Amer2 Protein Is a Novel Negative Regulator of Wnt/β-Catenin Signaling Involved in Neuroectodermal Patterning* [S]

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**Background:** Amer2 is a novel uncharacterized adenomatous polyposis coli (APC)-interacting protein.

**Results:** Amer2 interacts with components of the β-catenin destruction complex via APC, inhibits Wnt target gene expression, and alters neuroectodermal patterning.

**Conclusion:** Amer2 is a novel negative regulator of Wnt signaling involved in brain development.

**Significance:** A novel class of membrane-associated Wnt pathway regulators has been characterized.

Wnt/β-catenin signaling is negatively controlled by the adenomatous polyposis coli (APC) tumor suppressor, which induces proteasomal degradation of β-catenin as part of the β-catenin destruction complex. Amer2 (APC membrane recruitment 2; FAM123A) is a direct interaction partner of APC, related to the tumor suppressor Amer1/WTX, but its function in Wnt signaling is not known. Here, we show that Amer2 recruits APC to the plasma membrane by binding to phosphatidylinositol 4,5-bisphosphate lipids via lysine-rich motifs and that APC links β-catenin and the destruction complex components axin and conductin to Amer2. Knockdown of Amer2 increased Wnt target gene expression and reporter activity in cell lines, and overexpression reduced reporter activity, which required membrane association of Amer2. In Xenopus embryos, Amer2 is expressed mainly in the dorsal neuroectoderm and neural tissues. Down-regulation of Amer2 by specific morpholino oligonucleotides altered neuroectodermal patterning, which could be rescued by expression of a dominant-negative mutant of Lef1 that interferes with β-catenin-dependent transcription. Our data characterize Amer2 for the first time as a negative regulator of Wnt signaling both in cell lines and in vivo and define Amer proteins as a novel family of Wnt pathway regulators.

The canonical Wnt/β-catenin signaling pathway is involved in a variety of developmental and pathological processes, making it a central player in biology. Canonical Wnt signaling is propagated mainly by modulating the levels of the nucleocytoplasmic protein β-catenin, which is controlled by a β-catenin destruction complex composed of axin or axin-2/conductin and the adenomatous polyposis coli (APC)3 tumor suppressor protein, as well as the kinases glycogen synthase kinase 3β and casein kinase 1α. These kinases phosphorylate β-catenin, leading to its ubiquitination and subsequent degradation in proteasomes. APC acts as a scaffold for the complex by binding to β-catenin and to axin/conductin, which in turn recruit glycogen synthase kinase 3β. Binding of Wnt ligands to frizzled and LRP5/6 (LDL receptor-related protein 5/6) receptors results in inhibition of the β-catenin destruction complex, stabilization of β-catenin, and subsequent formation of T-cell factor–β-catenin complexes that activate transcription (1). Mutations of APC leading to constitutive stabilization of β-catenin and Wnt/β-catenin signaling are frequently observed in colorectal carcinomas. A variety of other tumor types are also characterized by permanent Wnt signaling activity (2).

Amer2 (APC membrane recruitment 2; FAM123A) was previously shown by our group to interact with APC via two domains that bind the Armadillo repeats of APC (3). These domains are conserved in the related proteins Amer1/WTX (Wilms tumor gene on the X-chromosome; FAM123B), which acts as a negative regulator of Wnt signaling (4, 5), and Amer3 (FAM123C), which has not been characterized so far (6). Amer1 is mutated in Wilms tumors and in the inherited disease osteopathia striata congenita with cranial sclerosis (7, 8). A recent expression analysis of the Amer gene family in the mouse showed rather widespread expression of Amer1, whereas Amer2 and, even more so, Amer3 were largely restricted to the nervous system (9). Apart from its APC-binding activity little is known about the regulation and function of Amer2. Here, we have performed a structural and functional analysis of Amer2 and found that it is linked to the β-catenin destruction complex by APC and has a negative regulatory role in Wnt signaling that depends on its membrane localization. Moreover, we reveal a role of Amer2 in controlling neuroectodermal patterning in Xenopus embryos by inhibiting Wnt signaling.

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3 The abbreviations used are: APC, adenomatous polyposis coli; Arm, Armadillo; mYFP, monomeric YFP; MO, morpholino; EGFP, enhanced GFP; xAmer2, Xenopus Amer2; NF, Nieuwkoop and Faber; Lef, lymphoid enhancer factor.
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**Experimental Procedures**

**DNA Constructs, siRNAs, and Morpholinos**—The following constructs have been described previously: pcDNA-FLAG-Amer2, pEGFP-Amer2, pcDNA-FLAG-Amer1, pEGFP-APC-Armadillo (Arm), and pEGFP-APC-Arm-N507K (3); pCMV-APC, monomeric YFP (mYFP)-APC, mYFP-β-catenin, mYFP-axin, mYFP-conductin, and monomeric red fluorescent protein dominant-active LR6p (10); pcDNA3.1-FLAG (11); and mYFP-APC1641 (12). To generate mYFP-β-catenin S33Y, point mutations were introduced into YFP-β-catenin by PCR. Amer2 deletion mutants were generated by restriction digests and PCR amplification. The splice variant Amer2-S1 was generated by PCR mutagenesis exchanging three nucleotides, leading to the mutation of the internal splice site without affecting the amino acid sequence. Amino acid residues 261–379 in pcDNA-FLAG-Amer2-S2 and amino acid residues 65–93 and 189–211 in pEGFP-Amer2ΔK1K2 were deleted. For expression of GST-Amer2(2–230), the cDNA was inserted into pGEX-4T3 (GE Healthcare). To generate the Amer2 morpholino (MO) site construct, the enhanced GFP (EGFP) open reading frame was subcloned by PCR from pEGFP (Clontech) into pCS2+, and a double-stranded oligonucleotide containing the Xenopus Amer2 (xAmer2) MO-binding sequence was inserted 5′ of EGFP into pCS2+-EGFP. The sequences of the siRNAs are as follows: GFP siRNA, 5′-GCUAACCUGUCCAGG-CCA-3′; Amer2-1 siRNA, 5′-GUCACCUCUAUCUGCCU-3′; and Amer2-2 siRNA, 5′-GCCAACCAUGAGAAGUGU-3′. siRNAs were purchased from Eurogentec. The standard control MO and the Amer2 MO (5′-CCTTTACCT-CAGTTACAATTATA-3′) were purchased from Gene Tools.

**Antibodies**—The rabbit anti-APC-M serum was a kind gift from I. Nächtle (University of Dundee, Dundee, Scotland, UK). Commercial antibodies were purchased from Cell Signaling (mouse anti-Myc), Sigma (rabbit anti-FLAG, mouse anti-FLAG M2, and mouse anti-GST), Roche Applied Science (mouse anti-GFP, mixture of clones 7.1 and 13.1), Epitomics (rabbit anti-Myc, mouse anti-FLAG), Santa Cruz (rabbit anti-GFP, mixture of clones 7.1 and 13.1), and Abcam (rabbit anti-GFP, ab290). Secondary antibodies (Jackson Immunoresearch Laboratories) were either Cy2 and Cy3 conjugates for immunofluorescence and HRP conjugates for Western blotting.

**Primers and in Situ Probes**— Primer pairs for RT-PCR are as follows: human Amer2, 5′-AGGGAAGCAGACAGGAGAGCAGGTCGCT-3′ and 5′-AAAGACACCTCTCCTGTCACCTGCT-3′; xAmer2, 5′-CCTCTTCTCCTGGAGGAGCTATC-3′ and 5′-CTTCTCTCCTGGAGGAGCTATC-3′; Xnr3 (Xenopus nodal-related 3), 5′-TAACACACCCATGGAGATATC-3′ and 5′-CAGACCACCCATGGAGATATC-3′; Nkx2.5 (Krox20, krox20, krox20), 5′-GAGCTCTGTTGATCTGCTG-3′ and 5′-GGGAGGAAACTGATGCTG-3′; and ornithine decarboxylase, 5′-GTGAGGACGCAAGGATGCTG-3′ and 5′-CGCAGGACGCAAGGATGCTG-3′. The Amer1, conduit, LGR5 (leucine-rich repeat-containing G-protein-coupled receptor 5), and GAPDH primers were described previously (5).

The xAmer2 probe was generated from the cDNA clone IRAkP961P02150Q in pCMV-Sport6. The En-2 (engrailed 2), Rx1, and Sox2 probes were described previously (13–16).

**Cell Culture and Transfections**— Cells were cultured and transfected as reported (5). HEK293T-pBAR/Renilla and RKO-pBAR/Renilla cells (4) were cultured in medium supplemented with 2 μg/ml puromycin (Sigma).

**Preparation of Protein Lysates, Subcellular Fractionation, Immunoprecipitations, and Western Blotting**—Lysates, co-immunoprecipitations, and Western blotting were performed as described previously (3). To obtain cytosolic fractions, subcellular fractionation of cells was carried out using the ProteoJET membrane protein extraction kit (Fermentas) according to the manufacturer’s instructions. Protein levels were quantified using AIDA Image Analyzer Version 3.52 (Raytest). For Xenopus experiments, Western blotting was carried out as reported (17).

**Immunofluorescence Microscopy**—For immunofluorescence staining, cells were grown on glass coverslips, fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with DMEM/FCS, and stained with the indicated antibodies and appropriate secondary antibodies. Photographs were taken with a CCD camera (Visitron, Munich, Germany) on an Axioplan 2 microscope (×63 objective; Zeiss, Oberkochen, Germany) using MetaMorph software (Molecular Devices). Images were processed using Adobe Photoshop CS software.

**Lipid Binding Assay**—GST and GST-Amer2(2–230) were expressed and purified from Escherichia coli BL21 as described (3). Membrane lipid strips (Echelon) were incubated with GST and GST-Amer2(2–230) at a concentration of 1 μg/ml at 4°C overnight and detected by mouse anti-GST antibodies.

**RT-PCR**—RNA isolation, cDNA synthesis, and PCR was performed as reported (5). For Xenopus RT-PCR, RNA was extracted from five embryos using the High Pure RNA isolation kit from Roche Applied Science. For cDNA synthesis, 500 ng of total RNA was reverse-transcribed with Moloney murine leukemia virus (Promega). mRNA levels were quantified using AIDA Image Analyzer Version 3.52.

**Reporter Assays**—T-cell factor-β-catenin-dependent reporter assays were performed in HEK293T or RKO cells stably expressing a β-catenin-responsive firefly luciferase reporter (pBAR) along with Renilla luciferase as an internal control (4) using 300 ng of Amer2 plasmids and 200 ng of monomeric red fluorescent protein dominant-active LR6p or 150 ng of mYFP-β-catenin S33Y as reported (5). Where indicated, Wnt3A-conditioned medium was added for 4–6 h. For knockdown of Amer2, cells were transfected with 40 pmol of siRNA. Cells were harvested after 24 h (overexpression experiments) or after 48 h (knockdown). Firefly luciferase values (pBAR) were normalized to Renilla values. All experiments were performed in duplicates and reproduced at least twice.

**Frog Handling and Microinjections**—Eggs were fertilized in vitro, cultured, and injected as described previously (17). Embryos were injected either in both dorsal blastomeres at the four-cell stage for RT-PCR experiments or in one anterior-dorsal blastomere at the eight-cell stage for krox20/En-2 and Rx1/En-2 in situ hybridizations. For Sox2 in situ hybridization experiments and for specificity testing of the xAmer2 MO by Western blotting, embryos were injected in both blastomeres at the two-cell stage. The injection amounts were 0.2, 0.4, or 0.8
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pmol of Amer2 MO or control MO, 100 pg of β-catenin RNA, 100 pg of lacZ DNA, 500 pg of LefΔBD RNA, and 100 pg of Amer2-pCS-MOsite-EGFP or Amer2-pCS-EGFP. RNA for microinjections was prepared using the mMESSAGE mMACHINE kit (Ambion).

In Situ Hybridization—Embryos were fixed in 0.1 M MOPS (pH 7.2), 1 mM EGTA, 1 mM MgSO4, and 3.7% formaldehyde and stained for β-galactosidase using X-Gal as a substrate. Whole-mount in situ hybridization was performed as described previously (17).

Sequence Alignment—Sequence alignment was performed with ClustalW using the xAmer2 sequences provided in the NCBI Database.

RESULTS

APC Interaction and Membrane Binding of Amer2—Human Amer2 consists of 671 amino acids, including APC-binding domains A1 and A2 and N-terminal clusters of lysines termed K1 and K2 conserved between Amer2 and Amer1 (Fig. 1A). In co-immunoprecipitation experiments, Amer2 interacted with the Armadillo repeat domain of APC but not with a point mutant (N507K) of this domain known to interfere also with Amer1 binding (Fig. 1B), in line with previous yeast two-hybrid data (3). A corresponding mutation in Drosophila E-APC was shown to reduce its membrane association (18). An N-terminal fragment of Amer2 retaining APC-binding domain A1 was able to recruit APC to the plasma membrane, whereas a fragment lacking this domain failed to do so (Fig. 1C). These data confirm that the putative APC-binding domains of Amer2 are functional and that Amer2 behaves similarly to Amer1 with respect to binding and membrane recruitment of APC. As Amer2 binds to APC, a negative regulator of Wnt signaling, we were interested to determine whether Amer2 can interact with other components of the destruction complex. We found that β-catenin, axin, or conductin co-immunoprecipitated with Amer2 when a fragment of APC containing binding domains for these factors (APC1641) (12) was coexpressed (Fig. 1D). Thus, Amer2 can associate with key components of the β-catenin destruction complex via APC.

Next, we analyzed the subcellular distribution of Amer2 mutants. Full-size Amer2 and the N-terminal fragment Amer2(2–230) were associated with the plasma membrane of transiently transfected cells (Fig. 1E), whereas the C-terminal fragment Amer2(230–671) was present in the cytoplasm and mainly in the nucleus. Importantly, deletion of the N-terminal lysine clusters (Amer2ΔK1,K2) resulted in loss of membrane association of Amer2 (Fig. 1E). We have shown previously that Amer1 associates with the plasma membrane by binding to phosphatidylinositol 4,5-bisphosphate, the predominant phospholipid at the plasma membrane (19), via these lysine motifs (3, 20). Indeed, recombinant Amer2(2–230) bound to membrane-spotted phosphatidylinositol phosphates, including phosphatidylinositol 4,5-bisphosphate, suggesting that it interacts with the plasma membrane in a similar fashion as Amer1 (Fig. 1F).

Repression of Transcriptional Activity of Wnt Pathway by Amer2—We next investigated whether Amer2 has a role in Wnt signaling. In human tissues, Amer2 is strongly expressed in the nervous system but is also detectable in muscle and epithelial tissues, as well as in cancer cell lines (supplemental Fig. S1, A and B). siRNA-mediated knockdown of Amer2 in HEK293T cells resulted in up-regulation of expression of conductin/axin-2 and LGR5, well defined target genes of Wnt signaling (Fig. 2, A and B) (21, 22). Moreover, Amer2 knockdown led to an increase in cytosolic β-catenin in HEK293T and RKO cells (Fig. 2C and data not shown) but did not affect expression of Amer1, which can serve as a specificity control (supplemental Fig. S1C). Amer2 knockdown also stimulated the activity of a T-cell factor-β-catenin-dependent luciferase reporter construct after activation by Wnt3A treatment in HEK293T and RKO cells (Fig. 2, D and E). Conversely, overexpression of Amer2 reduced reporter activity stimulated by Wnt3A or by transfection of a dominant-active LRP6 receptor, whereas overexpression of Amer2 lacking the membrane association motifs as defined in Fig. 1E (Amer2ΔK1,K2) had no effect (Fig. 2, F and G). Amer2 did not repress reporter activity stimulated by transfection of stabilized β-catenin S33Y (Fig. 2H), indicating that it cannot act by simple sequestration of β-catenin, e.g. at the plasma membrane. In line with this, Amer2 knockdown did not alter the levels of membrane-associated β-catenin in RKO cells (data not shown). These data indicate that Amer2 is a negative regulator of Wnt/β-catenin signaling acting at the plasma membrane probably by inducing degradation of β-catenin.

Amer2 occurs in two splice variants: Amer2-S1, which corresponds to the full-length sequence, and Amer2-S2, which lacks the conserved part of APC-binding domain A1 but retains domain A2. Amer2-S2 still interacted efficiently with APC, recruited it to the plasma membrane, and suppressed T-cell factor-β-catenin-dependent reporter activity (supplemental Fig. S2), suggesting that one APC-binding domain suffices for Amer2 activity.

Amer2 Controls Wnt-dependent Neuroectodermal Patternning in Xenopus—To determine Amer2 function in an in vivo model system, we analyzed the Xenopus ortholog of Amer2, xAmer2, which shows 49% amino acid identity to human Amer2 (supplemental Fig. 3A). xAmer2 mRNA was detected by in situ hybridization in the animal ectoderm at early gastrula stages (Fig. 3A). During neurulation, xAmer2 was expressed in the dorsal neuroectoderm, with the strongest signal detectable in the anterior neural plate (Fig. 3B). In tadpole stages, xAmer2 mRNA was detectable most prominently in the brain, eye, otic vesicle, and cranial ganglia (Fig. 3C). From stage 30 onward, xAmer2 expression was clearly seen in the forebrain, midbrain, and hindbrain but was excluded from the boundaries between these domains (Fig. 3C).

RT-PCR analysis detected xAmer2 transcripts specifically at the onset of gastrulation at stage 10.5. xAmer2 transcription was down-regulated during gastrulation (Nieuwkoop and Faber (NF) stages 11 and 12) and was detectable again from stage 16 onward, with a marked increase in embryos of stage 28 and older (supplemental Fig. 3B). Interestingly, we observed two splice variants of xAmer2 similar to those of human Amer2. These variants showed differential expression at stages 10.5 and 16 but were both expressed from stage 19 onward (supplemental Fig. 3, A and B).
To carry out loss-of-function studies, we designed a morpholino antisense oligonucleotide to knock down xAmer2 (supplemental Fig. 4A). First, we investigated whether xAmer2 acts as a negative regulator of Wnt/β-catenin signaling in vivo. Injection of the xAmer2 MO but not of a control MO resulted in a significant up-regulation of Xnr3 (Fig. 4A), which is a direct target
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gene of the Wnt/β-catenin pathway (23). Overexpression of β-catenin served as a positive control (Fig. 4A). These results suggest that xAmer2 acts as an endogenous antagonist of Wnt/β-catenin signaling in Xenopus embryos.

Wnt/β-catenin signaling is active in an anterior-posterior gradient in the neuroectoderm and contributes to determine posterior fates (24). The strong expression of xAmer2 in the anterior neural plate prompted us to analyze if xAmer2 plays a role in neural development. In xAmer2 MO-injected embryos, we observed a dose-dependent down-regulation of the eye field marker Rx1 and the mid-hindbrain boundary marker En-2 (Fig. 4B). At low doses (0.2 pmol of xAmer2 MO), 60% of the
embryos showed a reduction and 23% showed a loss of Rx1 and En-2 expression. At high doses (0.4 pmol of xAmer2 MO), 17% of the embryos showed a reduction and 39% showed a loss of the markers on the injected side. In addition, in 22% of the embryos, both markers were completely lost on both sides of the embryo (Fig. 4B), which was somewhat unexpected, as xAmer2 is not a secreted protein. Overexpression of xAmer2 was early embryonic lethal (data not shown), precluding gain-of-function or rescue experiments in neurula stage embryos. To investigate if the observed xAmer2 loss-of-function phenotypes are caused by an overactivation of Wnt/β-catenin signaling, we co-injected a dominant-negative mutant of Lef1 that lacks the β-catenin-binding motif (LefΔBD) (25, 26) with 0.4 pmol of xAmer2 MO. LefΔBD efficiently rescued Rx1 and En-2 expression (Fig. 4B), indicating that the loss of eye field and brain markers in xAmer2 morphants indeed reflects de-inhibition of Wnt/β-catenin signaling. Co-injection of LefΔBD with the control MO induced anteriorized phenotypes as indicated by enhanced expression of Rx1 and En-2 (Fig. 4B). Similar phenotypes have been reported after overexpression of Wnt antagonists (24).

xAmer2 knockdown also led to a dose-dependent down-regulation of the hindbrain marker krox20 and, at higher doses, to the complete loss of krox20 expression also on the uninjected side, similar to the results obtained for Rx1 and En-2 (supplemental Fig. 4B). Even these severe phenotypes were fully rescued by co-injection of LefΔBD (supplemental Fig. 4B). To rule out defects in neural induction in general, we tested the expression of the pan-neural marker Sox2, which was not affected by injection of even higher doses of the xAmer2 MO (0.8 pmol) (supplemental Fig. 4C).

In summary, loss of xAmer2 leads to massive disruption of neuroectoderm patterning but not to defects in neural induction. Anterior-posterior patterning of the neuroectoderm is Wnt/β-catenin-dependent, and the ability of LefΔBD to rescue xAmer2 morphant phenotypes clearly demonstrates that these phenotypes are caused by overactivation of Wnt/β-catenin signaling.

**DISCUSSION**

We have characterized the APC-interacting protein Amer2 for the first time as a novel negative regulator of Wnt/β-catenin signaling acting at the plasma membrane. Amer2 associates with key components of the β-catenin destruction complex and suppresses Wnt signaling dependent on its membrane localization. Moreover, analysis in Xenopus shows that Amer2 is a negative regulator of Wnt-dependent neural patterning in vivo. The mechanism by which Amer2 interferes with Wnt signaling is presently unclear. Amer2 associates with the β-catenin destruction complex components APC, axin/conductin, and β-catenin, indicating that it is involved in β-catenin degradation, similar to Amer1 (4). In line with this, Amer2 knockdown resulted in increased β-catenin levels. As the function of Amer2 requires its membrane localization, it might act by allowing activation of the destruction complex at the plasma membrane by a yet unknown mechanism. There is evidence that phosphorylation of β-catenin occurs at the plasma membrane (27). In principle, Amer2 might also act by sequestering β-catenin at the plasma membrane via APC. However, the fact that Amer2

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**FIGURE 2.** Amer2 negatively regulates Wnt/β-catenin signaling at the plasma membrane. A and B, mRNA expression of the Wnt target genes conductin (A) and LGR5 (B). RT-PCR was performed with HEK293T cells transiently transfected with control siRNA (GFP siRNA (siGFP)) and siRNA sequences targeting Amer2 for degradation (Amer2-1 (siAmer2-1) and Amer2-2 (siAmer2-2) siRNAs). GAPDH RT-PCR was used for normalization. Numbers between the panels represent the levels of conductin, Amer2 and LGR5 normalized to GAPDH as determined by densitometry. C, Wnt3A treatment or siRNA-mediated knockdown of Amer2 increased β-catenin levels in cytosolic fractions of HEK293T cells as determined by Western blotting. α-Tubulin staining served as a normalization control. Numbers between the panels represent the levels of β-catenin normalized to α-tubulin as determined by densitometry. D, siRNA-mediated knockdown of Amer2 resulted in up-regulation of β-catenin-dependent transcription. HEK293T cells stably expressing a β-catenin-responsive firefly luciferase reporter were transfected with GFP siRNA and Amer2-1 or Amer2-2 siRNA and stimulated with Wnt3A-conditioned medium (Wnt3A-CM) for 4 h. Fold stimulation of luciferase activity is shown. E, experiment performed as described for D with RKO cells stably expressing the reporter. F and G, overexpression of Amer2 down-regulated the β-catenin-dependent reporter in HEK293T cells upon stimulation with Wnt3A (F) or cotransfection of FLAG–dominant-active LRP6 (daLRP6; G), which was abolished in the Amer2ΔK1,K2 mutant, which was unable to bind to the plasma membrane. Western blots for Amer2 and dominant-active LRPs6 from a representative experiment are shown below the graphs. H, overexpression of Amer2 did not down-regulate the β-catenin-dependent reporter in HEK293T cells cotransfected of stabilized β-catenin S33Y from a representative experiment. In D–H, error bars indicate S.E. Statistical analysis was done using Student’s unpaired t test. Statistically significant differences are indicated: *, p < 0.05; **, p < 0.005. n.s., non-significant difference; n, number of independent experiments analyzed.
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A

![Graph A](Image)

**FIGURE 4.** xAmer2 negatively regulates Wnt signaling in vivo. A, depletion of xAmer2 by xAmer2 MO (cf. supplemental Fig. 4A) increased expression of the Wnt target gene Xnr3. Xenopus embryos were injected in both dorsal blastomeres at the four-cell stage with 0.4 pmol of xAmer2 MO or control MO or with 100 pg of β-catenin RNA as a positive control, and Xnr3 mRNA levels were determined by RT-PCR at NF stage 10.5. Ornithine decarboxylase (ODC) RT-PCR was used for normalization. Numbers between the panels represent the levels of Xnr3 normalized to ornithine decarboxylase as determined by densitometry. B, depletion of xAmer2 by xAmer2 MO affected anterior-posterior patterning of the neuroectoderm, consistent with a negative function in Wnt signaling. Embryos were injected in one anterior-dorsal blastomere at the eight-cell stage with 100 pg of lacZ DNA plus 0.2 or 0.4 pmol of Amer2 MO or control MO without or with 500 pg of LefΔBD RNA as indicated. Embryos were stained for lacZ expression of the markers on the injected side compared with the uninjected side of the same embryo. Images show embryos representative of the observed phenotypes. Injected sides are oriented to the right in all images. The colored insets represent phenotypes as follows: blue, enhanced Rx1 and En-2; green, equal-normal expression of markers; yellow, markers reduced/anteriorly shifted at the injected side; orange, markers lost at the injected side; red, markers lost at both the injected and uninjected sides of the embryo. The graph shows the statistics of at least three independent experiments. Numbers below the graph represent analyzed embryos.

cannot suppress β-catenin-dependent reporter activity stimulated by stabilized β-catenin argues against this possibility.

We noticed that knockdown of Xenopus Amer2 by morpholino oligonucleotides led to massive disruption of neuroectodermal patterning as revealed by down-regulation of Rx1, En-2, and krox20. Anterior-posterior patterning of the neuroectoderm is already initiated during gastrulation (24). In line with a function during this developmental stage, xAmer2 becomes specifically up-regulated at the onset of gastrulation. The ability of LefΔBD to rescue xAmer2 morphant phenotypes clearly demonstrates that these phenotypes are caused by overactivation of Wnt/β-catenin signaling due to Amer2 depletion. In support of this, down-regulation of En-2 and krox20 can be observed after overexpression of a Lef-β-catenin fusion construct immediately prior to gastrulation (stage 9) (28) and in embryos overexpressing the Wnt pathway activator Dvl (29). Wnt/β-catenin signaling acts partially indirectly in inducing posterior neural fates (24) by up-regulation of FGFs (28). Up-regulation of FGF might similarly account for the non-cell-autonomous effects that we observed in xAmer2 morphants.

Amer2 shares sequence similarities with Amer1 in the APC-binding and membrane association motifs (3, 20). Our data show that these motifs are functional, leading to the recruitment of APC to the plasma membrane by Amer2. Amer1 can directly interact with β-catenin via repeats of the amino acid sequence REA (5). Amer2 lacks the REA repeat region but can indirectly associate with β-catenin via APC. Interestingly, the REA repeats of Amer1 are not conserved in non-mammals such as frog and fish, implying that Amer1 exerts its negative regulatory role in Wnt signaling in these species via indirect interaction with β-catenin, similar to Amer2 (5).

Our findings allow us to define Amer proteins as a novel family of Wnt pathway regulators that act at the plasma membrane by inhibiting β-catenin. It would be of interest to analyze the function of the third putative member of this family, Amer3 (FAM123C), in which the conserved APC-binding (but not membrane association) motifs are preserved (6).

The similarities in structure and function of Amer1 and Amer2 proteins suggest that they may be functionally redundant in vivo. However, the two proteins have overlapping but distinct expression profiles in the mouse, and a recent knock-out study showed that Amer1 is critical for differentiation of mesenchymal progenitor cells (9, 30), whereas our Xenopus experiments indicate that Amer2 is relevant for development of neural tissues, in line with its restricted expression pattern. Thus, Amer proteins appear to have tissue-specific functional roles. Given the fact that Amer1/WTX mutations are found in human diseases, including bone disorders and cancer (7, 8), one can speculate that mutations in Amer2 might also contribute to human diseases. One example supporting the importance of Amer2 in neuronal function was reported recently: Amer2 is differentially expressed in the hippocampi of cocaine- and alcohol-addicted patients (31). Altogether, our studies provide evidence for a role of Amer2 as a novel Wnt pathway inhibitor in vivo involved in neuronal development.

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