diversin and AF9 were analysed at two different time points by confocal microscopy for the presence of nuclear Diversin.

ners of Diversin, we performed a yeast-two-hybrid screen. Since proteins with ankyrin repeats interact with their binding partners in a rather promiscuous way [12], we used the carboxy-terminal fragment of Diversin (amino acid 270–712) as bait, and screened a human fetal brain cDNA library. Among the twelve positive clones, 10 included different sequence fragments of human AF9/MLLT3. The other two clones contained sequences belonging to HCLS1-associated X-1 (HAX1), an anti-apoptotic protein involved in caspase-9 repression [13,14]. AF9 is a member of the TF2F domain transcription factors [15], and one of the most common fusion partners of the mixed-lineage leukemia protein (MLL). To confirm the interaction between Diversin and AF9, Flag-tagged Diversin (FLAG.Diversin) and V5-tagged AF9 (V5.AF9) were transiently coexpressed in HEK 293T cells. Immunoprecipitation of FLAG.Diversin using anti-Flag-M2 beads immobilized V5.AF9 but not V5-tagged GFP (V5.GFP) (Fig. 1A). In contrast, immunoprecipitation of a Flag-tagged control protein (FLAG.NPHP1) did not immobilize V5.AF9.

Mouse IMCD cells express both AF9 and Diversin endogenously. When Diversin was precipitated with a Diversin-specific antibody, endogenous AF9 was present in the precipitate. No AF9 was detectable after precipitation with an antibody directed against GFP (Fig. 1B).

To delineate the interaction domain, truncations of both proteins were generated and co-immunoprecipitated. In agreement with the AF9 clones isolated in the yeast-two-hybrid screen, FLAG.Diversin precipitated an amino-terminal fragment of V5.AF9 (amino acids 1–147) (Fig. 1C). Conversly, a truncation of Diversin, encompassing the first 417 amino acids, precipitated AF9, suggesting that the binding site for AF9 is located between amino acid 270 and 417 of Diversin (Fig. 1D).

The interaction between Diversin and AF9 occurs in the nucleus

AF9 reportedly shows an exclusive nuclear localization [16]. Accordingly, compartment-specific immunoprecipitation revealed that the interaction between AF9 and Diversin only occured in the nucleus (Fig. 2A). We mutated the five nuclear localization signals in the Diversin molecule (ΔNLS); this mutant did not co-immunoprecipitate with AF9 (Fig. 2B). The nuclear interaction was further supported by immunofluorescence, using CFP-tagged AF9 and YFP-tagged Diversin. Although Diversin was predomi-

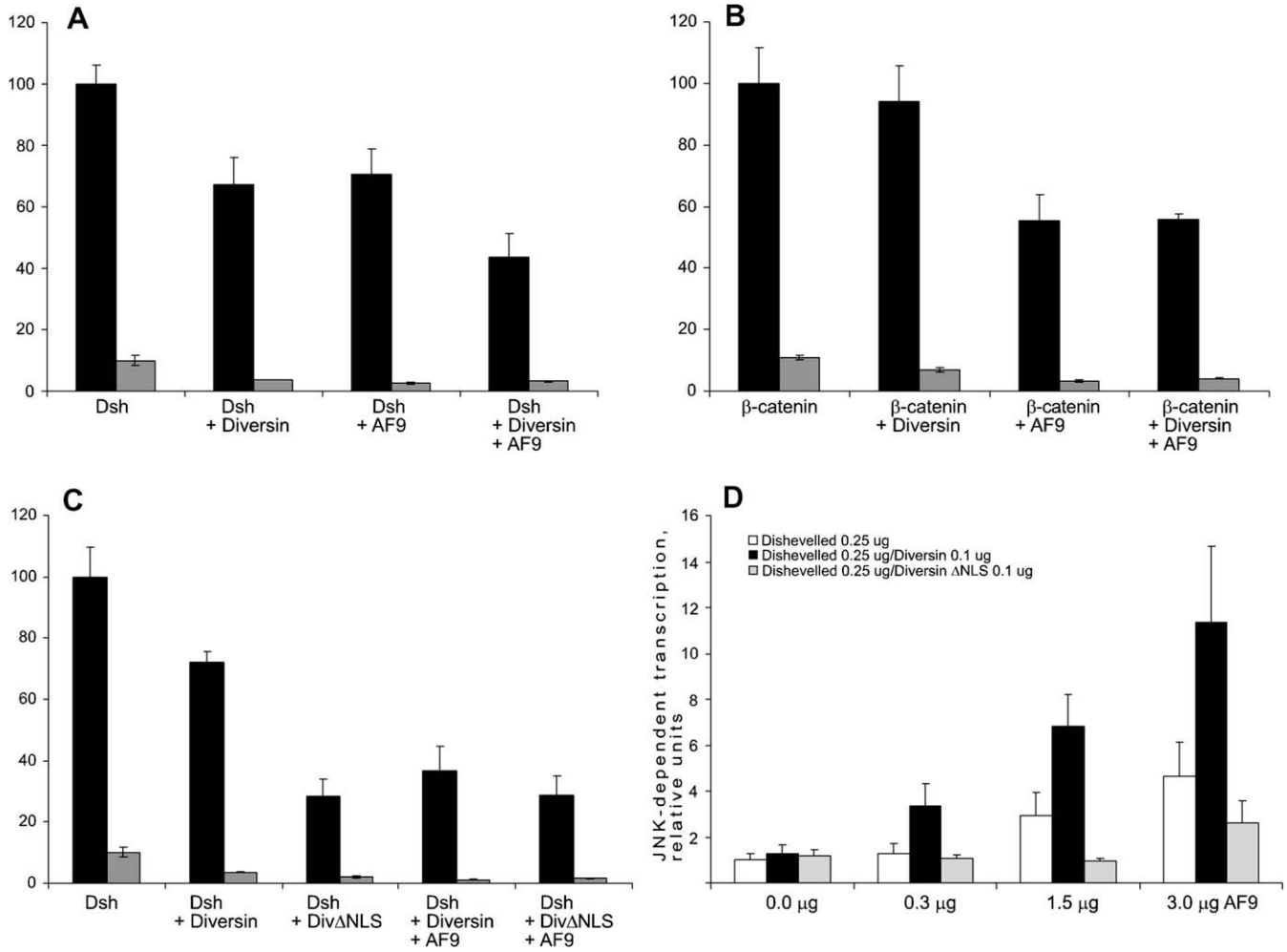

Fig. 3. AF9 inhibits canonical Wnt signaling, and augments the Diversin-mediated JNK activation. (A) HEK 293T cells were transfected with the depicted plasmids to activate TCF/LEF-dependent gene expression (TOPflash assay). Diversin inhibited Dshevelled (Dsh)-mediated stimulation to ca. 65%; a similar effect was achieved by expression of AF9. If both proteins were combined, TCF activity was reduced to 45% of the Dsh-stimulated cells. (B) Diversin did not affect β-catenin-stimulated TOPflash activation, while AF9 reduced the activity to 55% of β-catenin-stimulated cells. (C) The NLS-deficient mutant of Diversin (ΔNLS) was more effective than wild-type Diversin to inhibit Dsh-mediated TOPflash activation. In the presence of AF9, Dsh-mediated gene transcription was inhibited; this inhibition was not affected by the presence of DiversinΔNLS. Gray bars (A–C): A FOPFlash construct was included in all experiments as a negative control. (D) JNK-dependent transcription was measured by luciferase reporter activity in HEK 293T cells. Increasing amounts of AF9 augmented the Diversin- and Dishevelled-mediated JNK activation. At least 4 independent experiments were performed, each in triplicates. Error bars indicate standard deviation.

nantly present in the cytosol, the nuclear fraction of Diversin co-localized with AF9 (Fig. 2C). This co-localization was not observed with the NLS-lacking mutant of Diversin (Fig. 2D). The nuclear presence of Diversin was quantified (Fig. 2E).

AF9 blocks canonical Wnt signaling, but augments JNK-dependent gene expression

Diversin binds Dvl, and recruits CK1ε and Axin to target cytoplasmic β-catenin for degradation [8]. Therefore, we determined the effect of AF9 on TCF/LEF-dependent gene activation. Both Diversin and AF9 inhibited Dvl-mediated TOPflash activation by approximately 30%, while the combination of Diversin and AF9 reduced the Dvl-mediated TOPflash activation by approximately 60% (Fig. 3A). Diversin had little effect on β-catenin-mediated TOPflash activation, suggesting that Diversin inhibits canonical Wnt signaling mainly upstream of β-catenin. AF9 reduced both Dvl and β-catenin-mediated TOPflash by approximately 30% to 45%, suggesting that AF9 acts in the nucleus at the level of β-catenin (Fig. 3B). JNK-dependent gene activation is an essential component of noncanonical Wnt signaling, and Diversin augments the Dvl-mediated activation of JNK [8]. We found that AF9 markedly enhances Dvl-mediated JNK activation, and augments the stimulatory effect triggered by Diversin. Adding increasing amounts of AF9 augmented the ability of Diversin to enhance JNK-dependent gene transcription, suggesting that both proteins form a functional complex to regulate JNK-dependent gene transcription (Fig. 3D).

The nuclear localization of Diversin promotes JNK-dependent gene transcription

Diversin, lacking the nuclear localization sequences (Diversin ΔNLS), potently inhibited Dvl-mediated TOPflash activation (Fig. 3C), suggesting that Diversin blocks canonical Wnt signaling in the cytoplasm independent of the nuclear translocation of Diversin. This observation suggests that the additive effect of AF9 and Diversin on TOPflash inhibition likely occurs in two different cellular compartments; Diversin blocks canonical Wnt signaling in the cytoplasm, while the action of AF9 is confined to the nucleus. The stimulatory effect of Diversin on AF9-mediated JNK-dependent gene transcription, however, was found to be abolished when using Diversin ΔNLS, suggesting that Diversin translocates to the nucleus to modulate JNK-dependent gene transcription (Fig. 3D).

Discussion

The requirement for nuclear translocation of β-catenin and the modulation of canonical Wnt signaling by transcriptional regulators in the nucleus is firmly established. The histone acetyltransferase CREB binding protein (CBP) and its related protein p300/CBP synergize with β-catenin to stimulate gene expression [17]; BCL9 interacts with β-catenin to recruit the transcriptional coactivator Pygopus [18], while the lucine zipper tumor suppressor 2 antagonizes Wnt signaling by facilitating the nuclear export of β-catenin [19]. Other binding partners such as the hypermethylated in cancer 1 (HIC1) gene product attenuate Wnt signaling by sequestering TCF-4 and β-catenin [20]. The role of gene transcription is less well defined in noncanonical Wnt signaling. While the morphogenesis of *Drosophila* wing cells and their actin-based appendages is mainly mediated by rearrangement of the cytoskeleton, at least some tissues require JNK-dependent gene activation to establish a normal polarity [4,5].

We discovered that the PCP protein Diversin contains several nuclear localization sequences, and is present in the nucleus, where it co-localizes and interacts with AF9. The putative transcription factor AF9 is the most frequent fusion partner of MLL [21–23], and associates with several other nuclear proteins, including MPc3 [16], a member of the polycomb group (PcG) proteins that form large multi-protein complexes involved in gene silencing. Additionally, Af9/Enl bound to RNA polymerase II is able to recruit the histone H3 methyltransferase Dot1, which in turn leads to transcriptional activation or repression [24,25]. Deletion of AF9 in mice causes perinatal lethality associated with abnormalities of the axial skeleton [26]. Murine AF9 is expressed in the central nervous system, including the subventricular zone [27]. Interestingly, a balanced reciprocal t(4;9) (q34;p22) translocation, disrupting one AF9 allele, was reportedly associated with a delay in neuromotor development [28,29], suggesting that AF9 participates in transcriptional networks during neuronal development.

AF9 augments the inhibitory effect of Diversin on canoncical Wnt signaling. Our findings suggest that Diversin and AF9 act sequentially, Diversin in the cytoplasm and AF9 in the nucleus. However, the capacity of Diversin to modulate JNK-dependent gene transcription largely requires the translocation of Diversin to the nucleus and is supported by nuclear AF9. Taken together, our findings provide further evidence that components of the PCP signaling cascade translocate to the nucleus to modulate gene expression. The requirement for nuclear Diversin/AF9-dependent signaling may be tissue-dependent. For example, BCL9 is ubiquitously expressed, but synergizes with β-catenin only in lymphoid cells [30]. It is therefore conceivable that AF9 modulates and diversifies the transcriptional responses in canonical and noncanonical Wnt signaling in a cell-type specific manner.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.012.

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