Arginine methylation of G3BP1 in response to Wnt3a regulates β-catenin mRNA

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Summary
Wnt/β-catenin signaling is essential for normal mammalian development. Wnt3a activates the Wnt/β-catenin pathway through stabilization of β-catenin; a process in which the phosphoprotein Dishevelled figures prominently. Protein arginine methylation in signaling complexes containing Dishevelled was investigated. Mass spectrometry of a prominent arginine-methylated, Dishevelled-associated protein identified the Ras GTPase activating protein-binding protein 1 G3BP1. Stimulation of totipotent mouse embryonic F9 cells with Wnt3a provoked increased methylation of G3BP1. We show that G3BP1 is a novel Ctnnb1 mRNA binding protein. Methylation of G3BP1 constitutes a molecular switch that regulates Ctnnb1 mRNA in response to Wnt3a. Thus, the protein arginine methylation that targets G3BP1 acts as a novel regulator of Ctnnb1 mRNA.

Key words: Wnt, β-catenin mRNA, G3BP1, Arginine methylation, Dishevelled, Frizzled, RNA binding protein, Protein arginine methyl transferase

Introduction
Wnt/β-catenin signaling has an essential role in mammalian development and its deregulation leads to human carcinomas (Polakis, 2000; Moon et al., 2002; Logan and Nusse, 2004; Moon et al., 2004; Angers and Moon, 2009). Wnt ligands bind to Frizzled proteins (Bhanot et al., 1996; Liu et al., 2001), which are G-protein coupled receptors (GPCRs) that are necessary for downstream signaling. Wnts regulate both canonical (Wnt/β-catenin) as well as non-canonical (planar cell polarity and Wnt, Ctnnb1)mRNA, which is catalyzed by GSK3β-catalyzed phosphorylation provokes ubiquitylation and proteasome-mediated degradation of β-catenin. Wnt3a acts to oppose the ability of the Dishevelled3 (Dvl3)–KSRP complex to destabilize Ctnnb1 mRNA, as well as degradation of β-catenin, which is catalyzed by GSK3β phosphorylation. The ability of Wnt to enhance post-transcriptional stabilization of Ctnnb1 mRNA, followed by translation (Bikkavilli and Malbon, 2010) and reduced protein degradation, stimulates rapid accumulation of intracellular β-catenin. Accumulation of β-catenin provokes its translocation into the nucleus, stimulating activation of lymphoid enhancer factor and T cell factor (Le/Tcf)-sensitive gene transcription (Behrens et al., 1996; Molenaar et al., 1996).

Wnt-stimulated Ctnnb1 gene expression is a post-transcriptionally-regulated mechanism (Gherzi et al., 2006; Bikkavilli and Malbon, 2010). Transcripts of post-transcriptionally regulated genes display AU-rich elements (AREs) in their 3′-untranslated regions (3′-UTRs). AREs act as binding sites for RNA-binding proteins, which either stabilize or destabilize the mRNA transcripts that bind to them. Although only a speculation, it seems likely that RNA-binding proteins might also bind to Ctnnb1 mRNA. Such RNA-binding proteins also display predominant sites of a unique form of post-translational modification: arginine methylation (Bedford and Clarke, 2009; Lee and Stallcup, 2009). Nitrogen atoms of arginine residues within the protein can be post-translationally methylated, catalyzed by a class of enzymes called protein arginine methyl transferases (PRMTs) (Bedford and Richard, 2005; Bedford and Clarke, 2009; Lee and Stallcup, 2009). Previously, we identified a post-transcriptional mechanism of Wnt-dependent regulation of Ctnnb1 mRNA that operated through the Dvl3–KSRP complex (Bikkavilli and Malbon, 2010). As a strategy to more efficiently identify potential RNA-binding proteins involved in the regulation of Ctnnb1 mRNA, we tested for Wnt-stimulated protein arginine methylation events that might reveal novel RNA-binding proteins. Using such a strategy, we identified Ras GTPase activating protein-binding protein 1 (G3BP1). G3BP1 is shown to be a novel Dishevelled-associated protein that is methylated in response to Wnt3a and also binds and regulates Ctnnb1 mRNA. G3BP1 methylation is also shown to constitute a molecular switch that regulates Ctnnb1 mRNA in response to Wnt3a.

Results
G3BP1 is a Dishevelled-associated protein
To identify potential RNA-binding proteins involved in Wnt-dependent regulation of Ctnnb1 mRNA, we searched for Wnt-
dependent methylation products in Dvl3-based supermolecular signaling complexes (Yokoyama et al., 2010). The strategy was to treat totipotent mouse embryonic teratocarcinoma F9 cells with Wnt3a, isolate the Dvl3-based complexes using antibodies against Dvl3, and scan for arginine-methylated proteins. The Dvl3-based pull downs were subjected to SDS-PAGE and the methylated proteins within the signaling complexes were identified by immunoblotting with an antibody that specifically detects mono- and di-methyl arginine (Fig. 1A). With the exception of the light (~20 kDa) and heavy (~50 kDa) chains of IgG that would be expected, only one major methylated protein(s) (~70 kDa) was positively identified (Fig. 1A). Wnt3a, but not Wnt5a (not shown), showed a marked, progressive increase in arginine-methylation of this ~70 kDa protein. The area of the gel of interest was excised and subjected to either trypsin or chymotrypsin digestion. The resulting peptide fragments were analyzed by liquid chromatography and mass spectrometry (LC-MS) using an LTQ ion trap mass spectrometer equipped with a nano LC electrospray ionization (ESI) source. A methylated peptide was positively identified as that of Ras GTase activating protein-binding protein 1 (G3BP1, Fig. 1B,C). The ESI mass spectrum of a peptide revealed a doubly charged peak that corresponded to a monomethylated sequence of RGPGPRGGPSGGMR (amino acids 428–441 of G3BP1). A fragment ion (MS/MS) spectrum further showed that the first arginine (R433 of full-length G3BP1) was monomethylated by Wnt3a (Fig. 1D). The RRM domain and RGG rich recognition motif and an Arg-Gly-rich region of G3BP1 encodes an N-terminal Nuclear Transport Factor 2-like domain (NTF2 domain), an RNA binding protein that was recently identified as that of Ras GTase activating protein-binding protein 1 (G3BP1, Fig. 1B,C). The ESI mass spectrum of a peptide revealed a doubly charged peak that corresponded to a monomethylated sequence of RGPGPRGGPSGGMR (amino acids 428–441 of G3BP1). A fragment ion (MS/MS) spectrum further showed that the first arginine (R433 of full-length G3BP1) was monomethylated (Fig. 1B). The primary sequence of G3BP1 encodes an N-terminal Nuclear Transport Factor 2-like domain (NTF2 domain), an RNA recognition motif (RRM domain), and an Arg-Gly-rich region (RGG-rich region, Fig. 1D). The RRM domain and RGG rich regions function canonically in RNA binding of known RNA-binding proteins.

The pull-down data with antibodies against Dvl3 identified G3BP1 as a Dishevelled-associated protein. ectopic co-expression of HA–Dvl3 and Myc–G3BP1 in F9 cells was used to further interrogate this association. Immunoprecipitations performed on whole-cell lysates using anti-HA antibodies revealed G3BP1–Dvl3 association (Fig. 2A). We next tested the ability of the N- and C-terminal regions of G3BP1 to associate with Dvl3. HA-tagged Dvl3 was expressed in the absence or presence of Myc-tagged full-length (1–465), N-terminal (1–240) or C-terminal (241–465) G3BP1. Immunoprecipitation of Dvl3 was then performed using anti-HA antibodies. G3BP1 was found to associate with Dvl3-based complexes (Fig. 2B). The C-terminal (241–465) region of G3BP1 similarly associated with Dvl3-based complexes (Fig. 2B). The N-terminal (1–240) region of G3BP1, by contrast, failed to associate with Dvl3-based complexes (Fig. 2B).

We next assessed whether the G3BP1–Dvl3 association was Wnt sensitive. Stimulation of F9 cells overexpressing Myc-tagged G3BP1 with purified Wnt3a revealed a Wnt-dependent dissociation of G3BP1 from the Dvl3-based complexes (Fig. 2C). A transient increase in the amount of G3BP1 associated with the Dvl3-based complex was observed during the first hour of Wnt3a stimulation. Remarkably, following further stimulation with Wnt3a, the amount of G3BP1 associated with the Dvl3-based complex declined (Fig. 2C).

Role of PRMT1 in G3BP1 methylation

Arginine methylation of proteins can be catalyzed by protein arginine methyl transferase 1 (PRMT1), a ubiquitously expressed methyl transferase for histones and other nuclear proteins that bind nucleic acids (Bedford and Clarke, 2009; Lee and Stallcup, 2009). Because G3BP1 was prominently methylated by Wnt3a in the Dvl3-based complex, we evaluated whether PRMT1 was catalyzing the process. Pull-downs performed on F9 cell extracts expressing HA-tagged PRMT1 and Myc-tagged G3BP1 revealed PRMT1 association with Dvl3-based complexes (Fig. 3A). Pull-downs with antibodies against Dvl3 identified PRMT1–G3BP1 association in these complexes (Fig. 3A). Pull-downs with antibodies against Dvl3 identified PRMT1–G3BP1 association in these complexes (Fig. 3A). Pull-downs with antibodies against Dvl3 identified PRMT1–G3BP1 association in these complexes (Fig. 3A).
downs with control IgG antibodies or antibodies targeting either Dvl3 or glycogen synthase kinase 3β (GSK3β), by contrast, failed to show any association with PRMT1 (Fig. 3A). We probed next whether the PRMT1–G3BP1 association was Wnt sensitive. Stimulation of F9 cells (overexpressing Myc-tagged G3BP1 and HA-tagged PRMT1) with purified Wnt3a provoked a transient increase in the amount of G3BP1 associated with the PRMT1 (Fig. 3B). The Wnt3a-stimulated G3BP1–PRMT1 interaction peaked within 2 hours of treatment. The amount of G3BP1 associated with the PRMT1 declined thereafter (Fig. 3B). We next performed an in vitro methylation assay to test directly whether G3BP1 was a PRMT1 substrate. PRMT1 was isolated by immunoprecipitation from F9 cell extracts, whereas recombinant GST–G3BP1 (rG3BP1) was purified in bacteria. The in vitro reaction included the isolated PRMT1, purified rG3BP1 and radiolabeled S-adenosyl L-methionine (SAM) as a methyl group donor. PRMT1 isolated from cell extracts of F9 cells failed to methylate (Fig. 3C) or not of PRMT1 by Wnt3a probably explains the inability of PRMT1 isolated from unstimulated F9 cells to methylate (Fig. 3C) or not (Fig. 3D), GST–G3BP1 under these in vitro conditions. Pull-downs performed from cells expressing wild-type G3BP1 displayed methylation of G3BP1 and its mutants. F9 cells were transiently transfected with empty vector or Myc–G3BP1 for 24 hours. The cells were then treated with Wnt3a (20 ng/ml) for indicated periods of time followed by cell lysis and affinity pull-downs with anti-Dvl3 antibodies. Interaction of G3BP1 with Dvl3 was visualized by probing the blots with anti-Myc antibodies. (C) F9 cells were transiently transfected with empty vector or Myc–G3BP1 for 24 hours. The cells were then treated with Wnt3a (20 ng/ml) for indicated periods of time followed by cell lysis and affinity pull-downs with anti-Dvl3 specific antibodies followed by immunoblotting with anti-Myc antibodies. Top panel displays mean values ± s.e.m. obtained from three independent highly reproducible experiments; the lower panel displays representative blots. Asterisks indicate the bands of immunoglobulin heavy and light chains. Representative blots of three independent experiments that proved highly reproducible are displayed. **P<0.01 versus control (−Wnt3a).

G3BP1 negatively regulates Wnt/β-catenin signaling

To test whether G3BP1 expression modulated canonical Wnt/β-catenin signaling, Wnt-stimulated β-catenin accumulation and Lef/Tcf-sensitive gene transcription was probed. Knockdown of G3BP1 was effective, in response to treatment with small interfering RNAs (siRNAs). G3BP1 deficiency provoked a twofold increase in basal Cinn1 mRNA levels (Fig. 4A). Cells treated with scrambled siRNAs as a control, displayed no such increase (Fig. 4A). G3BP1 deficiency likewise provoked a twofold increase in β-catenin protein levels (Fig. 4B). More telling, G3BP1 deficiency was found to potentiate the ability of Wnt3a to stimulate β-catenin accumulation, the hallmark of canonical Wnt/β-catenin signaling (Fig. 4B). Knockdown of G3BP1 provoked not only an increase in β-catenin protein, but also a consequential increase in Wnt-stimulated Lef/Tcf-sensitive transcription (Fig. 4C). Overexpression of G3BP1 might be expected to attenuate canonical signaling. Indeed, increased expression of G3BP1 attenuated Wnt3a-stimulated β-catenin levels (Fig. 4D).
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Fig. 3. PRMT1 binds to and methylates G3BP1. (A) Ectopic PRMT1 interacts with G3BP1. F9 cells were transiently transfected with Myc–G3BP1 either alone or with HA–PRMT1 for 24 hours followed by cell lysis and affinity pull-downs with anti-HA antibodies. Interaction of G3BP1 with PRMT1 was visualized by probing the blots with anti-Myc antibodies. F9 cells were transiently transfected with HA–PRMT1 for 24 hours followed by cell lysis and affinity pull-downs with control IgG, anti-Dvl3, anti-GSK3β or anti-HA antibodies. The association of PRMT1 with different proteins was visualized by probing the blots with anti-HA antibodies. (B) F9 cells were transiently transfected with HA–PRMT1 and Myc–G3BP1 for 24 hours. The cells were then treated with Wnt3a (20 ng/ml) for indicated periods of time followed by cell lysis and affinity pull-downs with anti-HA specific antibodies followed by immunoblotting with anti-Myc antibodies. Top panel displays mean values ± s.e.m. obtained from three independent highly reproducible experiments; the bottom panel displays representative blots. Asterisks indicate the bands of Immunoglobulin heavy chain. Representative blots of three independent experiments that proved highly reproducible were displayed. *P<0.05 versus unstimulated control (–Wnt3a). (C) In vitro methylation assay for G3BP1. F9 cells were transiently transfected with empty vector or HA–PRMT1 for 24 hours. The cells were then treated with Wnt3a (20 ng/ml) for indicated periods of time followed by cell lysis and affinity pull-downs with anti-HA specific antibodies followed by an in vitro methylation assay using recombinant GST–G3BP1 as substrate and [3H]SAM as a methyl donor. Top panel displays the d.p.m. of the incorporated tritiated methyl groups measured from the excised bands of the SDS-PAGE gel and the bottom panel displays a representative fluorograph. (D) In vitro methylation assay for G3BP1 mutants. F9 cells were transiently transfected with HA–PRMT1 for 24 hours. The cells were then treated with Wnt3a (20 ng/ml) for 6 h followed by cell lysis and affinity pull-downs with anti-HA antibodies followed by an in vitro methylation assay using recombinant GST–G3BP1 and its mutants R433K or R445K as substrates and [3H]SAM as a methyl donor. Representative data of two independent experiments that proved highly reproducible were displayed. (E) In vivo methylation of G3BP1. F9 cells were transiently transfected with empty vector, Myc–G3BP1, or G3BP1 mutants for 24 hours. The cells were then metabolically labeled with L-[methyl-3H]methionine in the presence of protein translation inhibitors. After 3 hours, the cells were lysed and affinity pull-downs with anti-Myc antibodies were performed. The methylation statuses of G3BP1 and its mutants were then revealed by SDS-PAGE followed by fluorography. After fluorography, the gels were rehydrated, transferred to nitrocellulose membranes followed by immunoblotting with anti-Myc antibodies (bottom panel). Representative data of two independent experiments that proved highly reproducible were displayed.
G3BP1 binds Ctnnb1 mRNA in vivo

G3BP1 is known to bind the 3’-untranslated regions (3’-UTRs) of Myc or Tau mRNAs (Gallouzi et al., 1998; Liu et al., 1999; Tourriere et al., 2001; Atlas et al., 2004; Atlas et al., 2007). Ctnnb1 mRNA is present in Dvl3-based complexes (Bikkavilli and Malbon, 2010). Because it has domains necessary for RNA binding, G3BP1 was tested for its ability to bind Ctnnb1 mRNA.

The presence of Ctnnb1 mRNAs in the G3BP1 complex was probed. Myc–G3BP1 and its N-terminal or C-terminal complexes were isolated from cell lysates by immunoprecipitation with anti-Myc antibodies. RNA was isolated from the pull-downs and amplified by RT-PCR. Ctnnb1 transcripts were found in the G3BP1 complex (Fig. 5A). Pull-downs prepared from lysates of cells transfected with empty pCMV vector, by contrast, displayed no detectable Ctnnb1 mRNA (Fig. 5A). Pull-downs performed with cells expressing the C-terminal region of G3BP1 (241–465) also displayed Ctnnb1 transcripts (Fig. 5A). However, pull-downs performed with cells expressing the N-terminal region (1–240 amino acids) of G3BP1, did not display any Ctnnb1 mRNA (Fig. 5A). Therefore, G3BP1 appears to bind and regulate Ctnnb1 mRNA.

G3BP1 binds Ctnnb1 mRNA in vitro

The interaction of G3BP1 and Ctnnb1 mRNA was investigated in vitro, using northwestern blotting. Probing the blots of recombinant GST or GST–G3BP1 proteins that were transferred onto nitrocellulose membranes by western blotting with an in vitro transcribed digoxigenin (DIG)-labeled full-length Ctnnb1 UTR (2517–3536) demonstrated binding of the Ctnnb1 mRNA probe to G3BP1 (Fig. 5B). The Ctnnb1 mRNA probe also bound G3BP1 immunoprecipitated from cell lysates (Fig. 5C). However, the Ctnnb1 mRNA probe failed to bind either GST (Fig. 5B) or proteins from control (pCMV–Myc empty vector) immunoprecipitations (Fig. 5C). The Gapdh mRNA, tested as a control, did not bind G3BP1 (Fig. 5B).

Mapping of the 3’-UTR of Ctnnb1 mRNA for the binding site of G3BP1

The 3’-UTR of Ctnnb1 mRNA was probed for the site(s) to which G3BP1 binds. Ctnnb1 3’-UTR regions were truncated (2517–2587, 2588–3198, 3199–3536) and DIG-labeled RNA probes of each region were synthesized in vitro using T7 RNA polymerase. The binding of the probes to G3BP1 isolated from cells was then...
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**Fig. 5. G3BP1 binds Ctnnb1 mRNA.** (A) RNA immunoprecipitation assay was performed on F9 cell lysates expressing either empty vector, full-length G3BP1 (1–465), its N-terminal half (1–240) or its C-terminal half (241–465) with anti-myc antibodies. Ctnnb1 mRNA in the immunoprecipitates was quantified using quantitative PCR. The top panel represents mean values ± s.e.m. obtained from two independent experiments; the bottom panel displays representative gel picture of two independent experiments that proved highly reproducible. **P < 0.01 versus control (pCMV-Myc). (B,C) Northwestern analysis of interaction of G3BP1 with Ctnnb1 mRNA. Recombinant GST or GST–G3BP1 (B) or immunoprecipitated Myc–G3BP1 from F9 cell lysates (C) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Northwestern analysis was then performed either using DIG-labeled Ctnnb1 UTR or Gapdh UTR probes. The top panels represent northwestern blots whereas the lower panels display either Ponceau S staining (B) or immunoblotting with anti-Myc antibodies for the same blots. (D–F) Identification of G3BP1 binding region within the 3′-UTR of Ctnnb1 mRNA. Immunoprecipitated Myc–G3BP1 from F9 cell lysates were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes. Northwestern analysis was then performed using truncated versions of DIG-labeled Ctnnb1 UTR probes. The top panels represent northwestern blots whereas lower panels display immunoblots with anti-Myc antibodies. Representative data of two independent experiments that proved highly reproducible are displayed.
evaluated. RNA probes encoding the 2517–2857 and 2858–3198 regions of Ctnnb1 UTR bound G3BP1 (Fig. 5E). The 3199–3536 region of Ctnnb1 UTR, by contrast, failed to bind G3BP1 (Fig. 5E).

G3BP1 has been proposed to be an endoribonuclease selectively cleaving between ‘CA’ dinucleotides: an activity that requires a binding consensus sequence (Tourriere et al., 2001). Through in silico searches, we identified a putative G3BP1-binding consensus sequence within the 3′-UTR of Ctnnb1 mRNA (2885–2907 nucleotides). Therefore, RNA probes encoding additional truncations of the Ctnnb1 mRNA (2858–2968, 2969–3079, 3080–3198) were synthesized and tested for G3BP1 binding. The RNA probe encoding 2858–2968 region of the Ctnnb1 mRNA displayed the greatest G3BP1 binding (Fig. 5F). The in silico search also
Fig. 6. Arginine methylation of G3BP1 impairs its binding to Ctnnb1 mRNA and Dvl3. (A) RNA immunoprecipitation assay was performed on F9 cells lysates expressing either empty vector, wild-type G3BP1, its methylation-deficient mutants (R433K, R445K) or its methylation-mimicking mutants (R433F, R445F) with anti-Myc antibodies. The amount of Ctnnb1 mRNA in the immunoprecipitates was then quantified using quantitative PCR. **P<0.01 versus control (pCMV-Myc). (B) Northwestern analysis of unmethylated or methylated GST–G3BP1 and Ctnnb1 mRNA. Equal amounts of unmethylated or methylated (with PRMT1 isolated from unstimulated cells or cells treated with Wnt3a for 6 hours and [3H]SAM) GST–G3BP1 were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes. Northwestern analysis was then performed on the blots using DIG-labeled Ctnnb1 UTR. The binding of Ctnnb1 mRNA to unmethylated GST–G3BP1 was taken as 100%. The top panel represents mean values ± s.e.m. obtained from three independent experiments; the bottom panels display northwestern blots and the corresponding fluorograph. *P<0.05; **P<0.01 versus control (unmethylated GST–G3BP1). (C) F9 cells were transiently transfected with either pCMV–Myc, Myc–G3BP1 or its methylation-deficient mutants (R433K, R445K) for 24 hours followed by cell lysis and affinity pull-downs with anti-Dvl3 antibodies. Interaction of G3BP1 and its mutants with Dvl3 was made visible by probing the blots with anti-Myc antibodies. Top panel displays mean values ± s.e.m. obtained from three independent experiments; bottom panel displays representative blots. **P<0.01 versus control (WT). (D) F9 cells were transiently transfected with methylation-deficient mutants of Myc–G3BP1 (R433K, R445K) for 24 hours. The cells were then treated with Wnt3a (20 ng/ml) for indicated periods of time followed by cell lysis and affinity pull-downs with anti-Dvl3 antibodies followed by immunoblotting with anti-Myc antibodies. Top panel displays mean values ± s.e.m. obtained from three highly reproducible experiments; the bottom panel displays representative blots. *P<0.05; **P<0.01 versus corresponding unstimulated control (=Wnt3a). (E) F9 cells were transfected either with Myc–G3BP1 or its methylation-deficient mutants (R433K, R445K) for 24 hours and the lysates were assayed for Lef/Tcf-sensitive transcription following stimulation with Wnt5a for 7 hours. Top panel displays mean values ± s.e.m. obtained from three independent highly reproducible experiments; bottom panel displays representative blots. #P<0.01 versus control (pCMV-Myc).

Arginine methylation of G3BP1 is a molecular switch, provoking dissociation from Ctnnb1 mRNA and Dishevelled-based supermolecular complexes

Does methylation of G3BP1 alter its binding of Ctnnb1 mRNA? Myc-tagged wild-type G3BP1 and G3BP1-methylation-deficient (R433K, R445K) and methylation-mimicking [R433F, R445F (Mostaql Huq et al., 2006; Weber et al., 2009; Guo et al., 2010)] mutants of G3BP1 were used to address this question. Cells were transiently transfected with either wild-type or mutant forms of G3BP1 and cell lysates were later subjected to pull-downs with anti-Myc antibodies. Isolation of RNA (from Myc pull-downs) and amplification by RT-PCR was performed next. The relative amounts of Ctnnb1 transcripts in the G3BP1 complexes were then established using quantitative real-time PCR. Ctnnb1 transcripts were identified in the wild-type G3BP1 pull-downs (Fig. 6A). By contrast, Ctnnb1 transcripts were nearly undetectable in the pull-downs from cells expressing R433F mutant of G3BP1 (a ‘methylation-mimicking mutant’, Fig. 6A). Binding of Ctnnb1 mRNA to G3BP1 was unaffected by the R445F mutation, being similar to either wild-type or methylation-deficient mutants (R433K, R445K, Fig. 6A). To further test the role of arginine methylation in the association of G3BP1 with Ctnnb1 mRNA, we examined whether methylation of GST–G3BP1 affects its ability to bind Ctnnb1 mRNA in vitro. For these experiments, methylated GST–G3BP1 was prepared in vitro using HA–PRMT1. Methylation was assessed through the use of tritiated-S-adenosyl methionine ([3H]SAM) in the methylation assay buffer. Equal amounts of unmethylated and methylated GST–G3BP1 were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Northwestern analysis was then performed on the membranes using a DIG-labeled full-length Ctnnb1 UTR. Consistent with the RNA immunoprecipitation data (Fig. 6A), Wnt3a-stimulated PRMT1-mediated methylation of GST–G3BP1 provoked a sharp decrease in the ability of G3BP1 to bind Ctnnb1 mRNA (Fig. 6B). Methylation of GST–G3BP1 by PRMT1 isolated from untreated cells provoked a small decrease in the ability of G3BP1 to bind Ctnnb1 mRNA (Fig. 6B). Arginine methylation of G3BP1 appears to be a molecular switch: in response to methylation at R433, the ability of G3BP1 to bind Ctnnb1 mRNA was sharply attenuated (Fig. 6A,B).

G3BP1 is a Dishevelled-associated protein (Fig. 2) that is methylated upon Wnt3a stimulation (Fig. 3). We assessed whether G3BP1 methylation influences its ability to bind Dvl3. F9 cells overexpressing either Myc-tagged wild-type G3BP1 or methylation-deficient mutants (R433K and R445K) of G3BP1 were used to address this question. Pull-downs performed on cell lysates using anti-Dvl3 antibodies show greater association of the methylation-deficient mutants (R433K and R445K) of G3BP1 than wild-type G3BP1 to Dvl3 (Fig. 6C). Wnt3a stimulation of cells that overexpress wild-type G3BP1 provoked loss of G3BP1 from the Dvl3-based supermolecular complex (Fig. 2). Wnt3a stimulation of cells overexpressing methylation-deficient mutants (R433K and R445K), by contrast, did not release G3BP1 from the Dvl3-based supermolecular complex (Fig. 6D). These observations suggest that arginine methylation of G3BP1 provokes the release of G3BP1 from the Dvl3-based signaling complex.

Wnt3a and methylation-deficient (R433K and R445K) mutants of G3BP1 after canonical Wnt/β-catenin signaling? Lef/Tcf-sensitive gene transcription was assessed in cells expressing R433K or R445K G3BP1. Overexpression of wild-type G3BP1 decreased Wnt3a-stimulated Lef/Tcf-sensitive transcription (Fig. 6E). However, expression of the methylation-deficient mutants of G3BP1 attenuated the Wnt3a-stimulated Lef/Tcf-sensitive transcription further (Fig. 6E). In tandem, these observations make a compelling case for protein methylation as the regulator for G3BP1 binding of Ctnnb1 mRNA, as well as its docking to Dvl3-based signalsomes that catalyze canonical signaling.

Discussion

Dishevelled is a multi-functional scaffold protein that has a critical role(s) during Wnt signaling. Dishevelled, through its common domains such as DIX, PDZ and DEP domains, provides docking sites for many proteins and constitutes a large supermolecular assembly (Yokoyama et al., 2010). In the present study, G3BP1 was identified as a novel Dishevelled-associated protein. G3BP1 associates with Dishevelled through its C-terminus, which displays arginine methylation (RGG) motifs. Wnt3a stimulated robust methylation of G3BP1. Methylation of G3BP1 also provoked reduced association with the Dishevelled-based complexes.

G3BP1 was discovered as a Ras GTPase activating (Ras GAP) SH3-binding protein (Parker et al., 1996). The critical role of G3BP3s during mammalian and invertebrate development is highlighted in several studies using either knockout mice (Zekri et
We show herein a previously unidentified role for protein arginine methylation in canonical Wnt/β-catenin signaling, focusing upon Ctnnb1 mRNA. Ctnnb1 mRNA is regulated post-transcriptionally (Gherzi et al., 2006; Bikkavilli and Malbon, 2010). Now we show a post-translational modification-mediated regulation of Ctnnb1 mRNA. Under basal conditions, the Dvl3–G3BP1 complex actively mediates downregulation of Ctnnb1 mRNA. Wnt3a stimulation provokes methylation of G3BP1 by PRMT1, releasing Ctnnb1 mRNA from this regulatory degradation. We propose that protein arginine methylation is a Wnt3a-sensitive ‘molecular switch’ that fosters decreased binding of Ctnnb1 mRNA to G3BP1, which accompanies loss of G3BP1 from the Dvl3-based signalosome (Fig. 7). The molecular details of these altered interactions of G3BP1 and Ctnnb1 mRNA remain to be discerned; however, their functional role on canonical Wnt signaling is clearly important.

**Materials and Methods**

**Constructs**

Mouse Dvl2 and Dvl3 isoforms were engineered in-house with GFP2 and HA tags. cDNAs of G3bp1 and its fragments (1–240 and 241–465) were subcloned into the EcoRI and NotI sites of pCMV-Myc plasmid in-frame with the Myc tag sequence. Mouse Prmt1 cDNA was subcloned into EcoRI and KpnI sites of pCMV-HA vector in-frame with the HA tag sequence. DNA fragments of mouse Ctnnb1 3′-UTR (NM_007614, 2517–3536, 2517–2857, 2858–3198, 3199–3536, 2858–2968, 2969–3079, 3080–3198), and mouse Gapdh 3′-UTR (NM_008084, 1011–1230) were subcloned into KpnI and EcoRI sites of pDNA3.1 vector. Site-directed mutagenesis was performed on Myc–G3BP1 plasmid using Quick Change Site Directed Mutagenesis kit (Stratagene) to obtain Myc–G3BP1 mutants (R433K, R445K, R433F and R445F). For generating GST-tagged G3BP1, cDNAs encoding G3BP1 and its mutants were subcloned into EcoRI and NotI sites of pGEX4T1 plasmid in-frame with the GST protein. The primers used for cloning were summarized in supplementary material Table S1.

**Cell culture**

Mouse F9 teratocarcinoma cell stocks were obtained from ATCC (Manassas, VA) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% (F9 cells) heat-inactivated fetal bovine serum (Hyclone, South Logan, UT) at 37°C in a 5% CO2 incubator. The F9 cells stably expressing Rfz1 and pTOPFLASH (M50) luciferase reporter were generated as described earlier (Bikkavilli et al., 2008) and used as a standard in all experiments. The use of this stable cell line as the starting point for transient transfections reduced variability and offered greater consistency by reducing the number of plasmids required for simultaneous transfections.

**Commmunoprecipitation and immunoblotting**

For communoprecipitation experiments, F9 clones stably expressing rat Frizzled1 were transiently transfected for 4 hours with 6 μg of plasmid vectors in 100 mm culture dishes. After 24 hours, the cells were lysed in 1 ml of lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml phenylmethylsulphonyl fluoride). The lysates were cleared by centrifugation at 20,000 g for 15 minutes, twice. The supernatants were transferred into new tubes and protein concentrations were determined by the Lowry method (Lowry et al., 1951). Immunoprecipitations were performed using either rat anti-HA high affinity antibody (Roche), mouse monoclonal anti-Dvl3 (sc 8027, Santa Cruz), mouse monoclonal anti-Myc antibodies (M4439, Sigma), or mouse anti-GSK3β (610201, BD Transduction Laboratories) and Protein A–Sepharose CL-4B (17-0780-01, GE Life Sciences). For immunoblotting, total lysates (30–60 μg of protein/lane) were subjected to electrophoresis using 10% SDS-PAGE. The resolved proteins were transferred electrophoretically to nitrocellulose membrane ‘blots’. The blots were incubated with primary antibodies overnight at 4°C and immunocomplexes were made visible with a secondary antibody coupled to horseradish peroxidase and developed using the enhanced chemiluminescence method. Antibodies were purchased from the following sources: anti-HA high affinity antibody (Roche Applied Science, Indianapolis, IN), anti-β-catenin, anti-Myc, and anti-β-actin antibodies were from Sigma. Mouse anti-GSK3β was from BD Transduction Laboratories. Mouse monoclonal anti-monomethyl and anti-dimethyl arginine was from Abcam (ab412).

**In-gel tryptic digestion and mass spectrometry analysis**

Following SDS-PAGE analysis of Dvl3 immunocomplexes, the gel band corresponding to the molecular range of 50–80 kDa was excised, destained, reduced, alkylated and digested with either trypsin or chymotrypsin (Promega Gold, Mass Spectrometry Grade) essentially as described previously (Shevchenko et al., 1996) with minor modifications. The resulting concentrated peptide extract was diluted...
into a solution of 2% acetonitrile (ACN), 0.1% formic acid (FA) (Buffer A) for 40% to 80% buffer B over 5 minutes and held constant for 3 minutes. Finally, the gradient was changed from 80% buffer B to 100% buffer A over 1 minute, and then held constant at 100% buffer A for 15 more minutes. The application of a 1.8 kV distal voltage electrosprayed the eluted peptides directly into a Thermo LTQ ion trap mass spectrometer equipped with a custom nanoLC electrospray ionization source. Full mass (MS) spectra were recorded on the peptides over a 400–2000 m/z range, followed by five tandem mass (MS/MS) spectra sequentially generated in a data-dependent manner on the first, second, third, fourth and fifth most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectra were acquired on the MS using the data-dependent method with modifications: +16 on Met, +57 on Cys, +14 on Arg and Lys. Only peptides with a P value of at least 0.02 were analyzed further. Peptides with possible methylated arginines and lysines were manually verified.

In vitro methylation assays

In vitro methylation assay using bacterially expressed GST–G3BP1 was performed as described earlier (Tini et al., 2009). Briefly, F9 cells were transiently transfected with pCMV–Myc–G3BP1 or its mutants (R433K, R445K) (6 μg) in 100 μm culture dishes. After 24 hours of transfection, the cells were lysed in a lysis buffer (1% PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin and 1 μg/ml phenylmethylsulphonyl fluoride) after treatment either with or without Wnt3a for 3 minutes. The lysates were then used to immunoprecipitate PRMT1 with anti-α-HA antibodies and Protein-A-Sepharose CL-4B (17-0780-01, GE Life Sciences) at 4°C for 16 hours. After 16 hours, the immunoprecipitates were washed three times in RIPA buffer (20 mM Tris-2-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100) and once in methylation reaction buffer (50 mM Tris-2-HCl, pH 8.5, 20 mM KCl, 10 mM MgCl₂, 1 mM β-mercaptoethanol, 100 mM succrose). Finally, the HA–Sepharose containing bound PRMT1 was incubated with 10 μl of methylation reaction buffer containing 4 μg of GST-G3BP1 or its mutants and 1 μCi S-adenosyl-L-[methyl-³H] methionine (NEN Radiochemicals, 250 μCi/μl, 925 MBq), at 30°C for 1 hour. After 1 hour, the reactions were stopped by addition of equal volume of SDS sample loading buffer, boiled and separated on a SDS-PAGE gel. The gel was then fixed (45% methanol, 10% acetic acid in water, 30 minutes), amylated (Autofluor, National Diagnostics, 2 hours), dried and fluorography was performed.

In vivo methylation assays

In vivo methylation assay for G3BP1 was performed as described previously (Tini et al., 2009). Briefly, F9 cells were transiently transfected with pCMV–Myc–G3BP1 or its mutants (R433K, R445K) (6 μg) in 100 mm culture dishes and grown to confluency (24 hours). After 24 hours of transfection, the cells were washed once with PBS and protein translation was inhibited by incubating with 100 μg/ml cycloheximide (1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml phenylmethylsulphonyl fluoride). The lysates were then utilized for immunoprecipitations using anti-α-HA antibodies and Protein-A–Sepharose CL-4B (17-0780-01, GE Life Sciences) for 16 hours at 4°C. After 16 hours, the immunoprecipitates were washed three times in RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100) and once in methylation reaction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100) for 4 hours. After a 2 hour centrifugation, the supernatants were transferred to new tubes and 30 μl of ConA–Sepharose was added to each tube and rotated at 4°C for another hour. Finally, after a brief centrifugation, the supernatants were transferred to new tubes and their protein concentration was determined. Immunoblotting was performed with the samples and β-catenin accumulation was analyzed by probing the blots with anti-β-catenin antibodies and normalized by probing the same blots with anti-actin antibodies.

**Cytosolic β-catenin accumulation assay**

To separate the cytosolic β-catenin from membrane-associated β-catenin, lysates were treated with Concanavalin-A–Sepharose (ConA, Amersham Biosciences, Uppsala, Sweden), as described previously. A 1 ml wash of growth medium was used. Briefly, confluent F9 cultures were treated with Wnt3a for 4 hours (Fig. 3B, E, F) and lysed in a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM Na₃PO₄, 50 mM K₂HPO₄, 10 mM Na₂MoO₄, 2 μM Na₂VO₃, 1% Triton X-100, 0.5% NP40, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml phenylmethylsulphonyl fluoride). The lysates were transferred into 1.5 ml Eppendorf tubes and rotated at 4°C for 15 minutes following by centrifugation at 20,000 g for 15 minutes. The supernatants were transferred into new tubes, their protein concentrations were determined and the concentration was adjusted to 2.5 mg/ml with lysis buffer 60 μl of ConA–Sepharose was added to each tube and rotated at 4°C for 1 hour. After a brief centrifugation, the supernatants were transferred to new tubes and 30 μl of ConA–Sepharose was added to each tube and rotated at 4°C for another hour. Finally, after a brief centrifugation, the supernatants were transferred to new tubes and their protein concentration was determined. Immunoblotting was performed with the samples and β-catenin accumulation was analyzed by probing the blots with anti-β-catenin antibodies and normalized by probing the same blots with anti-actin antibodies.

**RNAi**

Double-stranded RNAs (siRNAs) targeting mouse Gephyr (5′-CAGAGAUAGGAUGUCUGGGGCUU-3′) and control siRNAs (5′-UCUGUAGUUGAAAAACUGAUCGAC-3′) were procured from Invitrogen (Invitrogen, Carlsbad, CA). F9 cells expressing Ralf1 were treated with 100 nM siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, 100 nm siRNA was incubated with 5 μl Lipofectamine 2000 for 20 minutes in 200 μl Optimem (Invitrogen) and the mixture was transferred into 1 ml of growth medium in a 12-well plate in which F9 cells expressing Ralf1 were cultured to 80% confluence. After siRNA treatment for 48 hours, the cells were assayed for Cnn1b mRNA levels, β-catenin stabilization or Lef1/Tcf-sensitive gene transcription.
Luciferase assays
F9 cells stably expressing Rfx1 and super 8xTOPFLASH (M50) luciferase reporter were seeded into 12-well plates. Following incubation with siRNAs for 48 hours at 37°C, the cells were treated with or without recombinant Wnt3a for 7 hours (R&D systems, Minneapolis). Cells were then directly lysed on the plates by addition of 1× cell culture lysis reagent (Promega, Madison, WI). Lysates were collected into chilled microfuge tubes on ice and centrifuged at 20,000 g for 5 minutes. The supernatant was transferred into a new tube and directly assayed as described below. 20 μl of the lysate was mixed with 100 μl of luciferase assay buffer (20 mM Tricine, pH 7.8, 1.1 mM MgCO₃, 4 mM MgSO₄, 0.1 mM EDTA, 0.27 mM coenzyme A, 0.67 mM luciferin, 33 mM DTT and 0.6 mM ATP) and the luciferase activities were measured with a luminometer (Berthold Lumat LB 9507). The samples were assayed in triplicate and the luciferase activities were normalized by protein content of the samples and are represented in the figures.

Data analysis
Typically, data were compiled from at least three independent replicate experiments, each performed on separate cultures and on separate occasions. We calculated and display the responses as ‘fold change’ (over the untreated control). Comparisons of data among such groups were performed using the Student’s t-test for assessing variance. Statistical significance (P<0.05) is denoted with either an asterisk or a pound symbol. For a few of the experiments replicated independently with very low variance, duplicates were deemed adequate. Each of these instances are indicated in their respective figure legends.

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