STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE WNT INHIBITOR APC MEMBRANE RECRUITMENT 1 (Amer1)*

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Amer1/WTX binds to the tumor suppressor APC and acts as an inhibitor of Wnt signaling by inducing β-catenin degradation. We show here that Amer1 directly interacts with the armadillo repeats of β-catenin via a domain consisting of repeated arginine-glutamic acid-alanine (REA) motifs, and that Amer1 assembles the β-catenin destruction complex at the plasma membrane by recruiting β-catenin, APC and Axin/Conductin. Deletion or specific mutations of the membrane binding domain of Amer1 abolish its membrane localization and abrogate negative control of Wnt signaling, which can be restored by artificial targeting of Amer1 to the plasma membrane. In line, a natural splice variant of Amer1 lacking the plasma membrane localization domain is deficient for Wnt inhibition. Knockdown of Amer1 leads to the activation of Wnt target genes preferentially in dense compared to sparse cell cultures suggesting that Amer1 function is regulated by cell contacts. Amer1 stabilizes Axin and counteracts Wnt-induced degradation of Axin, which requires membrane localization of Amer1. The data suggest that Amer1 exerts its negative regulatory role in Wnt signaling by acting as a scaffold protein for the β-catenin destruction complex and promoting stabilization of Axin at the plasma membrane.

The canonical Wnt/β-catenin signaling is a key pathway in embryonic development and disease. Aberrant activation of Wnt signaling leads to the development of tumors (1,2). The binding of Wnt ligands to the transmembrane receptors Frizzled (Fz) and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) leads to the stabilization of cytoplasmic β-catenin, which enters the nucleus and activates target gene expression by interacting with DNA-binding proteins of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family (3,4). In the absence of Wnts, β-catenin is degraded in the proteasome after its ubiquitination by the E3 ligase β-TrCP, which recognizes β-catenin phosphorylated at specific N-terminal residues. β-catenin phosphorylation is accomplished by the coordinated action of casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3) and takes place in a multiprotein complex assembled by the scaffold proteins adenomatous polyposis coli (APC) and Axin or its homologue Axin2/Conductin (4,5). Because Axin binds to β-catenin, GSK, CK1 and APC, and APC has multiple binding sites for β-catenin, it is generally believed that one function of this β-catenin destruction complex is to bring β-catenin into close vicinity to the kinases thereby allowing efficient phosphorylation (6).

It is generally thought that β-catenin phosphorylation and degradation is a constitutive process keeping the amount of β-catenin in the cytoplasm and nucleus low in the absence of Wnts. Wnt binding to the receptor complex leads to the recruitment of Axin to the plasma membrane and phosphorylation of LRP5/6 at cytoplasmic PPPSPxS motifs (7-10). Phosphorylated PPPSPxS motifs can directly inhibit the enzymatic activity of GSK3 thereby preventing β-catenin phosphorylation and leading to its stabilization (11-13). Inhibition of GSK3 might also result from its sequestration in
multivesicular bodies generated following endocytosis of the Fz-LRP6 receptor complex (14). A consequence of prolonged Wnt receptor activation is the degradation of Axin, which may contribute to sustained β-catenin stabilization (8,15-18).

There is evidence that phosphorylation of β-catenin can be dynamically regulated at the plasma membrane. Thus, it was recently reported that dephosphorylated β-catenin appears at the plasma membrane shortly after exposure to Wnt ligands and before it enters the nucleus (19). Phosphorylated β-catenin was shown to be primarily present at cell-cell contacts rather than in the cytoplasm, and increased cell-cell adhesion promoted β-catenin phosphorylation and turnover (20). How the plasma membrane and cell-cell contacts modulate the β-catenin degradation in conjunction with Wnt signaling is unclear.

Amer1 (APC membrane recruitment 1) is a plasma membrane-associated protein of 1135 amino acids conserved in vertebrates. It was previously identified as an interaction partner of APC in a yeast two hybrid screen using the armadillo repeat domain of APC as bait (21). Amer1 contains three binding domains for APC that share no obvious sequence similarity to each other (A1-A3; Fig. 1) (21). Amer1 is associated with the plasma membrane via two N-terminal phosphatidylinositol(4,5)bisphosphate (PtdIns (4,5)P2) binding domains (M1, M2; Fig. 1), and can recruit APC from microtubules to the plasma membrane (21). We have recently shown that Amer1 is involved in stimulating LRP6 phosphorylation, which requires its membrane association (22). Amer1 is identical to the tumor suppressor WTX shown to be mutated in a fraction of Wilms tumors, which are pediatric kidney cancers (23). The WTX gene is also mutated in the hereditary disease OSCS (osteopathia striata congenita with cranial sclerosis) (24). Functional studies in Zebrafish and Xenopus indicate that Amer1/WTX is a negative regulator of Wnt signaling in development, and in vitro studies show that it is required for β-catenin ubiquitination and degradation (25). Moreover, proteomic analysis indicates that Amer1 is present in complexes with APC, β-catenin, Axin and β-TrCP (25). The mechanisms by which Amer1 regulates β-catenin turnover and the relevance of the membrane binding function of Amer1 in this process are not known. In this paper we provide evidence for a direct interaction of Amer1 with β-catenin and show that Amer1 recruits the β-catenin destruction complex to the plasma membrane, which is essential for Amer1’s negative role in Wnt signaling. Furthermore we demonstrate a role for Amer1 in the stabilization of Axin.

**EXPERIMENTAL PROCEDURES**

**Cell culture, drug treatment and transfection.** All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria) at 37°C in a humidified atmosphere of 10% CO2. HEK293T cells stably expressing pBAR/Renilla (25) were kindly provided by R.T. Moon. For the cell density experiment HeLa cells were seeded at 100,000/well in six-well plates and transfected the next day. The cells were trypsinized 24 hours after transfection. One half of the cells were plated in one well of a six-well plate (high cell density) and the other half split into three wells of a six-well plate (low cell density). 48 hours later the cells were harvested for Western Blot or RT-PCR experiments. To block protein synthesis cells were treated with 100 µM cycloheximide (Sigma) after transfection. Wnt3A conditioned medium was produced from mouse L cells stably expressing Wnt3A (American Type Culture Collection CRL-2647) and added 16 hours after transfection. Plasmid transfections were performed using either polyethylenimin for HEK293T cells or TransIT-TKO (Mirus, Madison, WI, USA) for MCF-7 cells. siRNAs were transfected using either Oligofectamine (Invitrogen) when transfected alone or Lipofectamine (Invitrogen) when cotransfected with plasmid DNA.

**Plasmids and siRNAs.** The following plasmids have been described previously: pEGFP-Amer1, pcDNA-Flag-Amer1, pEGFP-APC-Arm, pcDNA-Flag-APC-Arm (21); pCMV-APC, mRFP-daLRP6 (26); pcDNA3.1-Flag, pcDNA-Flag-Conductin (27); pcDNA-Myc-β-catenin (28). pEGFP-Conductin(455-782) was kindly provided by M. Hadjihannas and pcDNA-Flag-Axin by A. Kikuchi. Deletion mutants of Amer1 were generated by restriction digests or PCR amplification. To obtain the
Amer1 lysine mutants the following lysines were mutated to alanines by PCR mutagenesis: lysine 83, 181 and 183 for Amer1(3µLys); lysine 54, 58, 83, 181 and 183 for Amer1(5µLys); lysine 54, 58, 79, 83, 166, 181 and 183 for Amer1(7µLys). pEGFP-NES-Amer1(7µLys) was created by replacing an oligonucleotide coding for a nuclear export sequence (NES) from MAPKK (NLVDLQKLEELDEEQ) (29) between the EGFP and Amer1 coding sequences of pEGFP-Amer1(7µLys). mRFP-TMD-Amer1ΔN was generated by replacing the LRP6 coding sequence of mRFP-daLRP6 (26) with the transmembrane domain of the LDL receptor (residues 781-849) (10) fused to the Amer1(207-1135) coding sequence. To generate pEGFP-Amer1-S1 three single nucleotide changes (147 A>C, 150 T>C, 153 G>A) were introduced leading to the ablation of the internal splice donor site without changing the amino acid sequence. pEGFP-Amer1-S2 and pEGFP-Amer1ΔREA contain in frame deletions of residues 50-326 and 551-649, respectively. Details of the plasmids are available upon request. The sequences of the siRNA oligonucleotides are: siGFP, 5’-GCTACCTGT-TCCATGGCCA-3’; siAmer1a, 5’-GGGAGT-ACCCTGAACAAA-3’; siAmer1b, 5’-CCTCTATGCCAAGCCAAA-3’; siAmer1-S1, 5’-CCACCAGCTACTGAGAAAA-3’; siAmer1-S2, 5’-GGCCCGTTGTCGAGACCA-3’; siAxin, 5’-GGTTGTGCGATTAAAGGTG-3’ (25). All siRNA oligonucleotides were purchased from Eurogentec.

Preparation of protein lysates, immunoprecipitations, and Western blotting. Cells were washed once with PBS and lysed in Triton-X-100 buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM DTT and 1 mM PMSF) at 4°C for 10 minutes. Lysates were cleared at 16,000 g for 10 minutes. For coimmunoprecipitation, lysates were incubated for 4 hours at 4°C with the appropriate antibody and Protein A/G PLUS agarose beads (Santa Cruz Biotechnology Inc.). Immunoprecipitates were collected, washed four times with low salt NET buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, 10 mM NaF) and eluted with SDS sample buffer. For Western blotting (30) proteins were visualized with a luminoimager (LAS-3000, Fuji) using Enhanced Chemiluminescence reagent (Perkin Elmer) and quantified using the AIDA image analyser software v. 3.52 (Raytest, Straubenhardt, Germany).

Immunofluorescence microscopy. Immunofluorescence stainings were performed as described previously (21). Photographs were taken with a CCD camera (Visitron, Munich, Germany) on a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany, 63x objective) and MetaMorph software (Molecular Devices).

Antibodies. The mouse monoclonal antibody against Amer1 has been described before (21). Note that this antibody only recognizes the Amer1-S1 splice variant. Commercial antibodies were obtained from Abcam (mouse anti-APC, Ali; rabbit anti-RFP), BD Transduction Laboratories (mouse anti-β-catenin), Cell Signaling (rabbit anti-Axin1, C76H11; rabbit anti-Axin2, 76G6; rabbit anti-LRP6, C5C7; mouse anti-Myc, clone 9B11), Roche (mouse anti-GFP, mixture of clones 7.1 and 13.1), Santa Cruz Biotechnology Inc. (rabbit anti-β-catenin, clone H102), Serotec (rat anti-α-Tubulin, clone YL1/2) and Sigma (mouse anti-β-Actin, clone AC-15; rabbit anti-Flag, mouse anti-Flag). Secondary antibodies coupled to horseradish peroxidase or Cy3 were purchased from Jackson ImmunoResearch.

Reporter Assays. TCF/β-catenin reporter assays were carried out in HEK293T cells stably expressing a β-catenin responsive firefly luciferase reporter (pBAR) along with a Renilla luciferase as an internal control (25). Cells were seeded in 12-well plates and transfected with 200 ng daLRP6 plus 300 ng Amer1 for overexpression experiments or with 20 ng daLRP6 together with 40 pmol siRNA for knockdown experiments. Cells were harvested 24 hours (overexpression experiments) or 48 hours (knockdown experiments) post-transfection. Firefly and Renilla luciferase activities were determined according to standard procedures and firefly luciferase values were normalized to Renilla values. All experiments were performed in duplicates and were reproduced at least twice.

Yeast two-hybrid screen. Yeast two-hybrid and β-galactosidase assays were performed in
the L40 yeast strain using pBTM116 as a bait vector and a mouse embryonic day 10.5 cDNA library in pVP16 as described previously (27).

Injection and analysis of X. laevis embryos. RNA for microinjections was prepared using the mMessage mMachine Kit (Ambion, Austin/TX, USA). Injection amounts were 10 pg XWnt8 RNA, 200 pg hAmer1, hAmer1ΔN and hAmer1(7µLys). Embryos were obtained by in vitro fertilisation and cultured as described previously (31). Embryos were injected at the four-cell stage in both ventral blastomeres, cultured till they reached stage 24-26 according to the normal table of Nieuwkoop and Faber (32) and scored for the presence of a secondary body axis. Results from each experiment were normalized to the percentage of secondary body axes induced by XWnt8 in embryos from the same egg batch.

RT-PCR. Total cellular RNA was isolated with the RNaseasy mini kit (Qiagen) and possible genomic contaminations were removed by treatment with DNase I. Single stranded cDNA was synthesized from 1 µg total cellular RNA using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene) according to manufacturer’s instructions. RT-PCR was performed according to standard protocols using the following sense and antisense primers: Axin (5’-GCCGAGGGAAGCTGGAGGAGCGGCAGCGCGTGCGC-3’; 5’-CTTCTTGGCATTCTTTTGGACGCGACCCACCTCTCCT-3’); Amer1 (5’-GCGAATTCGGAGACCAAAGGATGAAGCTGCTCAG-3’, 5’-CCTTGCTCTTTGAGCAGCGGATACTGC-3’); Conductin (5’-GCAAACCTTTGCGCCAACCCGGGAGTG-3’, 5’-CTCTGGAGCTTCTTCTTACTGCG-3’); LGR5 (5’-CTTGGCCCTGAACTACAATGGAATATG-3’, 5’-AACGGTGTGCCTAACAAATGCCCT-3’) and GAPDH (5’-CCTGCTTCACACCCTCTTG-3’, 5’-CTTCAACCACCATGGAGAAG-3’).

RESULTS

Amer1 interacts with β-catenin via REA repeats and recruits it to the plasma membrane. Amer1/WTX was previously detected in protein complexes together with APC, β-catenin and Axin (25), suggesting that Amer1 interacts with the β-catenin destruction complex. However, the structural organization of these complexes is unclear except of our previous findings that three binding domains in Amer1 (A1-A3; Fig. 1) mediate direct interaction with APC in yeast two hybrid assays and that Amer1 forms endogenous complexes with APC (21). Immunoprecipitation experiments confirmed that also Amer1 and β-catenin form endogenous complexes (Fig. 2A). Results from a yeast two hybrid screening using a fragment of Amer1 as a bait (Amer1(531-716); Fig. 1) allowed us to specify the domains involved in the Amer1/β-catenin interaction. In the screen we isolated two overlapping clones of β-catenin spanning the central part of the armadillo repeat domain, indicating that this region of β-catenin can interact directly with Amer1 (Fig. 2B). The Amer1 bait used for screening contains ten copies of a repeated amino acid motif including the core sequence arginine-glutamic acid-alanine (REA) (Fig. 2C). Amer1 fragments lacking this REA repeat domain, such as Amer1(2-530) and Amer1(714-1135), did not interact with β-catenin in immunoprecipitations experiments, whereas N- and C-terminal halves of Amer1 retaining some of the REA repeats were still capable of β-catenin binding (Amer1(2-601), Amer1(601-1135); Fig. 1 and 2D). Internal deletion of the whole REA repeat region (Amer1REA) from full-size Amer1 completely abolished β-catenin interaction (Fig. 1 and 2D). Amer1 recruited coexpressed β-catenin from cytoplasm and nucleus to the plasma membrane, which was abolished when the REA repeats were deleted (Fig. 2E). Together, these data strongly suggest that the REA repeats mediate binding of Amer1 to the armadillo repeats of β-catenin. Amer1 repressed a TCF/β-catenin dependent transcriptional reporter activated by a dominant-active mutant of LRP6 (daLRP6; Fig. 2F) (8,26). Deletion of the REA repeats reduced but did not fully abolish this repression indicating that the REA repeats are required but not essential for inhibition of Wnt signaling.

Plasma membrane recruitment of Axin proteins by Amer1. We next analyzed the interaction of Amer1 with Axin/Conductin. Conducin co-immunoprecipitated with full-size Amer1 and with N-terminal Amer1 fragments spanning amino acids 2-601 but not 2-530 (Fig. 1; supplemental Fig. S1A). In addition, a C-terminal fragment of Amer1 from amino acids 834-1135 co-immunoprecipitated with
Conductin indicating that there are two regions within Amer1 capable of interacting with Conductin (Fig. 1; supplemental Fig. S1A). Amer1 also co-immunoprecipitated with Amer1 (22). Importantly, Amer1 recruited Conductin and Axin from their cytoplasmic localization to the plasma membrane (supplemental Fig. 1B) (22). These and our previous data (21) show that Amer1 interacts with key components of the β-catenin destruction complex APC, Axin proteins and β-catenin via different domains and recruits these proteins to the plasma membrane.

**Amer1 acts as a scaffold for the β-catenin degradation complex.** Next, we analyzed whether Amer1 interacts with APC, β-catenin and Axin/Conductin in individual complexes or simultaneously. When the Arm repeat domain of APC (which binds directly to Amer1 but not β-catenin and Conductin) (21) was immunoprecipitated, β-catenin and Conductin co-immunoprecipitated in the presence of Amer1 but not in its absence (Fig. 3, A and B). Thus, Amer1 binds simultaneously to APC and β-catenin or Conductin and thereby forms heterotrimeric complexes with these components. Similarly, when a fragment of Conductin lacking the β-catenin binding site (Conductin 455-782) (27) was immunoprecipitated, β-catenin and Conductin co-immunoprecipitated in the presence of Amer1 but not in its absence (Fig. 3C). Thus Amer1 can link β-catenin to Conductin. Vice versa, Conductin and APC can link β-catenin to Amer1 because the amount of β-catenin co-immunoprecipitated with full-length Amer1 was strongly increased when Conductin and APC were also present (Fig. 3D). Interestingly, the total amount of β-catenin in the lysates was reduced when Conductin and APC were present indicating degradation of β-catenin by these components (Fig. 3D). The data indicate that Amer1 may assemble the core components of the β-catenin destruction complex and thereby serve as a scaffold protein for degradation of β-catenin.

**Plasma membrane localization of Amer1 is required for repression of Wnt signaling.** We previously identified a region of Amer1 (ranging from amino acids 2-209) which mediates binding of Amer1 to the plasma membrane by directly interacting with PtdIns(4,5)P2 (Fig. 1) (21). This domain is enriched in highly conserved basic amino acids, in particular lysines known to mediate PtdIns(4,5)P2 binding in other proteins (33). We mutated three, five or all seven of the conserved lysines to alanine and tested cellular localization and activity of these mutants (see Experimental Procedures) (22). Depending on the number of lysines mutated a gradual loss of membrane localization and a concomitant enrichment in the cytoplasm and nucleus of the mutants was observed (Fig. 4A). When tested in TCF/β-catenin dependent reporter assays the mutants gradually lost the capacity for inhibiting reporter activity stimulated by daLRP6 (Fig. 4B), although they bound similarly to APC, β-catenin and Conductin (supplemental Fig. S2). Because these mutants were predominantly localized in the nucleus and not the cytosol this experiment does not rule out that Amer1 might be functional when present in the cytoplasm and that membrane localization per se is not important. Therefore, the Amer1(7µLys) mutant was fused to a nuclear export sequence (NES-Amer1(7µLys)) which efficiently prevented its accumulation in the nucleus and led to exclusive cytoplasmic localization of this mutant (22). This fusion did not restore inhibition of TCF/β-catenin dependent transcription by Amer1 (Fig. 4C), indicating that membrane localization is indeed important and that cytoplasmic localization of Amer1 does not suffice for repression of Wnt signaling. In line, fusion of Amer1 lacking the membrane targeting domain (Amer1ΔN) to the transmembrane domain from the low-density lipoprotein (LDL) receptor (TMD-Amer1ΔN) (10) restored inhibition of transcription (Fig. 4D).

To analyze whether membrane localization is also important for Amer1 activity in vivo we performed axis formation assays in *Xenopus*. Activation of Wnt signaling by injection of Wnt mRNA into prospective ventral cells at the 4-cell stage leads to a duplication of the body axis, which can be blocked by co-injection of pathway inhibitors such as Axin (34-36). Amer1 expression efficiently suppressed double axis formation induced by Xenopus Wnt8 (XWnt8) (Fig. 4E). This effect was greatly reduced in the Amer1(7µLys) mutant or the membrane deletion mutant of Amer1 (Amer1ΔN) (Fig. 4E). These data show that membrane localization of Amer1 is essential for its activity in the repression of Wnt signaling in vivo.
WTX/Amer1 was recently shown to exist in two splice variants, the full-length form (Amer1-S1) and a variant lacking a large part of the membrane association domain of Amer1 (Amer1-S2; Fig. 1) (24). Because splicing occurs within the coding sequence transfection of the full-length cDNA of Amer1 leads to co-expression of both splice variants. To distinguish the activities of the variants we generated expression constructs to individually express Amer-S1 and Amer1-S2 and also designed siRNA oligonucleotides for specific knockdown of the variants (see Experimental Procedures) (22). As expected Amer1-S1 was localized at the plasma membrane whereas Amer1-S2 was predominantly found in the cytoplasm and nucleus (22,24). Amer-S1 efficiently inhibited TCF/β-catenin-dependent transcription induced by daLRP6 whereas Amer1-S2 did not (Fig. 4F), although both variants interacted similarly with APC, β-catenin and Conductin (supplemental Fig. S2). Conversely, specific knockdown of endogenous Amer-S1 increased TCF/β-catenin-dependent transcription whereas knockdown of Amer1-S2 had no effect (Fig. 4G). These data show that Amer1 exists in two splice forms which differ by their intracellular localization and activity in Wnt signaling and indicate that membrane localization is essential for endogenous Amer1 activity.

**Amer1 represses Wnt signaling mainly under conditions of high cell density.** It was recently shown that Wnt signaling activity is reduced by increasing cell density and cell contact formation, which could be mediated by a membrane associated factor (20). Since membrane binding of Amer1 is essential for its activity as shown above we analyzed whether its function is modulated by cell density. As expected knockdown of Amer1 in HeLa cells induced the expression of the Wnt target genes LGR5 and Conductin (Fig. 5; lanes 1, 2 and 5, 6). Importantly, induction of LGR5 and Conductin by Wnt3A was strongly enhanced in dense but not in sparse cell cultures by knockdown of Amer1 (Fig. 5; lanes 3, 4 versus 7, 8). This indicates that Amer1 exerts its negative role in Wnt signaling primarily when cells have established cell-cell contacts under conditions of high cell density.

**Amer1 stabilizes Axin.** Axin is a limiting component for β-catenin degradation, and changes in Axin levels are thought to result in altered Wnt signaling (37). Prolonged treatment of cells with Wnt3A (>1 hour) was shown to reduce Axin levels, which might contribute to the stabilization of β-catenin and downstream activation of the Wnt pathway (17,18). We found that knockdown of Amer1 by two specific siRNAs led to the reduction of endogenous Axin protein but did not affect mRNA levels of Axin (Fig. 6, A and B). Conversely, expression of full-size Amer1 and the splice variant Amer-S1 resulted in increased levels of co-expressed Axin, whereas expression of Amer1-S2 or mutants deficient for membrane binding (Amer1(7µLys) and Amer1ΔN) had no effect (Fig. 6C). A deletion mutant of Amer1 retaining the Axin/Conductin binding site (Amer1(2-601)) also increased Axin levels whereas a mutant lacking this site (Amer1(2-530)) did not (Fig. 1 and 6D; supplemental Fig. S1A) (22). These data show that Amer1 controls the amounts of Axin and that membrane binding and association with Axin is required for this activity. To examine whether Amer1 alters Axin protein stability we analyzed Axin levels after cycloheximide-mediated inhibition of protein synthesis. While Axin levels decreased after cycloheximide treatment in a time-dependent manner, this was prevented when Amer1 was cotransfected (Fig. 6, E and F). This shows that Amer1 stabilizes Axin by increasing its half life. Importantly, expression of Amer1 but not Amer1ΔN prevented the Wnt3A induced reduction of endogenous Axin (Fig. 6G). Similarly, Amer1 prevented downregulation of exogenous Axin by daLRP6 (Fig. 6H). These data suggest that Amer1 exerts its negative effects on Wnt signaling at least in part by counteracting the destabilization of Axin. In line, knockdown of Axin abrogated the repression of a TCF/β-catenin dependent reporter by Amer1 (Fig. 6I).

**DISCUSSION**

Amer1/WTX has recently gained interest because of its role as a negative regulator of Wnt signaling and as a putative tumor suppressor. Our study reveals several novel features of Amer1 relevant to its function. First, we show that Amer1 binds directly to β-catenin via a novel interaction motif, the REA repeats. Second, Amer1 forms a multi-protein complex with components of the β-catenin destruction
complex, namely β-catenin, APC, and Axin proteins, and can recruit these factors to the plasma membrane. Third, Amer1’s function as an inhibitor of Wnt signaling is dependent on its membrane localization and cell density, and involves stabilization of Axin. Our data define the topology of the Amer1/WTX protein by showing different binding domains for the β-catenin destruction complex and delineating a functional role of membrane localization.

We have identified the REA repeats as a novel domain within Amer1 that binds to β-catenin. The REA repeats share no similarity with other β-catenin binding domains in known interaction partners such as TCFs, E-cadherin, and APC, although Amer1 binds to a similar region in β-catenin as these factors, namely the central part of the armadillo repeat region. The fact that Amer1 and β-catenin interaction is mediated by repeated motifs in both proteins suggests a unique arrangement which deserves more specific structural investigations. Of note, all of the known point mutations found in the WTX gene in Wilms tumor are predicted to lead to truncated proteins that stop before the REA repeats, with the most C-terminal truncation at position 578 being only three amino acids away from the first REA repeat (38). This suggests that tumors select for the loss of the REA repeats and stresses the importance of this domain. Of interest, however, while being conserved in the mammalian homologs of Amer1 such as mice, rats and dogs, REA repeats are absent in fish, frogs and chicken indicating that they are specific to mammals. This implies that Amer1 does not directly interact with β-catenin in non-mammalian species, and also suggests that β-catenin binding is not absolutely essential to Amer1 function. Indeed, deletion of the REA repeat region in Amer1 reduced its activity in inhibiting a TCF/β-catenin dependent reporter, but did not completely abolish it (Fig. 2F). Association of Amer1 with β-catenin might occur independently of the REA repeats, via the associated Axin and APC proteins, which increase β-catenin binding capacity of the Amer1 complex as shown in the co-immunoprecipitation experiments of Fig. 3D. It is likely that the evolutionary acquisition of direct interaction of Amer1 with β-catenin has a specific role in mammals and may serve to fine-tune Wnt signaling, possibly in a tissue-specific fashion.

Our mutational analyses show that Amer1 requires an intact membrane localization domain to repress TCF/β-catenin dependent reporters and Wnt-induced axis duplication in Xenopus, and to stabilize Axin. The activity of an Amer1 mutant lacking this domain could be restored by fusion to an unrelated membrane targeting sequence. We found that knockdown of Amer1 increases Wnt target gene expression more effectively in dense than in sparse cell cultures, both in the absence or presence of Wnts. It was recently shown that phosphorylated β-catenin prone to degradation is enriched at cell contacts under conditions of high cell density indicating a mechanism by which cell-cell contact formation impacts on Wnt signaling (20). Given its localization at the plasma membrane Amer1 might relay cell contact formation to the regulation of Wnt signaling. Since Amer1 is attached to the intracellular side of the plasma membrane such a relay function would require its interaction with yet to be determined transmembrane components.

Amer1 exists in two splice variants, termed S1 and S2 differing by the presence or absence of a large part of the membrane association domain of Amer1. We found by overexpression and specific knockdown of the individual splice forms that Amer1-S1 which associates with the membrane, but not Amer1-S2 deficient for membrane binding represses Wnt signaling. These data corroborate a critical function of membrane binding domain for endogenous Amer1. The genetic disease OSCS, in which Amer1 is mutated, shows clinical characteristics in line with overactive Wnt signaling. Interestingly, these mutations can occur in the alternatively spliced region thereby leading to truncations of Amer1-S1 but preserving Amer1-S2. The fact that the disease develops in the presence of Amer1-S2 is in line with our finding that this splice variant has no activity in suppressing Wnt signaling (24).

The mechanism by which Amer1 exerts its function is presently unclear. We propose that Amer1 acts as a scaffold protein for the β-catenin destruction complex and that it activates this complex in a yet unknown manner. Of interest, we found that Amer1 knockdown strongly reduces levels of endogenous Axin and that overexpression of Amer1 stabilizes Axin, which required membrane localization of Amer1 and interaction of the two proteins. We also found that Amer1 counteracts Wnt-induced
We recently found that Amer1 can also activate Wnt signaling by stimulating LRP6 phosphorylation by GSK3β and CK1γ (22). Of note this activity also requires association of Amer1 with the plasma membrane via PtdIns(4,5)P2 (22). Interestingly, a similar dual function as activators and inhibitors of the pathway has been described before for Axin and GSK3β (10,39). How these differential activities are controlled are not yet clear.

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FOOTNOTES

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The abbreviations used are: Amer1, APC membrane recruitment 1; APC, adenomatous polyposis coli; CK1, casein kinase 1; Fz, Frizzled; GSK3, glycogen synthase kinase 3; dLRP6, dominant-active LRP6; LDL, low-density lipoprotein; LEF, lymphoid enhancer factor; LRP5/6, low-density lipoprotein receptor-related protein 5/6; NES, nuclear export sequence; OSCS, osteopathia striata congenita with cranial sclerosis; PtdIns(4,5)P2, phosphatidylinositol(4,5)bisphosphate; TCF, T-cell factor; WTX, Wilms Tumor gene on the X-chromosome.

FIGURE LEGENDS

Figure 1. Schematic representation of Amer1 splice variants and deletion mutants analyzed in this paper. Amer1 is expressed as two splice variants, termed Amer1-S1 and Amer1-S2. The two N-terminal membrane localization domains (M1, M2) are highlighted by light grey shading, the three APC interaction domains (A1-3) by dark grey shading, the central REA repeat domain involved in β-catenin binding by hatching and the regions involved in the interaction with Axin/Conductin by bold lines. Splicing of Amer1-S2 and the internal deletion of the REA repeat region in the Amer1ΔREA mutant are indicated by thin lines.
Figure 2. Amer1 interacts with β-catenin and recruits it to the plasma membrane. (A) Co-immunoprecipitation of endogenous Amer1 and β-catenin from lysates of HEK293T and SW480 cells. Immunoprecipitations were performed with mouse anti-β-catenin or control IgG antibodies. Asterisk indicates non-specific band. (B) Schematic representation of the β-catenin clones found in a yeast two-hybrid screen with Amer1(531-716) as a bait. The armadillo repeats are depicted as grey squares. Values represent β-galactosidase units of a representative yeast two hybrid interaction analysis. (C) Amino acid sequence of the Amer1 REA repeat domain. REAH/Y motifs are highlighted in grey. (D) Amer1 interacts with β-catenin via its REA repeat domain. Co-immunoprecipitation of Myc-tagged β-catenin with EGFP-tagged Amer1 or Amer1 deletion mutants in HEK293T cells (see Fig. 1). Note that full-size Amer1 and N-terminal fragments are expressed as two splice variants Amer1-S1 and Amer1-S2 represented by the two bands on the Western blot (see Fig. 1). (E) Amer1 recruits β-catenin to the plasma membrane. Cotransfection of EGFP-tagged Amer1 or Amer1ΔREA (left panels, GFP fluorescence) with Myc-β-catenin (right panels, anti-β-catenin immunofluorescence) in MCF-7 cells. Scale bar is 20 µm. (F) Deletion of the REA repeat domain reduces the ability of Amer1 to repress a TCF/β-catenin dependent transcriptional reporter. HEK293T cells stably expressing a β-catenin responsive firefly luciferase were cotransfected with RFP-daLRP6 and the indicated EGFP-Amer1 constructs. Fold changes of luciferase activity are presented relative to control transfected cells. Error bars indicate standard deviations. Similar expression of daLRP6 and Amer1 constructs was verified by anti-LRP6 and anti-GFP Western blotting, respectively.

Figure 3. Amer1 acts as a scaffold for the β-catenin degradation complex. Protein interactions were determined by immunoprecipitations from transfected HEK293T cells. (A) Amer1 can simultaneously interact with β-catenin and APC. Myc-β-catenin only co-immunoprecipitated with EGFP-APC-Arm when Flag-Amer1 was present. (B) Amer1 can simultaneously interact with APC and Conductin. Flag-Conductin only co-immunoprecipitated with EGFP-APC-Arm when Flag-Amer1 was present. (C) Amer1 can simultaneously bind to Conductin and β-catenin. Myc-β-catenin only co-immunoprecipitated with EGFP-Conductin(455-782) when Flag-Amer1 was present. (D) APC and Conductin link β-catenin to Amer1. Coexpression of APC and Flag-Conductin strongly increased the amount of Myc-β-catenin co-immunoprecipitated with EGFP-Amer1.

Figure 4. The membrane localization of Amer1 is required for repression of Wnt signaling. (A) Fluorescence micrographs of MCF-7 cells transfected with EGFP-Amer1 constructs as indicated above the panels. Mutation of N-terminal lysine residues (Amer1(3µLys), Amer1(5µLys), Amer1(7µLys)) or deletion of the membrane targeting N-terminus (Amer1ΔN, amino acids 207-1135) leads to the delocalization of Amer1 from the plasma membrane and its subsequent accumulation in the cytoplasm and nucleus. Note that Amer1 can appear as a diffuse staining or as discrete dots in the nucleus. Scale bar is 20 µm. (B-F) HEK293T cells stably expressing a β-catenin responsive firefly luciferase were cotransfected with RFP-daLRP6 and the indicated EGFP-Amer1 constructs. Fold changes of luciferase activity are presented relative to control transfected cells. Error bars indicate standard deviations. Similar expression of daLRP6 and Amer1 constructs was verified by anti-LRP6 and anti-GFP Western blotting, respectively. (B) N-terminal lysine mutants of Amer1 are deficient in downregulating the activity of a TCF/β-catenin dependent transcriptional reporter. (C) Fusion of the Amer1(7µLys) mutant to a nuclear export sequence (NES-Amer1(7µLys)) does not restore inhibition of a TCF/β-catenin dependent reporter. (D) Fusion of Amer1ΔN to the transmembrane domain of the LDL receptor (TMD-Amer1ΔN) restores inhibition of a TCF/β-catenin dependent reporter. (E) Amer1 mutants lacking membrane association are defective in downregulating Wnt8-induced axis duplication in X. laevis embryos. The chart shows normalized average percentage of axis induction from at least three independent experiments. Error bars indicate standard error of the mean. n, number of embryos injected. (F, G) The membrane bound splice variant Amer1-S1 represses Wnt signaling whereas Amer1-S2 lacking the membrane targeting domain does not. Effect of overexpression (F) or knockdown (G) of Amer1 splice variants Amer1-S1 and Amer1-S2 on a TCF/β-catenin dependent reporter upon activation with daLRP6.
**Figure 5.** Amer1 exerts its negative regulatory activity on Wnt signaling mainly under conditions of high cell density. Induction of Wnt target genes Conductin and LGR5 after knockdown of Amer1 as analyzed by RT-PCR or Western blot (WB) analysis. siRNA transfected HeLa cells were seeded at either high or low cell density and incubated with Wnt3A for 3.5 hours (RT-PCR experiment) or 5 hours (Western blot experiment, WB) before cell lysis.

**Figure 6.** Amer1 increases the stability of Axin. (A) Knockdown of Amer1 reduces Axin levels. HEK293T or SW480 cells were transfected with the indicated siRNAs and endogenous Axin was analyzed by Western blotting. Asterisk indicates non-specific band. (B) Knockdown of Amer1 using siRNA does not affect Axin mRNA levels as shown by RT-PCR analysis in HEK293T cells. (C, D) Overexpression of Amer1 increases Axin levels, which requires membrane localization (C) and Axin binding (D) of Amer1. The indicated EGFP-Amer1 constructs were coexpressed with Flag-Axin in HEK293T cells. (E, F) Amer1 increases the half-life of Axin. (E) HEK293T cells were transfected with Flag-Axin with or without EGFP-Amer1 and incubated with 100 µM cycloheximide (CHX) for the indicated time points (h, hours). (F) Quantification of the experiment from (E) by densitometry. The levels of Axin protein at each time point were normalized to the starting level. (G) Overexpression of Amer1 prevents Wnt-induced downregulation of Axin. HEK293T cells were transfected with the indicated EGFP-Amer1 constructs, incubated with Wnt3A for four hours and endogenous Axin was analyzed by Western blotting. (H) Overexpression of Amer1 prevents downregulation of Axin induced by daLRP6. HEK293T cells were transfected with Flag-Axin, RFP-daLRP6 and EGFP-Amer1 as indicated. (I) Knockdown of Axin abolishes the negative regulatory effect of Amer1 on a TCF/β-catenin dependent reporter. The TCF/β-catenin reporter assay was performed as described in Fig. 2F.
Figure 4

A

|          | Amer1 | Amer1(3µLys) | Amer1(5µLys) | Amer1(7µLys) | Amer1ΔN |
|----------|-------|--------------|--------------|--------------|---------|
|          | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |

B

|          | fold stimulation |
|----------|------------------|
| LRP6     | ![Image](image6.png) |
| Amer1    | ![Image](image7.png) |
| daLRP6   | +                |
| Amer1    | +                |
| Amer1(3µLys) | +       |
| Amer1(5µLys) | +       |
| Amer1(7µLys) | +       |
| Amer1ΔN  | +                |

C

|          | fold stimulation |
|----------|------------------|
| LRP6     | ![Image](image8.png) |
| Amer1    | ![Image](image9.png) |
| daLRP6   | +                |
| Amer1    | +                |
| Amer1(7µLys) | +       |
| NES-Amer1(7µLys) | +       |

D

|          | fold stimulation |
|----------|------------------|
| LRP6     | ![Image](image10.png) |
| Amer1    | ![Image](image11.png) |
| daLRP6   | +                |
| Amer1    | +                |
| Amer1ΔN  | +                |
| TMD-Amer1ΔN | +       |

E

|          | axis duplication (%) |
|----------|----------------------|
| XWnt8    | ![Image](image12.png) |
| Amer1    | ![Image](image13.png) |
| Amer1(7µLys) | ![Image](image14.png) |
| Amer1ΔN  | ![Image](image15.png) |

F

|          | fold stimulation |
|----------|------------------|
| LRP6     | ![Image](image16.png) |
| Amer1    | ![Image](image17.png) |
| daLRP6   | +                |
| Amer1    | +                |
| Amer1-S1 | +                |
| Amer1-S2 | +                |

G

|          | fold stimulation |
|----------|------------------|
| LRP6     | ![Image](image18.png) |
| daLRP6   | +                |
| siGFP    | +                |
| siAmer1a | +                |
| siAmer1-S1 | +       |
| siAmer1-S2 | +       |
Figure 5

| Wnt3A | dense | sparse |
|-------|-------|--------|
| siAmer1a | +     | +      |
| siGFP  | +     | +      |

**RT-PCR**
- LGR5
- Conductin
- Amer1
- GAPDH

**WB**
- Conductin
- Amer1
- Actin

1 2 3 4 5 6 7 8
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