Hepatitis C virus NS5A protein interacts with β-catenin and stimulates its transcriptional activity in a phosphoinositide-3 kinase-dependent fashion

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Hepatitis C virus (HCV) infection is increasingly associated with the development of hepatocellular carcinoma (HCC). HCV is not thought to be directly oncogenic but, by modulating a range of cellular functions, may predispose patients to the development of liver tumours. However, the molecular mechanisms by which HCV infection might contribute to HCC remain to be characterized. In this regard, we showed previously that the HCV NS5A protein bound to the p85 regulatory subunit of phosphoinositide-3 kinase (PI3K), thereby stimulating the activity of the p110 catalytic subunit of the enzyme. One of the downstream consequences of this was the stabilization of the proto-oncogene, β-catenin, with a concomitant stimulation of its transcriptional activity. Here, we further analyse the mechanism by which NS5A mediates activation of β-catenin.

Although our previous data were consistent with a role for the PI3K downstream effector kinases, Akt and glycogen synthase kinase-3β, in NS5A-mediated activation of β-catenin, we demonstrate here that it is in fact independent of both of these kinases. Truncation analysis revealed that both the N and C termini of NS5A are required for full activation of β-catenin. Furthermore, we demonstrate that NS5A, either alone or in complex with p85, is able to bind directly to β-catenin; again both N and C termini contribute to this interaction. We propose that NS5A activates β-catenin via a novel mechanism that involves a direct interaction between the two proteins and is augmented by PI3K activity. This may contribute to the association between chronic HCV infection and the development of HCC.

INTRODUCTION

According to the World Health Organization, as many as 170 million individuals (3% of the global population) are infected with hepatitis C virus (HCV). In 85% of cases, HCV establishes a chronic infection, and a significant number of infected individuals progress to end-stage liver disease – cirrhosis and hepatocellular carcinoma (HCC). Indeed, HCV infection is increasingly associated with the development of HCC (Anzola, 2004), one of the most frequent (4%) of human tumours with an invariably fatal outcome unless treated by radical surgical resection. Despite this clear clinical link, the molecular mechanisms underpinning the development of HCC in HCV-infected individuals remain to be clearly defined.

HCV is the prototype member of the genus Hepacivirus in the family Flaviviridae. Its genome is a single-stranded, positive-sense RNA which encodes a single ORF, translated in a cap-independent manner by an internal ribosome entry site (IRES) located in the 5’ untranslated region, into a polyprotein of approximately 3000 aa. The polyprotein is proteolytically cleaved by host and viral proteases to form 10 mature proteins: the structural proteins (core, E1 and E2), a viroporin p7 and the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B).

NS5A is a pleiotropic protein. It functions as a component of an RNA replication complex on cytoplasmic membranes together with the other non-structural proteins (Gosert et al., 2003), and it also plays a role in modulating cellular signalling pathways with implications for viral pathogenesis (Macdonald & Harris, 2004). Of particular interest is our previous observation (Street et al., 2004), and that of He et al. (2002), that NS5A binds to, and activates, phosphoinositide 3-kinase (PI3K), resulting in activation of the downstream effector serine/threonine kinase Akt (PKB). We further showed that this resulted in the stabilization of the proto-oncogene β-catenin and concomitant stimulation of β-catenin-dependent transcription (Street et al., 2005), with implications for the development of HCC.

β-Catenin is a protein with two distinct functions. Firstly, it plays an essential role in cell–cell adhesion by binding type 1 cadherins and linking them to the actin cytoskeleton (Daugherty & Gottardi, 2007). A second pool of β-catenin is located in the cytoplasm and is subject to regulation by...
both the Wnt and PI3K signalling pathways. In the absence of either Wnt or PI3K signalling, β-catenin is part of a complex including axin, adenomatosus polyposis coli and glycogen synthase kinase-3β (GSK-3β). The latter phosphorylates β-catenin at its N terminus, targeting it for proteasomal degradation (Daugherty & Gottardi, 2007). GSK-3β can be either inactivated via the Wnt pathway (Moon et al., 2004) or, of particular relevance to HCV and NS5A, subject to inhibitory phosphorylation by the PI3K effector, Akt. GSK-3β inactivation prevents β-catenin phosphorylation and results in its stabilization within the cytoplasm. The increased pool of β-catenin can then translocate to the nucleus, associate with transcriptional coregulatory proteins such as the TCF family and activate transcription of a variety of genes such as c-myc (He et al., 1998) and cyclin D1 (Tetsu & McCormick, 1999). Dysregulation of β-catenin, by mutation of either β-catenin itself or one of the myriad of regulatory proteins that control its activity, is associated with a range of tumours, including both colorectal carcinoma (Gavert & Ben-Ze’ev, 2007) and HCC (de La Coste et al., 1998). Of note, β-catenin is also upregulated by other oncogenic viruses, including Epstein–Barr virus (EBV) (Morrison et al., 2003), Kaposis’s sarcoma herpes virus (Fujimuro et al., 2003), the papovavirus JC (Gan & Khalili, 2004), hepatitis B virus (Cha et al., 2004) and human T-lymphotropic virus 1 (Tomita et al., 2006).

Here, we investigate the mechanism by which NS5A stimulates the transcriptional activity of β-catenin. We show that, although this stimulation is dependent on PI3K, it is independent of either Akt or GSK-3β. We also demonstrate that NS5A is able to interact physically with both β-catenin and the p85 regulatory subunit of PI3K and we propose that NS5A therefore intercepts the PI3K–Akt–GSK-3β–β-catenin pathway at several stages in order to stimulate β-catenin activity.

**RESULTS**

**NS5A-mediated stimulation of β-catenin transcriptional activity is PI3K dependent but is independent of either GSK-3β or Akt**

We have previously shown that NS5A bound to and activated PI3K, an event which led to the downstream phosphorylation and activation of Akt (Street et al., 2004). Furthermore, NS5A induced the stabilization of β-catenin and an increase in β-catenin-dependent transcriptional activity in a manner that was PI3K dependent (Street et al., 2005). Although our previous data implicated the PI3K–Akt–GSK-3β pathway in NS5A-mediated stabilization of β-catenin, the mechanisms underpinning this effect remained to be determined. In order to investigate this further, we performed a more extensive analysis of the effects of NS5A on β-catenin activity. As Huh-7 cells (the only cell type to support HCV replication) exhibit high constitutive levels of GSK-3β phosphorylation (and therefore high levels of β-catenin activity) (Desbois-Mouthon et al., 2002), we routinely used Cos-7 cells for our assays. To measure β-catenin transriptional activity, Cos-7 cells were transfected with plasmids expressing β-catenin and NS5A, together with a luciferase reporter plasmid containing eight tandem copies of wild-type Tcf/Lef binding sites (M50) or a derivative (M51) in which the Tcf/Lef binding sites were disrupted by mutation; this plasmid acted as a negative control. As previously shown, expression of NS5A resulted in a threefold increase in β-catenin transcriptional activity (Fig. 1a, compare lanes 1 and 5). To test the role of the PI3K–Akt–GSK-3β pathway, cells were treated with specific inhibitors of these three kinases: LY294002 (PI3K inhibitor), AKTVIII (Akt inhibitor) or LiCl (GSK-3β inhibitor). This analysis revealed that the level of β-catenin activity was elevated threefold by treatment with LiCl (lane 2), consistent with the role of GSK-3β in phosphorylating β-catenin and targeting it for proteasomal degradation (Barker et al., 2000). Interestingly, in the presence of NS5A, treatment with LiCl had an additive effect, further elevating β-catenin activity to 7.5-fold higher than the basal level (lane 6). In contrast, treatment of NS5A-expressing cells with LY294002 resulted in a modest reduction of β-catenin activity, indicating at least a partial dependency on PI3K (lane 7) but, surprisingly, treatment with AKTVIII had no effect on NS5A-mediated activation of β-catenin (lane 8). Samples from the experiment described above were Western blotted to confirm expression of the FLAG-tagged β-catenin and NS5A (Fig. 1a). Additionally, as expected, phosphorylation of Akt was reduced by treatment with either LY294002 or AKTVIII, with no effect on total Akt levels (Fig. 1a, bottom panel).

The observation that NS5A and LiCl had additive effects on β-catenin activity suggests that NS5A mediates its effect in a GSK-3β-independent fashion. We predicted, therefore, that NS5A would stimulate β-catenin activity in Huh-7 cells, even though these cells exhibited a background of high basal β-catenin activity due to high levels of GSK-3β phosphorylation. We therefore repeated these experiments both in Huh-7 human hepatoma cells harbouring a genotype 1b culture-adapted subgenomic replicon (FK5.1) (Krieger et al., 2001), and in Huh-7 cells transiently transfected with plasmids expressing NS5A and β-catenin. As shown in Fig. 1(b), in FK5.1 cells, LiCl enhanced β-catenin activity but to a lesser extent than in Cos-7 cells (consistent with the low basal levels of GSK-3β activity), LY294002 had a similar inhibitory effect and AKTVIII had no effect. In Huh-7 cells transfected with plasmids expressing NS5A and β-catenin, the transcriptional activity of the latter was increased compared with control cells (Fig. 1c). We conclude from these data that NS5A-mediated activation of β-catenin requires PI3K activity but is independent of both Akt and GSK-3β.

**Mutants of β-catenin that are non-responsive to either GSK-3β or Akt are stimulated by NS5A**

GSK-3β phosphorylation of β-catenin requires a priming phosphorylation event by casein kinase I (CK1) at serine 45...
activated by NS5A. To test this, we transfected Cos-7 cells with plasmids expressing either N-terminally V5-tagged wild-type or S45A mutant β-catenin (Hagen et al., 2004) together with NS5A and either M50 or M51 reporters (Fig. 2a). The activity of the S45A mutant of β-catenin was twofold greater than wild-type β-catenin and was further stimulated approximately twofold by NS5A. Appropriate expression of β-catenin and NS5A was verified by Western blot.

The transcriptional activity of β-catenin has also been shown to be promoted by direct phosphorylation of serine 552 by Akt in a GSK-3β-independent fashion (Fang et al., 2007). To further confirm that NS5A-mediated stimulation of β-catenin was Akt independent, we tested whether NS5A could stimulate a β-catenin mutant in which an activating PKA-phosphorylation site (serine 675) (Taurin et al., 2006) was mutated to alanine (S675A). As seen for S552A, this mutant was stimulated twofold by NS5A. Appropriate expression of β-catenin and NS5A was verified by Western blot.

These data provide further evidence that NS5A-mediated stimulation of β-catenin transcriptional activity is both GSK-3β- and Akt-independent.
Both the N and C termini of NS5A are required for β-catenin stimulation

To map the region(s) within NS5A that is required for stimulation of β-catenin transcriptional activity, three truncation mutants of NS5A were utilized (Fig. 3a). NS5A(33–448) lacks the N-terminal amphipathic helix reported to be required for membrane association (Brass et al., 2002). Two other mutants, NS5A(144–448) and (1–270) lacked the N-terminal 143 or C-terminal 178 residues, respectively. All three mutants were transiently expressed and exhibited the expected cellular distribution; those lacking the amphipathic helix [NS5A(33–448) and (144–448)] showed a much more diffuse localization of NS5A due to a loss of membrane association compared with the characteristic cytoplasmic punctate staining observed with wild-type NS5A and NS5A(1–270) (Fig. 3b).

Interestingly, both wild-type NS5A and NS5A(33–448) showed similar stimulation of β-catenin transcriptional activity (approx. fivefold in these assays) (Fig. 3c). In contrast, NS5A(1–270) and NS5A(144–448) exhibited a partial phenotype, showing only a threefold stimulation of β-catenin compared with the baseline. In all cases, treatment with LiCl showed an additive effect (approx. twofold further stimulation), suggesting that they all functioned similarly. We conclude that regions at both the N and C termini of NS5A are required for maximal stimulation of β-catenin; however, either of these two regions was sufficient for partial stimulation.

NS5A interacts with both β-catenin and the p85 subunit of PI3K

We have demonstrated previously that NS5A interacts with the p85 regulatory subunit of PI3K (Street et al., 2004). This interaction was further shown to require residues 270–300 of NS5A. One explanation of the data presented here could be that NS5A also interacted directly with β-catenin, either independently or as part of a trimeric complex with p85. To test this, we first examined the ability of NS5A to bind to β-catenin in vivo using a co-immunoprecipitation assay. As shown in Fig. 4(a) (lanes 2 and 3), both wild-type NS5A and NS5A(33–448) efficiently co-precipitated with FLAG-tagged β-catenin. Consistent with the reduced stimulation of β-catenin transcriptional activity by NS5A(1–270) and (144–448), both of these mutants co-precipitated with FLAG–β-catenin to a lesser extent (Fig. 4a, lanes 4 and 5), in the latter case the interaction was...
extremely weak, suggesting that the N-terminal 144 residues of NS5A are most important for a stable interaction with β-catenin. We then asked whether these mutant NS5A proteins could also interact with p85. Both wild-type NS5A and NS5A(33–448) efficiently co-precipitated with HA-tagged p85 (Fig. 4b, lanes 2 and 3). Consistent with our previous data (Street et al., 2004), NS5A(1–270) failed to precipitate p85, as did the N-terminal truncation NS5A(144–448) (Fig. 4b, lanes 4 and 5). Appropriate expression of the precipitated proteins was demonstrated by Western blotting (see lower panels). We conclude that NS5A is able to bind to both β-catenin and the p85 subunit of PI3K, and that each of these interactions most probably involves several protein–protein contacts.

**NS5A does not form a trimeric complex with p85 and β-catenin**

The lack of involvement of either Akt or GSK-3β in NS5A-mediated activation of β-catenin suggested that NS5A might form a trimeric complex with both p85 and β-catenin. To address this, we initially immunoprecipitated FLAG–β-catenin from lysates of cells co-transfected with plasmids expressing either HA-p85, NS5A or both.

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Fig. 3. Both N and C termini of NS5A contribute to stimulation of β-catenin transcriptional activity. (a) Schematic diagram showing the structure of the NS5A truncations used in this study. Grey boxes, amphipathic helices; black boxes, low complexity sequences. (b) Cos-7 cells were transfected with pSG5-NS5A expression plasmids. At 48 h post-transfection, cells were fixed and permeabilized, and NS5A was visualized with a polyclonal sheep anti-NS5A serum followed by Alexa-fluor 594-conjugated secondary antibody. Bar, 10 μm. Representative confocal images are shown. (c) Plasmids expressing the wild-type or NS5A mutants (33–448, 1–270 and 144–448) were transfected with the FLAG-tagged wild-type β-catenin expression plasmid and luciferase reporters M50 or M51. Where appropriate, LiCl (10 mM) was added for 16 h prior to lysis and luciferase activity was measured as described in Fig. 1 (n=3). Lower panels: lysates from the transcription assay were blotted for the indicated proteins. Values significantly different from controls, as determined by one-way ANOVA and the Bonferroni test, are indicated (**P<0.01). Error bars indicate SEM.
Although it has been reported previously that β-catenin can directly interact with p85 (Espada et al., 2005), we were unable to see this interaction in this experiment. However, we did observe that, in the presence of HA-p85, the interaction between NS5A and β-catenin was disrupted (Fig. 5a, compare lanes 3 and 4). We then performed the complementary experiment and immunoprecipitated HA-p85 from lysates of cells co-transfected with plasmids expressing either FLAG–β-catenin, NS5A or both. As expected, NS5A co-precipitated with HA-p85 in both the presence and the absence of FLAG–β-catenin (Fig. 5b, lanes 3 and 4). However, we also observed that β-catenin co-precipitated with p85 only when NS5A was present, suggesting that NS5A promoted the interaction between p85 and β-catenin (Fig. 5b, lane 4). Taken together, the results of these experiments do not provide evidence for a trimeric complex of NS5A with both p85 and β-catenin; however, they do suggest that NS5A perturbs this pathway by participating in, and influencing, several protein–protein interactions.

**DISCUSSION**

We have shown previously that the HCV NS5A protein stabilized β-catenin in a PI3K-dependent fashion (Street et al., 2005), in common with other viruses associated with human tumours (Cha et al., 2004; Fujimuro et al., 2003; Gan & Khalili, 2004; Morrison et al., 2003; Tomita et al., 2006). PI3K is known to activate β-catenin via the Akt/GSK-3β pathway; consistent with this, our previous data showed both β-catenin activation and GSK-3β inhibition upon expression of the complete HCV polyprotein. However, they did not unambiguously demonstrate a causative link between these two observations, i.e. that GSK-3β inhibition directly resulted in β-catenin activation. Although NS5A-mediated activation of PI3K did result in concomitant activation of Akt (Street et al., 2004), we demonstrate here that neither Akt nor GSK-3β is in fact involved in NS5A-mediated stimulation of β-catenin. This conclusion is based on two main strands of evidence. Firstly, pharmacological inhibition of Akt did not prevent the NS5A-mediated stimulation of β-catenin, whereas inhibition of GSK-3β had an additive effect, further stimulating β-catenin transcriptional activity (Fig. 1). The latter demonstrates that NS5A was not acting via PI3K-mediated inhibition of GSK-3β. Secondly, we observed that there were no significant differences in the NS5A-responsiveness between wild-type β-catenin and mutant derivatives in which phosphorylation sites for either Akt or CK1 (acting upstream of GSK-3β) were disrupted (Fig. 2).

As we have shown previously that NS5A interacts with the p85 regulatory subunit of PI3K (thereby activating the p110 catalytic subunit) (Street et al., 2004), the data presented here suggest that this binding directly stimulates β-catenin without the involvement of downstream effectors. This hypothesis is supported by evidence in the literature which points to a direct interaction between p85 and β-catenin (Espada et al., 2005); this interaction was shown to stimulate transcriptional activity in a manner that was independent of...
both PI3K and GSK-3β activities. Consistent with this, we were able to observe a p85–β-catenin interaction (Fig. 5b); however, this could only be seen in the presence of NS5A, and was only apparent when the complex was precipitated with an anti-HA antibody (targeting the HA-p85), not with anti-FLAG (targeting the FLAG–β-catenin). There are two possible explanations for this observation: firstly, that a trimeric complex of NS5A with both p85 and β-catenin does exist, but its detection by co-precipitation with the anti-FLAG antibody is precluded for technical reasons (outlined below); secondly, that NS5A bound to both p85 and β-catenin independently, and the binding to p85 induced a conformational change allowing the subsequent interaction of p85 with β-catenin. Our data are more consistent with the latter explanation, as we clearly show independent interactions of NS5A with either β-catenin (Fig. 4a) or p85 (Fig. 4b). Furthermore, these data demonstrate that both interactions appear to involve several protein–protein contacts; deletion of residues 33–144 dramatically reduced the interaction of NS5A with β-catenin and abolished the interaction with p85. A similar result was seen with a C-terminal truncation that removed residues 270–448, implying that there are at least two contact points between these interacting partners. Our data suggest that the interaction of NS5A with β-catenin predominantly involves the N-terminal 144 residues of NS5A, with only limited contribution from residues 144–448. This is in agreement with a study published during the preparation of this manuscript (Park et al., 2009), in which the N-terminal 147 residues of NS5A were shown to be necessary and sufficient to interact with FLAG-tagged β-catenin.

Furthermore, although we cannot formally rule out the existence of a trimeric complex, the observation that in the presence of exogenous p85 the NS5A–β-catenin interaction was disrupted (Fig. 5a, lane 4) suggests that binding of either p85 or β-catenin to NS5A is in fact mutually exclusive. The absence of p85 from anti-FLAG immunoprecipitates might be due to technical issues, for example if the N-terminal FLAG-tag on β-catenin was inaccessible when the protein was complexed with p85. Alternatively, binding of the FLAG antibody might disrupt the ternary complex, whereas this could retain its integrity following binding of the HA antibody. In this regard, although the N-terminal SH2 domain of p85 has been implicated in binding to β-catenin (Woodfield et al., 2001), the binding site(s) within β-catenin for p85 has not been identified.

If NS5A is not recruiting activated PI3K to β-catenin, a key question is whether there is a downstream pathway involved in the stimulation of β-catenin. Although our data show that PI3K is not functioning via Akt and GSK-3β, there are many other downstream effectors and interacting partners for PI3K. PtdIns(3,4,5)P3 production by PI3K recruits and activates many pleckstrin homology (PH) domain-containing proteins; for example, a recent study identified an endosomal adaptor protein APPL1 which contains a PH domain and stimulated β-catenin activity (Rashid et al., 2009).
However, a more compelling explanation is suggested by the parallels between NS5A and the EBV latent membrane protein 1 (LMP1), which also activates β-catenin (Shackelford et al., 2003). LMP1 has been shown to inhibit an alternative pathway of β-catenin ubiquitination, involving the Siah-1 (seven in absentia homologue 1) ubiquitin ligase (Jang et al., 2005). Importantly, this mechanism is GSK-3β-independent and, indeed (like NS5A), LMP1 and LiCl exhibited an additive effect on β-catenin transcriptional activity. Furthermore, LMP1 was able to activate a mutant β-catenin that was unable to be phosphorylated by GSK-3β (S37A) (Jang et al., 2005). It will be intriguing to determine whether NS5A also modulates the Siah-1 pathway of β-catenin regulation; current efforts in our laboratory are investigating this topic.

**METHODS**

**DNA manipulation and constructs.** The expression plasmid for the genotype 1a (H77 isolate) (Yanagi et al., 1997) NS5A (pSG5.NS5A) has been described previously (Macdonald et al., 2003). Truncation mutants of NS5A were amplified by PCR with appropriate primers (sequences available on request) and PCR products were ligated into pSG5 (Green et al., 1988). The Tcf/Lef luciferase reporters M50 and M51 were provided by Randall Moon, Seattle. The V5-tagged β-catenin expression plasmids were provided by Thilo Hagen, Singapore (Hagen et al., 2004), and the FLAG-tagged β-catenin expression plasmids were provided by Zhimin Lu, Houston (Fang et al., 2007).

**Cell culture.** Cos-7 and Huh-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, 100 IU penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Medium for Huh-7 cells also contained 1% non-essential amino acids and 20 mM HEPES–KOH, pH 7.4.

**Luciferase assays.** Cos-7 cells were seeded 24 h prior to transfection (1 × 10⁶) into 12-well dishes. Cells were transfected using polyethylenimine (Polysciences) according to the manufacturer’s instructions. For luciferase assays, cells were transfected with either M50 or M51 (0.25 μg), pSG5 expression vectors (1 μg) and either V5- or FLAG-tagged β-catenin (0.25 μg). A Renilla luciferase construct (pRLTK) was used as an internal control for transfection efficiency. The total amount of DNA was kept constant with the use of empty vector DNA. Where appropriate, LiCl (10 mM) (Sigma), LY294002 (20 μM) or AKT8VIII (5 μM) (Calbiochem) was added for 16 h prior to harvest. Cells were harvested 40 h after transfection in 200 μl passive lysis buffer (PLB) and luciferase levels were measured using the Stop and Glo reagent (Promega) and a luminometer with a dual injector (BMG LABTECH). All assays were performed in triplicate, and each experiment was repeated a minimum of three times.

**Western blotting.** Lysates (in PLB) were denatured at 95 °C, subjected to SDS-PAGE, then transferred onto polyvinylidene difluoride membrane (Millipore) using a semi-dry blotting apparatus (Bio-Rad). After blocking with 5% dried skimmed milk in TBS-T (150 mM NaCl, 50 mM Tris/HCl pH 7.5, 0.1% Tween-20), primary and secondary antibodies (diluted in 5% dried skimmed milk in TBS-T) were added sequentially with thorough washing using TBS-T after each step. Blots were visualized using chemiluminescence and autoradiography. Antibodies used were a sheep polyclonal NS5A antiserum (Macdonald et al., 2003), anti-FLAG monoclonal antibody (Sigma), anti-V5 monoclonal antibody (AbD Serotec), anti-phospho-Akt and anti-Akt (Cell Signalling Technology), and anti-GAPDH (ABCAM).

**Immunoprecipitations.** Transfected cells were lysed in RIPA buffer [50 mM Tris/HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, supplemented with 2 mM EDTA, 200 μM Na₃VO₄, 50 mM NaF and complete protease inhibitor cocktail (Roche)]. Anti-FLAG M2 affinity beads (Sigma) or anti-αHA (Affinity Bioreagents) were used according to the manufacturers’ instructions. Briefly, the bead suspension was washed twice in RIPA buffer before blocking with 1 mg BSA ml⁻¹ for 30 min at 4 °C and 40 μl of a 50% bead suspension was added to the lysate (500 μg protein). The mixture was incubated on a blood mixer at 4 °C for 6 h, washed three times with lysis buffer, and bound proteins were analysed by SDS-PAGE and Western blotting.

**Immunofluorescence.** Cos-7 cells grown on glass coverslips were transfected as described above. At 48 h post-transfection, cells were fixed with ice-cold methanol for 10 min, followed by permeabilization with 0.1% Triton X-100 (v/v) in PBS at room temperature for 10 min. Primary antibody [diluted in 5% (v/v) in FCS/PBS as described below] was applied directly to coverslips following washing and then detected using appropriate Alexa-fluor-conjugated secondary antibodies (Invitrogen Molecular Probes) diluted in 5% FCS in PBS at 1/500. Cells were washed and mounted onto microscope slides using Citifluor (Agar Scientific). Labelled cells were viewed on a Zeiss 510-META laser scanning confocal microscope under an oil-immersion × 63 objective lens (numerical aperture=1.40). Alexa-Fluor 488 (494 nm excitation; 519 nm emission) was excited using an argon laser fitted with 488 nm filters. Images displayed are representative and displayed as single optical sections of 50 μm thickness.

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