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The ubiquitin specific protease 4 (USP4) is a new player in the Wnt signalling pathway

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Abstract

The canonical Wnt signalling pathway is essential for cell fate determination during embryonic development and for the maintenance of adult tissue homeostasis. Deregulation of Wnt signalling leads to developmental defects and is associated with various types of cancer. Here we have used an RNA interference (RNAi) library specifically targeting human deubiquitinating enzymes (DUBs) to screen for new regulators of the canonical Wnt signalling pathway. We found that suppression of the ubiquitin specific protease 4 (USP4) activates β-catenin dependent transcription. We also show that USP4 is a DUB with dual hydrolysing activity for K^{48}- and K^{63}-conjugated polyubiquitin chains and interacts with two known Wnt signalling components: the Nemo like kinase (Nlk) and the transcription factor (T-cell factor 4 [TCF4]). Overexpression of a catalytically active Nlk promotes nuclear accumulation of USP4 whereas a subpopulation of TCF4 is a substrate of USP4-dependent deubiquitination. Thus, modulation of USP4 expression may provide a new means to interfere with canonical Wnt signalling in a variety of physiological and pathological conditions.

Keywords: deubiquitinating enzyme (DUB) • Nemo-like kinase (Nlk) • T-cell factor 4 (TCF4) • ubiquitin • ubiquitin specific protease 4 (USP4) • Wnt signalling

Introduction

Post-translational modifications by ubiquitin have emerged as major regulatory events in essential cellular processes such as cell cycle progression, signal transduction, transcriptional regulation and apoptosis [1, 2]. In addition to the originally described role in proteasome-dependent degradation, where ubiquitin chains conjugated through K^{48} linkages are typically involved, it is now well established that mono- and poly-ubiquitination involving alternative lysines, *e.g.* K^{63}, can convey signals that modulate protein activity or localization [3]. Like most other post-translational modifications ubiquitination is also reversible. Deconjugation is performed by a family of cystein and metalloproteases, collectively known as deubiquitinating enzymes (DUBs). Approximately 100 putative DUBs have been identified in the human genome [4], but only a minority of them have been functionally characterized. Their roles are diverse, varying from housekeeping functions, such as maintaining the pool of free ubiquitin, to highly specialized regulatory activities on specific substrates and signalling pathways, making them potentially interesting as therapeutic targets [5, 6].

Ubiquitin-dependent modifications are critically involved in the regulation of a variety of signalling pathways, including for example the highly conserved Wnt pathway that controls multiple developmental processes and is deregulated in human cancers [7]. Three signalling cascades are known to be induced by different members of the Wnt family of glycoproteins: the canonical Wnt/β-catenin pathway [8, 9], the less well-characterized non-canonical planar cell polarity pathway [10] and the Wnt/Ca^{2+} pathway [11].

The expression of the key player in the canonical signalling cascade, β-catenin, is kept under strict control because deregulation may cause developmental defects and promote tumorigenesis. In the absence of Wnt interaction with the Frizzled cell surface receptor, β-catenin levels are kept low by the activity of the 'destruction complex' comprising the adenomatous polyposis coli (APC), casein kinase 1 (CK1), Axin and glycogen synthase kinase-3 (GSK-3) [12]. The CK1 and GSK-3β kinases of the destruction complex phosphorylate newly synthesized β-catenin that is then recognized by the F-box protein βTrCP, a component of the Skp1/Cullin/Fbox (SCF) multi-subunit ubiquitin ligase, which promotes β-catenin ubiquitination and proteasomal degradation. Wnt-dependent triggering of the Frizzled receptor prevents the phosphorylation of β-catenin, which promotes its accumulation and nuclear translocation. Nuclear

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β-catenin activates the family of T-cell factor (TCF) and lymphoid enhancer factor (LEF) transcription factors, initiating transcription of genes such as cyclin D1 and c-Myc [13, 14]. Additional regulatory events take place in the nucleus such as the Bcl9/Legless-dependent recruitment of Pygopus to β-catenin, which activates transcription by promoting nuclear import and nuclear retention of β-catenin [15]. The signalling pathway is also regulated by the Nemo-like kinase (Nlk). Nlk is the mammalian homologue the *Drosophila* Nemo (nmo) Ser/Thr kinase, that is involved in apoptosis [16] and in multiple developmental processes [17] and was initially identified as a protein required for planar cell polarity in the developing *Drosophila* eye [18]. Nlk can be activated by the upstream MAP kinase kinase kinase (MAPKKK) family member transforming growth factor-β activated kinase-1 (Tak1) [19–21]. Activated Nlk promotes the phosphorylation of TCF/LEF bound to β-catenin, causing an overall inhibition of transcription [22]. Nlk can also regulate the transcription of other transcription factors such as HMG2L1 [23], STAT3 [24], Myb [25] and NF-κB [26].

Here we have used an RNA interference (RNAi) library specifically targeting the family of human DUBs [27] to search for possible new regulators of the canonical Wnt signalling pathway. We found that knock-down of the ubiquitin specific protease 4 (USP4, previously known as UNP for ubiquitous nuclear protein) is associated with hyperactivation of β-catenin mediated transcription. We also show that USP4 interacts with Nlk and TCF4 and that TCF4 is a target of USP4 DUB activity. Together our results identify USP4 as a new player in the canonical Wnt signalling pathway.

Material and methods

Plasmid construction

The RNAi library targeting the expression of DUBs has been described previously [27]. In short it is based on the pSUPER vector [28] for expression of short hairpin (sh) RNAs targeting various DUBs. Each different DUB is targeted for knock-down from a pool of four different shRNA transcripts. The shRNA target sequences for USP4 in the pooled sample are; (*1*) 5′-TTGTGGAGCATGGCCTGTT-3′, (*2*) 5′-GCTCGACACAGTCGTAGAA-3′, (*3*) 5′-CAGCCTTTACCTGATGAGT-3 and (*4*) 5′-GCACTGCGCCTAGCAGAAA-3′. For cloning USP4 in overexpression vectors it was PCR amplified from human brain cDNA using Pwo polymerase (Roche Diagnostics, Indianapolis, IN, USA) and the primers 5′-ATC**AAGCTT**ATGGCGGAAGGTGGAGGCTGCC-3′ and 5′-ATC**GGATCC**TGTTGGTGTCCATGCTGCAAGCC-3′ (HindIII and BamHI sites in bold). The PCR product was cloned in frame with enhanced green fluorescent protein (EGFP) in the pEGFP-N2 plasmid (Clontech, Mountain View, CA, USA) generating USP4-GFP. To generate Glutathione S-transferase (GST)-USP4 for bacterial expression USP4 was PCR amplified and cloned in frame with GST in the pGEX-5X-1 vector (Amersham, Buckinghamshire, UK) using BamHI and NotI restriction sites. The His-tagged Nlk and TCF4 vectors were generated by PCR amplification and ligation into the pQE30 plasmid (Qiagen, Valencia, CA, USA) using BamHI/HindIII and BamHI/Asp718, respectively. The plasmids expressing FLAG-Nlk, FLAG-Nlk^{K155M}, HA-TCF4, Bcl9-FLAG, Pygopus2-FLAG, p300-HA were kindly provided by Professor Hans Clevers (Hubrecht Laboratory, Utrecht, The Netherlands).

Tissue culture and expression analyses

The human embryonic kidney (HEK) epithelial cell lines HEK293 or HEK293T, the colorectal cancer cell lines SW480, DLD1 and colo320 were all cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% foetal calf serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 10 U/ml penicillin and 10 μg/ml streptomycin. The cells were transfected either by a $CaPO_4$ method or by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For co-immunoprecipitation with FLAG-Nlk the cells were lysed in a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1% IGEPAL supplemented with complete mini protease inhibitor cocktail pills (Roche Diagnostics) and for co-immunoprecipitations with TCF4 the cells were lysed in a buffer containing 250 mM NaCl, 0.1% IGEPAL, 50 mM Hepes (pH 7.3) supplemented with complete mini protease inhibitor cocktail and lysis was completed by sonication. Precipitations were performed with indicated antibodies conjugated to GammaBind G Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK). The samples were fractionated in acrylamide Bis-Tris 4–12% pre-casted gradient gels (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and probed with indicated antibodies. The following antibodies were used: anti-HA (Y11), anti-GFP (FL) and anti-Nlk (C20) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-GFP (ab290) from Abcam (Cambridge, MA, USA), anti-TCF4 (6H5–3) from Upstate (Billerica, MA, USA), anti-FLAG (M2) from Sigma-Aldrich, anti-ubiquitin from Dako (Carpinteria, CA, USA), anti-USP4 (Bethyl Laboratories, Montgomery, TX, USA) and anti-HA (12CA5) from Roche (Indianapolis, IN, USA). The HA-Ub-VME probe was kindly provided by Dr Huib Ovaa (The Netherlands Cancer Institute, Amsterdam) and it was used in order to detect DUB activity in transiently transfected HEK293T cells 2 days after transfection. The experiment was performed as previously described [29] except that the time for labelling was reduced to 5 min. Dual luciferase assay quantifying levels of luciferase and renilla was performed according to manufacturers protocol (Promega, Madison, WI, USA) and values are expressed as luciferase relative to renilla.

In vitro binding experiments

GST and GST-USP4 were expressed after induction by 1 mM isopropyl-β-D-1-thiogalactopyranoside, 3.5 hrs 27°C, in the *E.coli* strain BL21. The cells were harvested and washed in ice-cold phosphate buffered saline (PBS) and subsequently resuspended in a buffer containing 50 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 0.5% IGEPAL supplemented with phenylmethylsulfonyl fluoride (PMSF) (0.18 mg/ml) (Sigma-Aldrich) and protease inhibitor cocktail (Roche Diagnostics) and lysed by three freeze-thaw cycles and sonication. The proteins were purified on glutathione sepharose (Amersham Biosciences, Buckinghamshire, UK). His-Nlk and His-TCF4 were expressed in the *E.Coli* strain M15 and lysates were prepared in a buffer containing 50 mM NaH_2PO_4 pH 8.9 and 0.5 M NaCl by three freeze-thaw cycles and sonication followed by clarification of lysates by centrifugation. *In vitro* binding was performed in a buffer containing 50 mM Tris pH 8.0, 0.1 mM EDTA, 150 mM NaCl, 0.1% IGEPAL, 0.5 mM dithiothreitol (DTT) supplemented with PMSF by rotating for 3 hrs at +4°C. Unbound material was washed away in the same buffer used for binding.

K^{48} and K^{63} ubiquitin-chain cleavage experiment

The ability of GST-USP4 to hydrolyse ubiquitin chains linked through K^{48} or K^{63} (Biomol, Exeter, UK) was investigated by incubation of 0.04 μg chains with increasing amounts of GST-USP4 in a buffer containing 20 mM Tris pH 7.5,

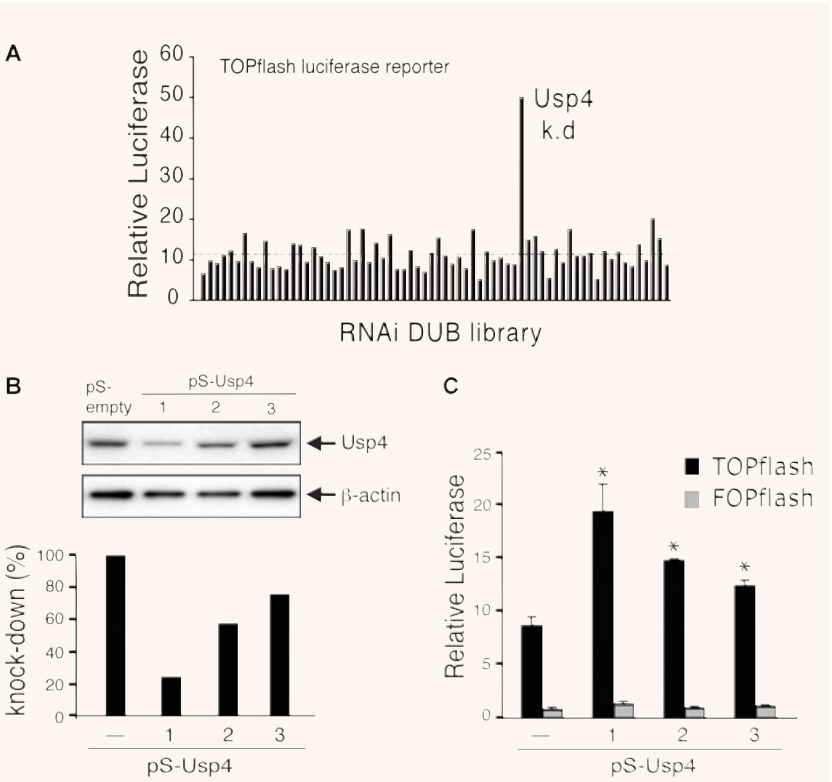

Fig. 1 DUB knock-down screen identifies USP4 as a new player in the canonical Wnt signalling pathway. (**A**) Luciferase assay on SW480 cells co-transfected with the Wnt-reporter TOPflash together with individual samples of the RNAi DUB library. Knock-down of the USP4, results in a hyperactivation of Wnt signalling activity. The relative levels of luciferase activity (luciferase/renilla), at day 3 after transfection, are shown. The dotted line represents the mean value (mean ± S.D. is 11 ± 6). (**B**) Individual evaluation of three independent knock-down vectors from the pS-USP4 pool transiently transfected in HEK293 cells, harvested 4 days after transfection. Western blot against USP4 (upper panel) and β-actin as loading control (lower panel). Densitometry showing the mean of two experiments in the graph below. (**C**) Luciferase assay in SW480 cells evaluating the capacity of the three individual pS-USP4 vectors to induce Wnt signalling. The FOPflash reporter serves as a control for non-Wnt specific induction. Values represent the relative luciferase (luciferase/renilla) ±S.D. Paired t-test with values <0.05 are indicated with asterisk.

150 mM NaCl, 2 mM EDTA and 2 mM DTT for 1 hr at 37°C. Incubation with GST or treatment with the cysteine protease inhibitor *N*-ethylmaleimide was used as control to illustrate the specificity of the reaction.

Fluorescence microscopy

For analysis by fluorescence microscopy cells were cultured and transfected on cover slips. The cells were fixed in 4% formaldehyde, washed in PBS and immunostained with the primary anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) and the secondary antimouse antibody labelled with Alexa Fluor 594 (Molecular Probes, Carlsbad, CA, USA). Antibodies were diluted in a buffer containing 50 mM Tris pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.5 Triton X-100. Cells were counterstained with Hoechst 33258 (Molecular Probes). Fluorescence was analysed using a Leiz-BMRB fluorescence microscope (Leica, Wetzlar, Germany) and images were captured with a Hamamatsu (Japan) cooled charge-coupled device (CCD) camera.

Results

USP4 is a new regulator of the canonical Wnt signalling pathway

Given the critical involvement of ubiquitin-dependent events in Wnt signalling, we suggested that one or more DUBs may act as regulators in the pathway. To test this possibility we used an RNAi library that targets the expression of human DUBs [27] to perform a loss-of-function screen in the colon cancer cell line SW480 where Wnt signalling is constitutively active due to a mutation in the APC gene. The library is based on the short hairpin expression vector pSUPER and specifically induces the knock-down of individual DUBs by independent pools of plasmids targeting different sites in each mRNA.

Wnt signalling was assessed using the previously described TOP and FOPflash reporter pair [30]. The TOPflash reporter responds to Wnt signalling whereas the FOPflash serves as a negative control due to mutations in the promoter region that prevent TCF binding. The TOPflash plasmid was co-transfected with the individual pools of the RNAi library and the levels of luciferase were measured 72 hrs after transfection. Only the pool targeting USP4 caused a marked change of relative luciferase activity, with an approximate 4.5-fold increase compared to the average activity of all samples (mean ± S.D. is 11 ± 6) (Fig. 1A).

Three of the four knock-down vectors present in the pooled sample used in the screen were tested for their ability to individually suppress the expression of endogenous USP4 in SW480 and HEK293 cells. The fourth vector targets a known splice variant of USP4 lacking exon 7 and was therefore excluded from further analysis. The hairpin 1 resulted in the best knock-down, with approximately 20% residual expression compared to the control (Fig. 1B). The TOP and FOPflash

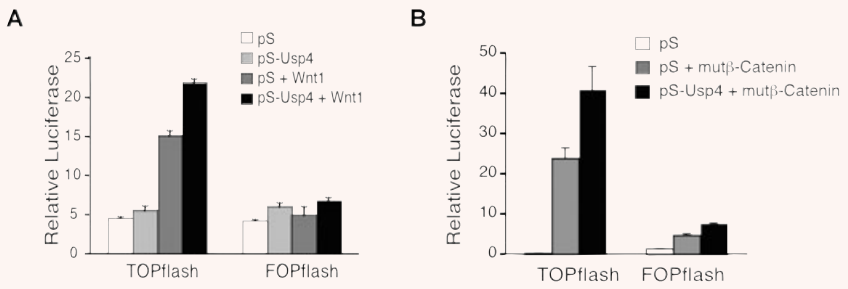

Fig. 2 USP4 acts in a late step of Wnt signalling. (**A**) Luciferase assay of transfected HEK293 cells. Wnt signalling was induced by co-transfection of a Wnt1 expressing plasmid as indicated. Values represent the luciferase values in relation to co-transfected renilla ± S.D. One representative experiment performed in triplicate. (**B**) TOPflash luciferase assay of transfected HEK293T cells where Wnt signalling was induced by co-transfected mutβ-catenin lacking sites for phosphorylation by GSK-3β, thus functioning as a constitutive activator of transcription. One representative experiment performed in triplicate.

reporter pair was then used to test if the hyperactivation of Wnt signalling could be reproduced with the individual vectors. As shown in Fig. 1C, the three shRNAs increased Wnt signalling with an efficiency directly proportional to the levels of USP4 knock-down (compare Fig. 1B and C). The pSUPER-USP4–1 target sequence consistently resulted in the strongest activation of Wnt signalling and was therefore used in subsequent experiments. Similar activation of Wnt-signalling upon USP4 knock-down was obtained in two additional colon cancer cell lines with mutated APC; DLD1 and colo320 (data not shown).

In order to investigate whether USP4 knock-down could regulate signalling in a cellular background where the Wnt pathway is intact we chose HEK293 cells that show low levels of activity in the absence of exogenous Wnt. Knock-down of USP4 alone did not affect the signalling in these cells (Fig. 2A). However, in agreement with the hyperactivation observed in colon cancer cells, knock-down of USP4 was associated with a significantly increased activity when signalling was induced by co-transfection of Wnt1 (Fig. 2A).

The capacity of USP4 knock-down to activate Wnt signalling in cells with mutated APC suggests that USP4 may act downstream of the destruction complex. To test this possibility, activation of Wnt signalling was induced in HEK293 cells by transient transfection of a β-catenin lacking GSK-3β phosphorylation sites (referred to as mutβ-catenin), which prevents ubiquitin-dependent degradation of β-catenin and induces constitutive activation of the pathway. Hyperactivation of the TOPflash reporter was observed also in the presence of mutβ-catenin suggesting that USP4 affects signalling downstream of β-catenin stabilization (Fig. 2B). A minor transcriptional activation on the FOPflash control reporter was occasionally detected in cells expressing either mutβ-catenin or shRNA USP4 vectors. This is likely to reflect experimental variability although additional mechanisms of transcriptional regulation cannot be formally excluded.

USP4 interacts with components of the Wnt signalling pathway

The finding that USP4 acts downstream of β-catenin stabilization motivated us to attempt to further narrow down its site of action. To this end, we selectively searched for potential USP4 interacting partners among Wnt pathway components acting at the level of transcriptional regulation. Co-immunoprecipitation experiments were performed in HEK293T cells expressing either USP4-GFP or GFP alone together with a panel of candidates including β-catenin, Bcl9, Pygopus2, the acetyltransferase p300 that is a known potentiator of β-catenin mediated transcription in vertebrates [31, 32], Nlk and the transcription factors LEF-1, TCF3 and TCF4. HA-TCF4 and FLAG-Nlk were successfully co-immunoprecipitated with USP4-GFP but not with GFP alone (Fig. 3A and B) whereas no interaction with the other components could be demonstrated (not shown). Both USP4-GFP and the catalytic mutant USP4^{C311S}-GFP were co-precipitated with HA-TCF4 and FLAG-Nlk (Fig. 3A and B), indicating that enzymatic activity is not required for these interactions. Furthermore, the interaction was reproduced by *in vitro* binding assays performed with bacterially expressed His-TCF4, His-Nlk and GST-USP4 (Fig. 3C), confirming that the interaction is direct and does not require additional partners expressed in mammalian cells.

The Nlk promotes nuclear localization of USP4

The knock-down phenotype suggests that USP4 may act as an inhibitor of Wnt signalling. To test this possibility we first verified the USP4 DUB activity using a previously described DUB functional probe that comprises an HA-tagged ubiquitin that is C-terminally modified with a vinyl methyl ester (HA-Ub-VME) [33]. Covalent linkage of the probe to an active DUB allows detection of the enzyme using an anti-HA antibody in Western blot. As expected USP4-GFP was functionally active, whereas mutation of the catalytic Cys residue (C311S) abrogated the activity (Fig. 4A).

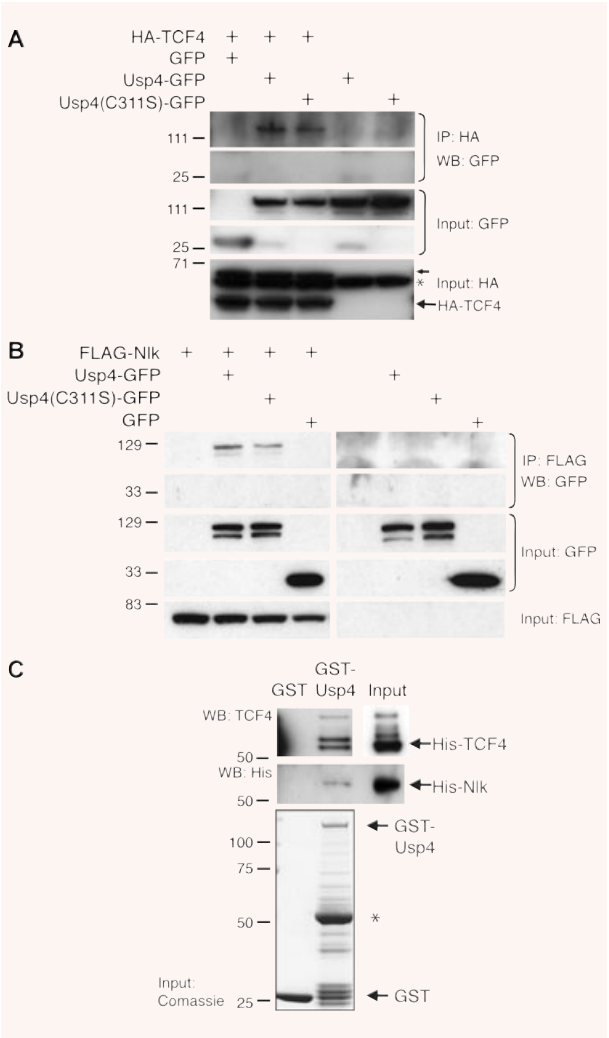

Fig. 3 USP4 interacts with components in the Wnt signalling pathway. (**A**) Western blot analysis of co-immunoprecipitation experiments where HA-TCF4 was co-transfected in HEK293T cells either with USP4-GFP, USP4^{C311S}-GFP or GFP as control. Immunoprecipitations from whole cell lysates were performed using an anti-HA antibody. The input in the experiment is shown in the lower panels. A slower migrating band specific for HA-TCF4 expression that reacts with the HA antibody is of unknown identity and appears above 75 kD, here marked with a small arrow. A background band appearing with the HA antibody in the input sample is indicated with an asterisk. (**B**) Western blot analysis of co-immunoprecipitation experiments where FLAG-Nlk was co-transfected in HEK293T cells either with USP4-GFP, USP4^{C311S}-GFP or GFP as control. Immunoprecipitations from whole cell lysates were performed using an anti-FLAG antibody. (**C**) Western blot analysis of *in vitro* binding experiments with GST, GST-USP4 and His-TCF4 or His-Nlk as indicated. The input of the purified GST and GST-USP4 is shown by coomassie staining in the lower panel. A dominant band of unknown origin above 50 kD in the GST-USP4 purified sample is indicated with an asterisk.

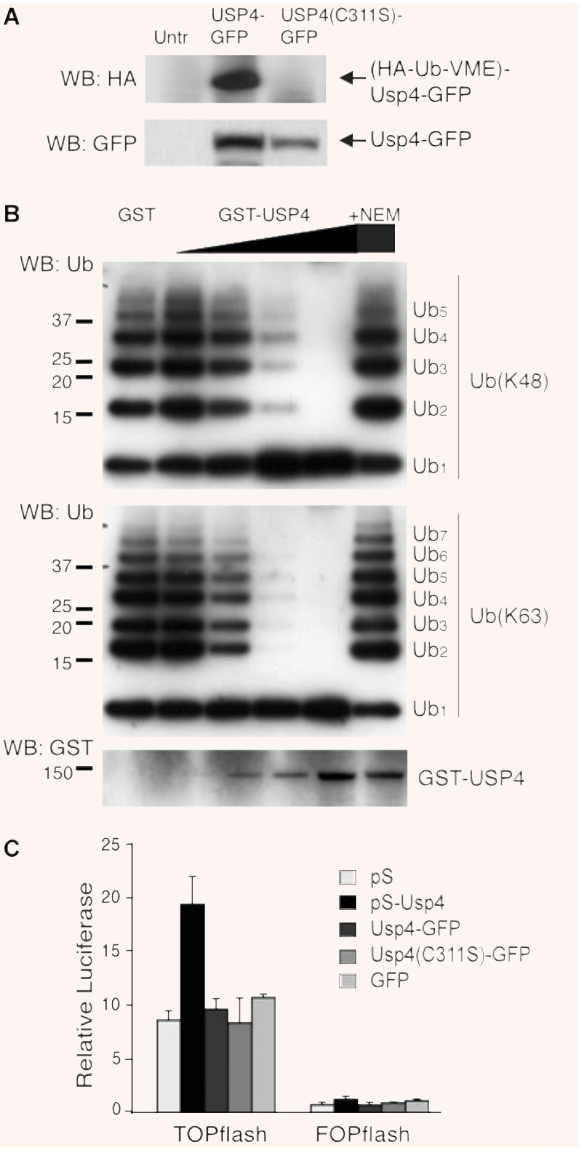

Fig. 4 USP4 cleaves both K^{48} and K^{63} ubiquitin chains and does not affect Wnt-signalling after overexpression. (**A**) Western blot analysis of HEK293T cells transiently transfected with USP4-GFP or USP4^{C311S}-GFP and subsequently labelled with the DUB active site probe HA-Ub-VME [33]. The upper blot is probed with an anti-HA antibody and illustrates DUB activity of USP4-GFP and the lower blot shows the same samples probed with an anti-GFP antibody to show the input in the labelling experiment. (**B**) Western blot analysis with an anti-ubiquitin antibody, of K^{48}- and K^{63}-polyubiquitin chains incubated *in vitro* for 1 hr with bacterially purified GST as a control, and increasing amounts of GST-USP4 as indicated. Treatment with *N*-ethylmaleimide abrogates cysteine protease activity and serves as a control. (**C**) Luciferase assay of SW480 cells transiently transfected with the TOP/FOPflash reporters and pSUPER-empty, pSUPER-USP4, USP4-GFP, Usp4^{C311S}-GFP or GFP as a control. Cells were harvested 3 days after transfection. One representative experiment in triplicate.

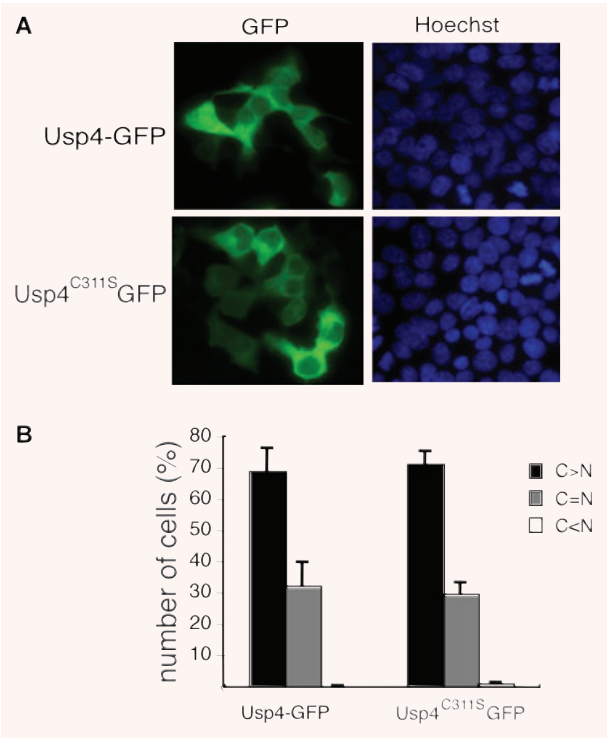

Fig. 5 USP4 localization is predominantly cytoplasmic. (**A**) Representative micrographs of HEK293T cells transiently transfected with USP4-GFP and the active site mutant USP4^{C311S}-GFP. GFP fluorescence to the left and Hoechst counter staining to the right (**B**) Quantification of cells in A by scoring for localization of the GFP; C > N (predominant cytoplasmic staining), C = N (equal in cytoplasm and nucleus), C < N (predominant nuclear staining). Values are expressed as percent of GFP fluorescent cells with a specific localization ± S.D. One representative experiment performed in triplicate.

We further tested the ability of USP4 to hydrolyse ubiquitin isopeptide bonds and the possible preference for either K^{48} or K^{63}-linked polyubiquitin chains. Interestingly both chain types were cleaved into ubiquitin monomers with equal efficiency after incubation with GST-USP4 (Fig. 4B). Next the activity of the TOP/FOPflash reporters was tested in SW480 cells expressing the catalytically active USP4-GFP. Surprisingly, overexpression of USP4-GFP did not affect the activity of the TOPflash reporter nor did we observe any dominant-negative effect after overexpression of the catalytic mutant USP4 (Fig. 4C). Thus, either the endogenous amounts of USP4 are sufficient to obscure any effect of overexpression, or rate limiting protein interactions or post-translational modifications of USP4 may be required for its capacity to act as an inhibitor of Wnt signalling.

USP4 harbours both nuclear import and export signals and it is therefore conceivable that, similar to other shuttling proteins [34], sub-cellular localization may regulate its activity. Phosphorylation is a common regulator of nuclear cytoplasmic shuttling and gene transcription [35, 36] and Nlk is known to promote Mad nuclear export in *Drosophila* and thereby attenuate bone morphogenetic protein signalling [37]. To investigate whether the USP4-Nlk interaction affects the sub-cellular localization of USP4, the fluorescence pattern of a transfected USP4-GFP plasmid was studied in HEK293 cells (Fig. 5A). A predominant cytoplasmic fluorescence was observed in approximately 70% of the cells (Fig. 5B), whereas the remaining 30% showed a more homogenous fluorescence in both nucleus and cytoplasm and predominant nuclear localization was virtually never observed. The catalytic mutant USP4^{C311S}GFP showed a similar localization pattern indicating that DUB activity is not critical for its localization. The effect of Nlk on this localization pattern was investigated by co-expression of USP4-GFP or USP4^{C311S}GFP with the wild-type FLAG-Nlk or the inactive kinase, FLAG-Nlk^{K155M} in HEK293T cells. A dramatic Nlk activity-dependent redistribution of USP4 was detected in cells co-transfected with FLAG-Nlk (Fig. 6A). Approximately 60% of the cells expressing the active kinase showed an equal distribution of USP4-GFP or USP4^{C311S}GFP between the cytoplasm and the nucleus and approximately 30% of the cells exhibited a predominant nuclear localization (Fig. 6B) whereas localization of USP4-GFP was not affected by expression of the catalytically inactive FLAG-Nlk^{K155M}. Thus, the activity of the Nlk appears to be required for a redistribution of USP4 within the cells of which approximately 30% of the cells accumulate USP4 in the nucleus.

USP4 is a DUB for TCF4

Although the interaction between USP4 and Nlk was readily detected, the interaction with TCF4 was experimentally more challenging. This could be due to suboptimal binding conditions or to the preference for post-translational modifications of one or both partners. To test this possibility we performed a reverse co-immunoprecipitation where USP4-GFP instead of HA-TCF4 was used as bait (compare to Fig. 3B). HA-TCF4 was readily co-precipitated under these conditions but appeared as two closely migrating bands, indeed suggesting the involvement of a post-translational modification (Fig. 7A). A side-by-side comparison of the input and the immunoprecipitated HA-TCF4 (Fig. 7B) clearly illustrates the size difference of the post-translationally modified form of HA-TCF4 that USP4 preferentially interacts with.

The interaction of USP4 with both Nlk and TCF4 suggests that they could be substrates of USP4 DUB activity. Although both proteins were found to be ubiquitinated (Fig. 8A and B) and targeted for proteasomal degradation, overexpression of USP4 did not induce detectable differences in the ubiquitination pattern or steady state levels of the proteins (not shown). However, knowing that USP4 preferentially interacts with a post-translationally modified form of TCF4 we tested whether this subpopulation of TCF4 proteins is specifically targeted by USP4 DUB activity. To this end, co-immunoprecipitations with an anti-GFP antibody were performed on HEK293T cells expressing HA-tagged ubiquitin (HA-Ub), HA-TCF4

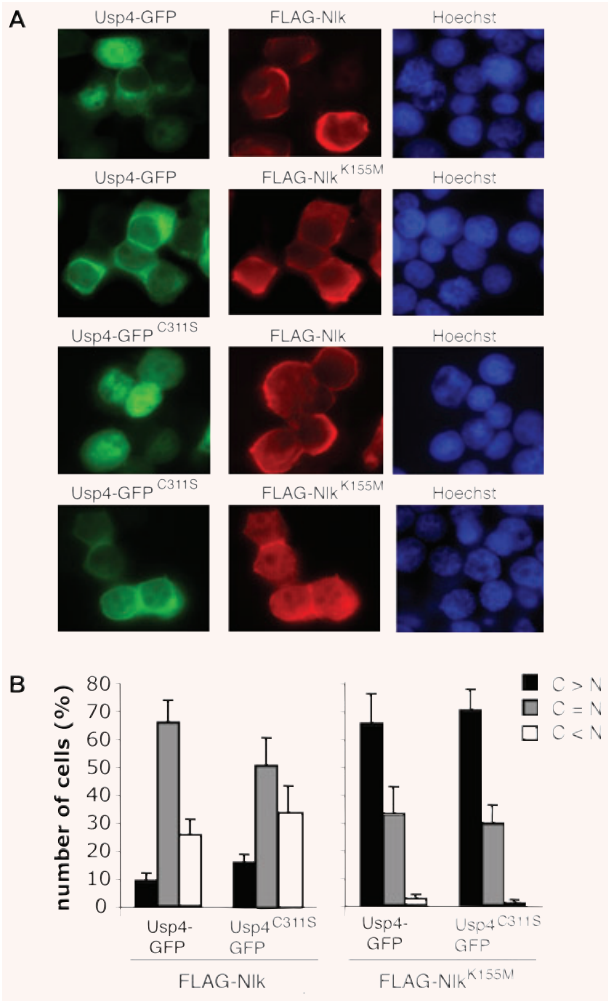

Fig. 6 The Nlk promotes the nuclear localization of USP4. (**A**) Representative micrographs of HEK293T cells transiently transfected with USP4-GFP or USP4^{C311S}-GFP together with either FLAG-Nlk or the catalytically inactive FLAG-Nlk^{K155M} as indicated. GFP fluorescence in green, FLAG immunostaining of the same cells in red and Hoechst staining in blue. (**B**) Quantification of cells in A by scoring for localization of the GFP fluorescence in FLAG-Nlk or FLAG-Nlk^{K155M} positive cells. C > N (predominant cytoplasmic staining), C = N (equal in cytoplasm and nucleus), C < N (predominant nuclear staining). Values are expressed as percent GFP fluorescent cells with a specific localization ± S.D. One representative experiment performed in triplicate.

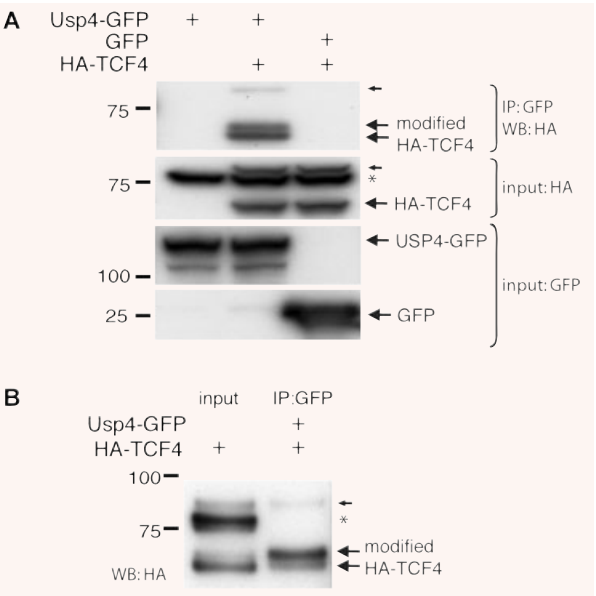

Fig. 7 USP4 interacts preferentially with a modified form of TCF4. (**A**) Western blot analysis of co-immunoprecipitation experiments where HA-TCF4 was co-transfected with USP4-GFP or GFP as control in HEK293T cells. Immunoprecipitations from whole cell lysates were performed using an anti-GFP antibody. The co-immunoprecipitated HA-TCF4 appears as two closely migrating bands (big arrows, upper panel). A slower migrating band specific for HA-TCF4 expression that reacts with the HA antibody is of unknown identity and appears above 75 kD, here marked with a small arrow. A background band appearing with the HA antibody in the input sample is indicated with an asterisk. Input sample identified with a GFP antibody in the two lower panels. (**B**) Western blot using an anti-HA antibody illustrating HA-TCF4 input material next to the HA-TCF4 that has been co-immunoprecipitation with USP4-GFP. A modified form of HA-TCF4 enriched after USP4-GFP immunoprecipitation is indicted.

and either USP4-GFP or USP4^{C311S}-GFP. The level of ubiquitinated TCF4 was then detected by Western blot analysis using an anti-HA antibody. A smear of high molecular weight species corresponding to ubiquitinated TCF4 was clearly detected in immunoprecipitates of cells expressing USP4^{C311S}-GFP (Fig. 8C). Thus, catalytically inactive USP4 appears to specifically associate with polyubiquitinated TCF4, indicating that it is a target of USP4 DUB activity.

Discussion

By using an RNAi library that allows selective knock-down of individual human DUBs we have shown that USP4 acts as a regulator of the Wnt signalling pathway. USP4 interacts with Nlk, a negative regulator of Wnt signalling, and a catalytically active Nlk promotes the nuclear accumulation of USP4. Furthermore, USP4 interacts with a post-translationally modified form of the transcription factor TCF4 that appears to be a substrate of USP4 DUB activity. Taken together our data identify USP4 as a new negative regulator of the canonical Wnt signalling pathway. It seems likely that USP4 will do so in the nucleus, probably by interfering with a transcriptional regulatory complex that includes TCF4. It should be noted that additional DUBs regulating Wnt signalling may still be discovered because our screen was specifically designed to detect DUBs acting downstream of the destruction complex and the library used [27] did not include newly discovered DUBs such as Trabid that was recently shown to regulate Wnt responses in both mammals and *Drosophila* [38].

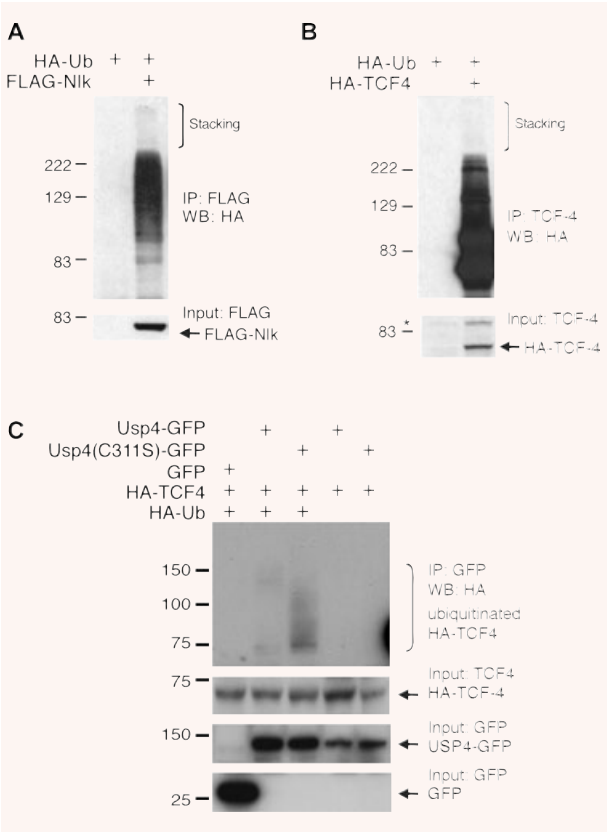

Fig. 8 USP4 deubiquitinates TCF4. (**A**) Western blot illustrating ubiquitinated Nlk. HEK293T cells were co-transfected with FLAG-Nlk and HA-ubiquitin as indicated. In order to accumulate ubiquitinated material the cells were treated with the proteasome inhibitor MG132 (10 μM) 4 hrs prior to immunoprecipitation with an anti-FLAG antibody. (**B**) Same as in A but co-transfection with the HA-TCF4 expression plasmid. (**C**) Co-transfections in HEK293T cells performed as indicated. Immunoprecipitation was performed 3 days after transfection with an anti-GFP antibody and the Western blot was probed against HA, illustrating the ubiquitinated TCF4 interacting with USP4^{C311S}-GFP. Input samples were loaded and probed as indicated to ensure expression of all plasmids.

Deregulation of the Wnt pathway and nuclear accumulation of β-catenin are hallmarks of many human tumours [8, 9]. Our data suggest that USP4 may counteract the oncogenic properties of activated Wnt signalling and act as a tumour suppressor. Interestingly, the USP4 gene is localized in a region on the short arm of chromosome 3 (3p21.3) that is often deleted in human epithelial tumours, such as carcinomas of the lung, breast, kidney and ovary [39–44], which show deregulated Wnt signalling [45–47]. The possibility that USP4 may act as a negative regulator of tumour cell growth is also supported by the reduced expression in a panel of human lung cancer cell lines [48]. However, USP4 was found among the most up-regulated genes in adrenocortical carcinomas [49] and overexpression studies suggest a role of USP4 in malignant transformation [50]. This may reflect a highly selective mode of action and stresses the importance of a careful dissection of USP4 function in different cell types.

We have shown that USP4 knock-down enhances Wnt signalling in cells where the pathway is activated either by mutations in APC (SW480 cells), by transfection of constitutively active β-catenin or by co-expression of the physiological Wnt1 ligand (HEK293 cells). However, overexpression of USP4 alone was not sufficient to impair signalling, suggesting that specific protein interactions or rate limiting post-translational modifications may be required for its inhibitory function. In agreement with this possibility we found that co-expression with a catalytically active Nlk results in nuclear accumulation of USP4, where it may interact with a post-translationally modified TCF4 species representing only a minor fraction of the transfected TCF4 detected in a total cell lysate. Previously reported modifications of TCF transcription factors include ubiquitination [51], sumoylation [52], phosphorylation [22] and acetylation [53]. Considering the size of the slow migrating band, phosphorylation or acetylation appears most probable; however, the true nature of this post-translational modification remains to be determined.

Our data suggest that USP4 represses Wnt signalling by acting downstream of β-catenin stabilization. Nlk is also a negative regulator, acting at a late step of the signalling cascade. Two different modes of action have been described: first, direct phosphorylation of TCF4, which impairs DNA binding [22], and second, induction of TCF4 ubiquitination and degradation through recruitment of the Nlk-associated ring finger protein, NARF [51]. We have found that Nlk interacts with USP4 and induces its accumulation in the nucleus. The cellular localization of USP4 has been a matter of debate because different reports have found it to be prevalently expressed either in the nucleus [50] or cytoplasm [48] of different cell types. This apparent discrepancy was recently resolved by the finding that USP4 contains nuclear import and export signals and has therefore nucleocytoplasmic shuttling properties [34]. Our data add to these observations and suggest that phosphorylation of USP4, or a yet unidentified USP4 interacting protein, may be required for its nucleocytoplasmic shuttling because only the catalytically active Nlk was capable of inducing nuclear accumulation. This is likely to be an important requirement for the effect of USP4 on Wnt signalling because we placed its target downstream of β-catenin stabilization and the other identified USP4 interacting protein, TCF4, is mainly a nuclear protein.

Although we could confirm that both Nlk and TCF4 are ubiquitinated and short-lived proteins, their steady state levels were not affected by USP4 expression (not shown). The finding that USP4 hydrolyses both K^{48}- and K^{63}-ubiquitin chains with equal efficiency is in this context particularly interesting because it suggests that regulation of Wnt signalling by USP4 may not necessarily involve the stabilization of specific substrates. It is tempting to speculate that the preferential interaction of USP4 with post-translationally modified TCF4 and the detection of ubiquitinated TCF4 in complexes containing the inactive USP4 may reflect the capacity of this DUB to regulate either the stability, or the activity of the transcription factor. It is noteworthy that K^{63}-linked polyubiquitin chains were shown to be a preferred

target of the recently identified positive regulator of Wnt signalling Trabid [38], supporting the possibility that this type of chains may play a yet unidentified role in the regulation of β-catenin mediated transcription.

In conclusion, our finding that USP4 knock-down results in hyperactivation of the canonical Wnt signalling pathway highlights a new regulatory mechanism that may play an important role in a variety of physiologic and pathologic conditions including cancer. Further work in this area is required to increase our understanding of the complex regulation of the canonical Wnt signalling pathway.

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References

1. **Glickman MH, Ciechanover A.** The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev*. 2002; 82: 373–428.
2. **Sigismund S, Polo S, Di Fiore PP.** Signaling through monoubiquitination. *Curr Top Microbiol Immunol*. 2004; 286: 149–85.
3. **Ikeda F, Dikic I.** Atypical ubiquitin chains: new molecular signals. 'Protein modifications: beyond the Usual Suspects' review series. *EMBO Rep*. 2008; 9: 536–42.
4. **Nijman SM, Luna-Vargas MP, Velds A, *et al.*** A genomic and functional inventory of deubiquitinating enzymes. *Cell*. 2005; 123: 773–86.
5. **Daviet L, Colland F.** Targeting ubiquitin specific proteases for drug discovery. *Biochimie*. 2007; 90: 270–83.
6. **Nicholson B, Marblestone JG, Butt TR, *et al.*** Deubiquitinating enzymes as novel anticancer targets. *Future Oncol*. 2007; 3: 191–9.
7. **Wodarz A, Nusse R.** Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol*. 1998; 14: 59–88.
8. **Clevers H.** Wnt/beta-catenin signaling in development and disease. *Cell*. 2006; 127: 469–80.
9. **Polakis P.** The many ways of Wnt in cancer. *Curr Opin Genet Dev*. 2007; 17: 45–51.
10. **Katoh M.** WNT/PCP signaling pathway and human cancer (review). *Oncol Rep*. 2005; 14: 1583–8.
11. **Kohn AD, Moon RT.** Wnt and calcium signaling: beta-catenin-independent pathways. *Cell Calcium*. 2005; 38: 439–46.
12. **Kimelman D, Xu W.** Beta-catenin destruction complex: insights and questions from a structural perspective. *Oncogene*. 2006; 25: 7482–91.
13. **He TC, Sparks AB, Rago C, *et al.*** Identification of c-MYC as a target of the APC pathway. *Science*. 1998; 281: 1509–12.
14. **Tetsu O, McCormick F.** Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature*. 1999; 398: 422–6.
15. **Townsley FM, Cliffe A, Bienz M.** Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. *Nat Cell Biol*. 2004; 6: 626–33.
16. **Mirkovic I, Charish K, Gorski SM, *et al.*** Drosophila Nemo is an essential gene involved in the regulation of programmed cell death. *Mech Dev*. 2002; 119: 9–20.
17. **Verheyen EM, Mirkovic I, MacLean SJ, *et al.*** The tissue polarity gene Nemo carries out multiple roles in patterning during Drosophila development. *Mech Dev*. 2001; 101: 119–32.
18. **Choi KW, Benzer S.** Rotation of photoreceptor clusters in the developing Drosophila eye requires the Nemo gene. *Cell*. 1994; 78: 125–36.
19. **Ishitani T, Ninomiya-Tsuji J, Nagai S, *et al.*** The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. *Nature*. 1999; 399: 798–802.
20. **Shin TH, Yasuda J, Rocheleau CE, *et al.*** MOM-4, a MAP kinase kinase kinase-related protein, activates WRM-1/LIT-1 kinase to transduce anterior/posterior polarity signals in C. elegans. *Mol Cell*. 1999; 4: 275–80.
21. **Smit L, Baas A, Kuipers J, *et al.*** Wnt activates the Tak1/Nemo-like kinase pathway. *J Biol Chem*. 2004; 279: 17232–40.
22. **Ishitani T, Ninomiya-Tsuji J, Matsumoto K.** Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/beta-catenin signaling. *Mol Cell Biol*. 2003; 23: 1379–89.
23. **Yamada M, Ohkawara B, Ichimura N, *et al.*** Negative regulation of Wnt signalling by HMG2L1, a novel NLK-binding protein. *Genes Cells*. 2003; 8: 677–84.
24. **Ohkawara B, Shirakabe K, Hyodo-Miura J, *et al.*** Role of the TAK1-NLK-STAT3 pathway in TGF-beta-mediated mesoderm induction. *Genes Dev*. 2004; 18: 381–6.
25. **Kanei-Ishii C, Ninomiya-Tsuji J, Tanikawa J, *et al.*** Wnt-1 signal induces phosphorylation and degradation of c-Myb protein via TAK1, HIPK2, and NLK. *Genes Dev*. 2004; 18: 816–29.
26. **Yasuda J, Yokoo H, Yamada T, *et al.*** Nemo-like kinase suppresses a wide range of transcription factors, including nuclear factor-kappaB. *Cancer Sci*. 2004; 95: 52–7.
27. **Brummelkamp TR, Nijman SM, Dirac AM, *et al.*** Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. *Nature*. 2003; 424: 797–801.
28. **Brummelkamp TR, Bernards R, Agami R.** A system for stable expression of short interfering RNAs in mammalian cells. *Science*. 2002; 296: 550–3.
29. **Ovaa H, Kessler BM, Rolen U, *et al.*** Activity-based ubiquitin-specific protease (USP) profiling of virus-infected and malignant human cells. *Proc Natl Acad Sci USA*. 2004; 101: 2253–8.
30. **Korinek V, Barker N, Morin PJ, *et al.*** Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science*. 1997; 275: 1784–7.
31. **Hecht A, Vleminckx K, Stemmler MP, *et al.*** The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *EMBO J*. 2000; 19: 1839–50.

32. **Takemaru KI, Moon RT.** The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J Cell Biol*. 2000; 149: 249–54.
33. **Borodovsky A, Ovaa H, Kolli N, *et al.*** Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chem Biol*. 2002; 9: 1149–59.
34. **Soboleva TA, Jans DA, Johnson-Saliba M, *et al.*** Nuclear-cytoplasmic shuttling of the oncogenic mouse UNP/USP4 deubiquitylating enzyme. *J Biol Chem*. 2005; 280: 745–52.
35. **Hood JK, Silver PA.** In or out? Regulating nuclear transport. *Curr Opin Cell Biol*. 1999; 11: 241–7.
36. **Yang SH, Sharrocks AD, Whitmarsh AJ.** Transcriptional regulation by the MAP kinase signaling cascades. *Gene*. 2003; 320: 3–21.
37. **Zeng YA, Rahnama M, Wang S, *et al.*** Drosophila Nemo antagonizes BMP signaling by phosphorylation of Mad and inhibition of its nuclear accumulation. *Development*. 2007; 134: 2061–71.
38. **Tran H, Hamada F, Schwarz-Romond T, *et al.*** Trabid, a new positive regulator of Wnt-induced transcription with preference for binding and cleaving K63-linked ubiquitin chains. *Genes Dev*. 2008; 22: 528–42.
39. **Guillen-Ahlers H.** Wnt signaling in renal cancer. *Curr Drug Targets*. 2008; 9: 591–600.
40. **Imreh S, Klein G, Zabarovsky ER.** Search for unknown tumor-antagonizing genes. *Genes Chromosomes Cancer*. 2003; 38: 307–21.
41. **Kok K, Naylor SL, Buys CH.** Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. *Adv Cancer Res*. 1997; 71: 27–92.
42. **Maitra A, Wistuba II, Washington C, *et al.*** High-resolution chromosome 3p allelotyping of breast carcinomas and precursor lesions demonstrates frequent loss of heterozygosity and a discontinuous pattern of allele loss. *Am J Pathol*. 2001; 159: 119–30.
43. **Mohinta S, Wu H, Chaurasia P, *et al.*** Wnt pathway and breast cancer. *Front Biosci*. 2007; 12: 4020–33.
44. **Zabarovsky ER, Lerman MI, Minna JD.** Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. *Oncogene*. 2002; 21: 6915–35.
45. **Dehan E, Ben-Dor A, Liao W, *et al.*** Chromosomal aberrations and gene expression profiles in non-small cell lung cancer. *Lung Cancer*. 2007.
46. **Uematsu K, He B, You L, *et al.*** Activation of the Wnt pathway in non small cell lung cancer: evidence of dishevelled overexpression. *Oncogene*. 2003; 22: 7218–21.
47. **Virmani AK, Rathi A, Sathyanarayana UG, *et al.*** Aberrant methylation of the adenomatous polyposis coli (APC) gene promoter 1A in breast and lung carcinomas. *Clin Cancer Res*. 2001; 7: 1998–2004.
48. **Frederick A, Rolfe M, Chiu MI.** The human UNP locus at 3p21.31 encodes two tissue-selective, cytoplasmic isoforms with deubiquitinating activity that have reduced expression in small cell lung carcinoma cell lines. *Oncogene*. 1998; 16: 153–65.
49. **Velazquez-Fernandez D, Laurell C, Geli J, *et al.*** Expression profiling of adrenocortical neoplasms suggests a molecular signature of malignancy. *Surgery*. 2005; 138: 1087–94.
50. **Gupta K, Chevrette M, Gray DA.** The Unp proto-oncogene encodes a nuclear protein. *Oncogene*. 1994; 9: 1729–31.
51. **Yamada M, Ohnishi J, Ohkawara B, *et al.*** NARF, an Nemo-like kinase (NLK)-associated ring finger protein regulates the ubiquitylation and degradation of T cell factor/lymphoid enhancer factor (TCF/LEF). *J Biol Chem*. 2006; 281: 20749–60.
52. **Yamamoto H, Ihara M, Matsuura Y, *et al.*** Sumoylation is involved in beta-catenin-dependent activation of Tcf-4. *EMBO J*. 2003; 22: 2047–59.
53. **Waltzer L, Bienz M.** Drosophila CBP represses the transcription factor TCF to antagonize Wingless signalling. *Nature*. 1998; 395: 521–5.