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Comparison of substrate specificity of the ubiquitin ligases Nedd4 and Nedd4-2 using proteome arrays

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Target recognition by the ubiquitin system is mediated by E3 ubiquitin ligases. Nedd4 family members are E3 ligases comprised of a C2 domain, 2–4 WW domains that bind PY motifs (L/PPxY) and a ubiquitin ligase HECT domain. The nine Nedd4 family proteins in mammals include two close relatives: Nedd4 (Nedd4-1) and Nedd4L (Nedd4-2), but their global substrate recognition or differences in substrate specificity are unknown. We performed *in vitro* ubiquitylation and binding assays of human Nedd4-1 and Nedd4-2, and rat-Nedd4-1, using protein microarrays spotted with ~8200 human proteins. Top hits (substrates) for the ubiquitylation and binding assays mostly contain PY motifs. Although several substrates were recognized by both Nedd4-1 and Nedd4-2, others were specific to only one, with several Tyr kinases preferred by Nedd4-1 and some ion channels by Nedd4-2; this was subsequently validated *in vivo*. Accordingly, Nedd4-1 knockdown or knockout in cells led to sustained signalling via some of its substrate Tyr kinases (e.g. FGFR), suggesting Nedd4-1 suppresses their signalling. These results demonstrate the feasibility of identifying substrates and deciphering substrate specificity of mammalian E3 ligases.
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Introduction

Ubiquitylation of proteins targets them for degradation or other cellular fates, such as endocytosis, vesicular sorting, histone modifications and others, and has been implicated in numerous human diseases (Schwartz and Ciechanover, 2008). The ubiquitylation pathway involves the action of three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase), which is responsible for substrate recognition. Two major classes of E3s are the RING E3s and the HECT E3s, with HECT E3s directly transferring ubiquitin from their HECT domain onto Lys residues on the substrate (Glickman and Ciechanover, 2002). A prominent family within the HECT class of ubiquitin ligases is the Nedd4 family (Rotin and Kumar, 2009; Yang and Kumar, 2009). Nedd4 (neuronal precursor cell developmentally downregulated 4) proteins are comprised of an N-terminal C2 domain, 2–4 WW domains that bind PY motifs (LPxY or PPxY—(Staub *et al*, 1996; Kanelis *et al*, 2001, 2006; Kasanov *et al*, 2001)) and a C-terminal HECT domain. Although the yeast *Sachharomyces cerevisiae* contains a single Nedd4 member, Rsp5, mammals possess nine Nedd4 members, with Nedd4(Nedd4-1) and Nedd4L(Nedd4-2) being most closely related to each other (Kamynina *et al*, 2001b).

One of the major obstacles to studying E3 ubiquitin ligases is the identification of their substrates, and many studies to date have focused on description of a few specific substrates. For example, Nedd4-2 was identified as a regulator of the epithelial Na^+ channel, ENaC (Abriel *et al*, 1999; Harvey *et al*, 2001; Kamynina *et al*, 2001a), as well as of other ion channels (Abriel and Staub, 2005). ENaC contains a PY motif in each of its three subunits, and mutations in these PY motifs cause Liddle syndrome, a hereditary hypertension caused by elevated ENaC activity and numbers at the plasma membrane (Firsov *et al*, 1996; Abriel *et al*, 1999; Lu *et al*, 2007). This increase in ENaC numbers is believed to result from the inability of the PY motif-mutated channel to bind Nedd4-2, leading to defective channel

endocytosis (Lu *et al*, 2007). Indeed, mice bearing the Liddle syndrome mutation in ENaC (Pradervand *et al*, 1999), or lacking Nedd4-2 expression (Shi *et al*, 2008), exhibit salt-induced hypertension similar to that observed in Liddle syndrome patients.

Nedd4-1 was recently shown to regulate cellular and animal growth by affecting the cell surface levels of the IGF-1R, through (indirectly) regulating the amount of Grb10 (Cao *et al*, 2008), but not PTEN (Cao *et al*, 2008; Fouladkou *et al*, 2008; Yang *et al*, 2008). In addition, it was also shown to regulate stability of Cbl-b (Yang *et al*, 2008), CNrasGEF (Pham and Rotin, 2001), MTMR4 (Plant *et al*, 2009) and several other proteins (see below), as well as sorting of LAPTM5 to lysosomes (Pak *et al*, 2006). Both Nedd4-1 and Nedd4-2 were also shown to regulate budding of viruses that contain PY motifs in their late domains, such as Ebola, rabies, HTLV-1, MMuLV and RSV (Morita and Sundquist, 2004).

Despite the identification of several targets for Nedd4-1 and Nedd4-2, global identification of their substrates on a proteomic scale was lacking, nor was it known how they differ in their substrate specificity. We have recently developed a high-throughput (HTP) method to identify *in vitro* ubiquitylation and binding substrates for Rsp5 using yeast protoarrays (protein chips) (Gupta *et al*, 2007). Here we used this technology to identify ubiquitylation substrates and binding partners for human Nedd4-1 (hNedd4-1) and hNedd4-2 (both possessing four WW domains), as well as rat rNedd4-1 (that lacks WW3 domain) using proteome arrays spotted with ~8200 human proteins, and compared the substrate specificity of these closely related Nedd4 family members.

Results

Ubiquitylation screens with Nedd4 proteins using human proteome arrays

To identify substrates for mammalian Nedd4 proteins on a proteomic scale, we focused on human Nedd4-1 and Nedd4-2 (hNedd4-1 and hNedd4-2, both possessing four WW domains), as well as on rat Nedd4-1 (rNedd4-1), which lacks the third WW domain (Figure 1A). The rat Nedd4-1 is almost identical to mouse Nedd4-1 (95% identity). The WW3 domain was previously shown to provide higher affinity interaction (low μM range) to the PY motifs (L/PPxY) of several substrates (e.g. ENaC and Comm), and was shown to bind well to both PPxY and LPxY (Kanelis *et al*, 2006). Rat and human Nedd4-1 are orthologues, sharing 82% sequence identity. hNedd4-2 is encoded by a separate gene from hNedd4-1 and has several splice isoforms: the one used in our study, which contains all the known domains of Nedd4 proteins, shares 67% sequence identity with hNedd4-1 (Figure 1A).

To identify and compare the *in vitro* substrates for these closely related family members, we used proteome arrays (protoarrays, Invitrogen) spotted in duplicates on PATH slides with 8222 unique human GST fusion proteins generated in insect cells (plus 1326 controls). Before performing the ubiquitylation assay on proteome arrays, we tested ubiquitylation by purified Nedd4 proteins (Figure 1B) of control GST fusion proteins spotted on PATH slides (Figure 2A), using several known Nedd4 substrates, as well as proteins that do not bind Nedd4 (negative controls). As seen in Figure 2A, purified hNedd4-1 incubated with purified E1, E2 (UbcH5b), FITC-labelled ubiquitin (FITC–Ub) and ATP yielded strong ubiquitylation of the known Nedd4 substrates (which contain PY motifs) but not of the negative controls that lack PY motifs. Similar results were obtained with hNedd4-2 and rNedd4-1. Once established, we performed the ubiquitylation assays on the protoarrays, each assay carried out on duplicate slides (Figure 3 and Supplementary Figure S1) to ensure reproducibility. Indeed, as seen in Figure 3A for hNedd4-1, a close-up look at a sub-array region revealed a similar pattern of ubiquitylation on the replicate slides.

As described in the Materials and methods section, a positive hit was defined as a protein for which ubiquitylation was >2 s.d. above mean background in all four spots (i.e. duplicate spots in the each of the two arrays). Using this definition, we identified 154 hits for hNedd4-1, 107 for hNedd4-2 and 92 for rNedd4-1 (Supplementary Table SI). We termed these the 'relaxed' data sets, as described earlier (Gupta *et al*, 2007). From these, we selected the top hits ('high confidence' data set), approximately 50 proteins that were most highly ubiquitylated (Table I).

Features of the top hits

The most obvious common feature of the top hits is the presence of PY motif(s) in most of the substrates for all three Nedd4 proteins (Table I), in line with the known recognition of this motif by the WW domains of Nedd4 proteins. The presence of a PY motif alone in the protein, however, is not sufficient to promote its ubiquitylation, because ~700 of the proteins present on the array possess a PY motif (and Lys residues), yet only ~50 were strongly ubiquitylated. This suggests that this *in vitro* on-chip ubiquitylation assay exhibits substrate specificity. This conclusion is also supported by the finding of differences in substrate preference between the Nedd4-1 and Nedd4-2 proteins (see below). An analysis of the top hits that contain PY motifs (most of the top hits) revealed that for hNedd4-1 and hNedd4-2, both PPxY and LPxY sequences were recognized, whereas for rNedd4-1, which lacks WW3 (a domain previously shown to bind well to LPxY (Kanelis *et al*, 2006)), most substrates primarily contained a PPxY sequence (Figure 4). In addition, a hydrophobic residue (often Phe) seemed to be a preferred residue at the 'x' position in the PY motif (L/PPxY).

Given that only a few substrates for mammalian Nedd4 proteins have been identified to date (see Discussion section), most of the substrates we identified here are new, and include numerous signalling proteins, ion channels, proteins involved in the ubiquitin system, in RNA processing and other cellular functions. A major goal of this study was to compare substrate specificity between hNedd4-1 and hNedd4-2. Table II lists substrates that were recognized by both of these closely related E3s, as well as those unique to each. Interestingly, we found several tyrosine kinases and other signalling proteins among the substrates of hNedd4-1, whereas hNedd4-2 seemed to ubiquitylate several ion channels. Although Nedd4-1 from rat and human are orthologues, hNedd4-1 possesses WW3 domain, which is absent from rNedd4-1. As seen in Supplementary Table SII, there were several substrates that

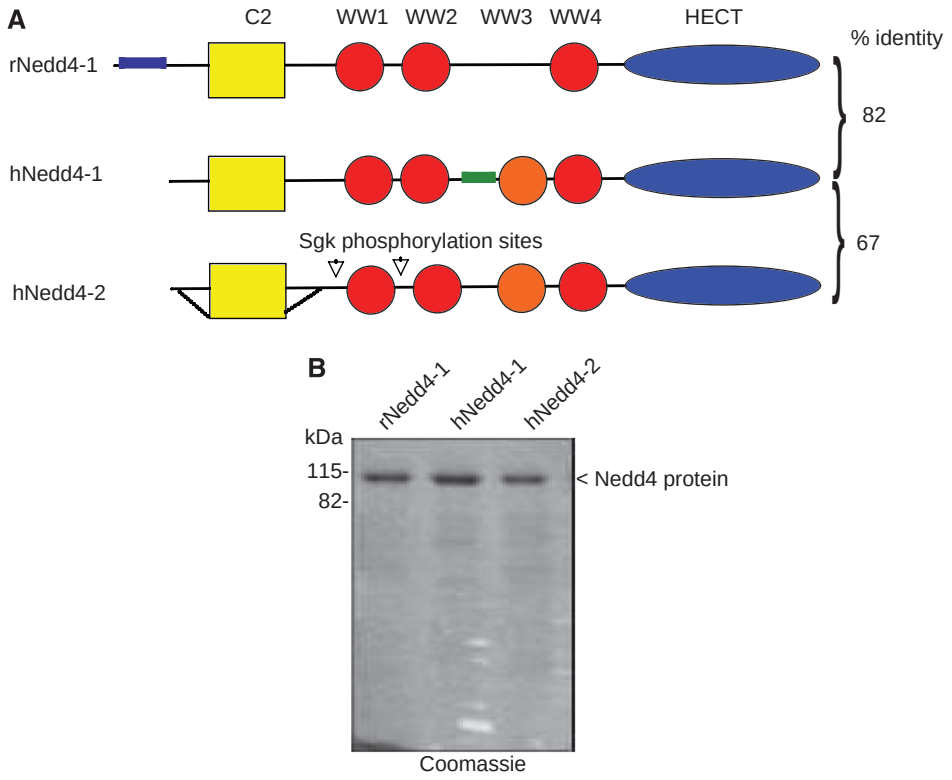

Figure 1 Nedd4 proteins. (**A**) Schematic representation of rNedd4-1, hNedd4-1 and hNedd4-2 (not to scale), with % amino-acid identity between them depicted at the right hand side. The presence of WW3 domain in hNedd4-1 and hNedd4-2, but not rNedd4-1, should be noted. Additional unique regions are shown as blue and green thick lines. (**B**) Coomassie staining of purified Nedd4 proteins used for the protoarray screen.

were recognized by hNedd4-1 but not by rNedd4-1, suggesting that WW3 domain may contribute to the interactions with these substrates.

Validation of hits by *in vivo* ubiquitylation analysis

To validate the ubiquitylation hits and compare substrate specificity *in vivo*, we performed *in vivo* ubiquitylation analysis on several (5 or 6 each) of the top hits for Nedd4 proteins. For this, HEK293T cells were transfected with the indicated Flag-tagged substrate and either wild-type (WT) or catalytically inactive Nedd4 protein (Nedd4(CS), bearing a Cys-to-Ser mutation in the HECT domain), and in some cases also His-Ub. We also tested control proteins that were not hits in our screen. Lysates from the transfected cells were boiled in SDS to remove putative substrate-associated proteins. The SDS was diluted and substrates were immunoprecipitated with Flag antibodies and blotted with anti-ubiquitin or anti-His antibodies to detect substrate ubiquitylation. Parallel lysates (without boiling in SDS) were used for co-immunoprecipitation (co-IP) experiments to verify binding between Nedd4 proteins and their substrates. As exemplified in Figures 5 and 6 and summarized in Table III, the five controls tested (non-hits) indeed were not ubiquitylated in HEK293T cells, nor did they bind any of the Nedd4 proteins (Figure 5D–F and Table III). In contrast, WBP2 (previously shown to bind WW domains (Chen *et al*, 1997)) and the transcription factor Ying-Yang1 (YY1) (Figure 5A–C and Table III), both hits for Nedd4-1 and Nedd4-2 proteins, were indeed strongly ubiquitylated by these Nedd4s and were also able to bind them. Interestingly, several proteins that were unique substrates for Nedd4-1 or Nedd4-2 in our screen seem to be unique substrates *in vivo* as well (Figure 6). For example, RTF1 (large subunit of RNA Pol II) was ubiquitylated by hNedd4-1 but not hNedd4-2, and conversely, the calcium channel CACNB1 and the activin receptor ACVR1B were *in vivo* substrates for hNedd4-2 but not hNedd4-1 (Figure 6B and and Table III). Moreover, annexin A9 (AXNA9) and aquaporin AQP9 were both unique substrates for rNedd4-1 in our screen as well as in our *in vivo* validation study (Figure 6A and Table III). Although we did not detect ubiquitylation of MAP2 (which was a rNedd4-1 substrate in our screen) *in vivo*, it nevertheless co-immunoprecipitated with rNedd4-1 in cells (Table III). Overall, all the proteins that were not hits in the screen were not ubiquitylated *in vivo* either, whereas ~70% of our hits (substrates) were found to be substrates *in vivo* as well, under our experimental conditions.

To further validate the physiological importance of some of our hits, we studied signalling downstream of several growth factor receptors that were substrates for Nedd4-1 in our screen, using both RNA-mediated knockdown in HEK293T cells and our recently generated Nedd4-1$^{-/-}$ MEFs (Fouladkou *et al*, 2008). We first focused on FGF signalling as FGFR1 and FGFR2 (Table I and Figure 6) were identified as Nedd4-1 substrates in our screen. As seen in Figure 7A, knockdown of hNedd4-1 in HEK293T cells transfected with FGFR1 led to FGF-mediated sustained activation of the FGFR1 in the Nedd4-1 knockdown

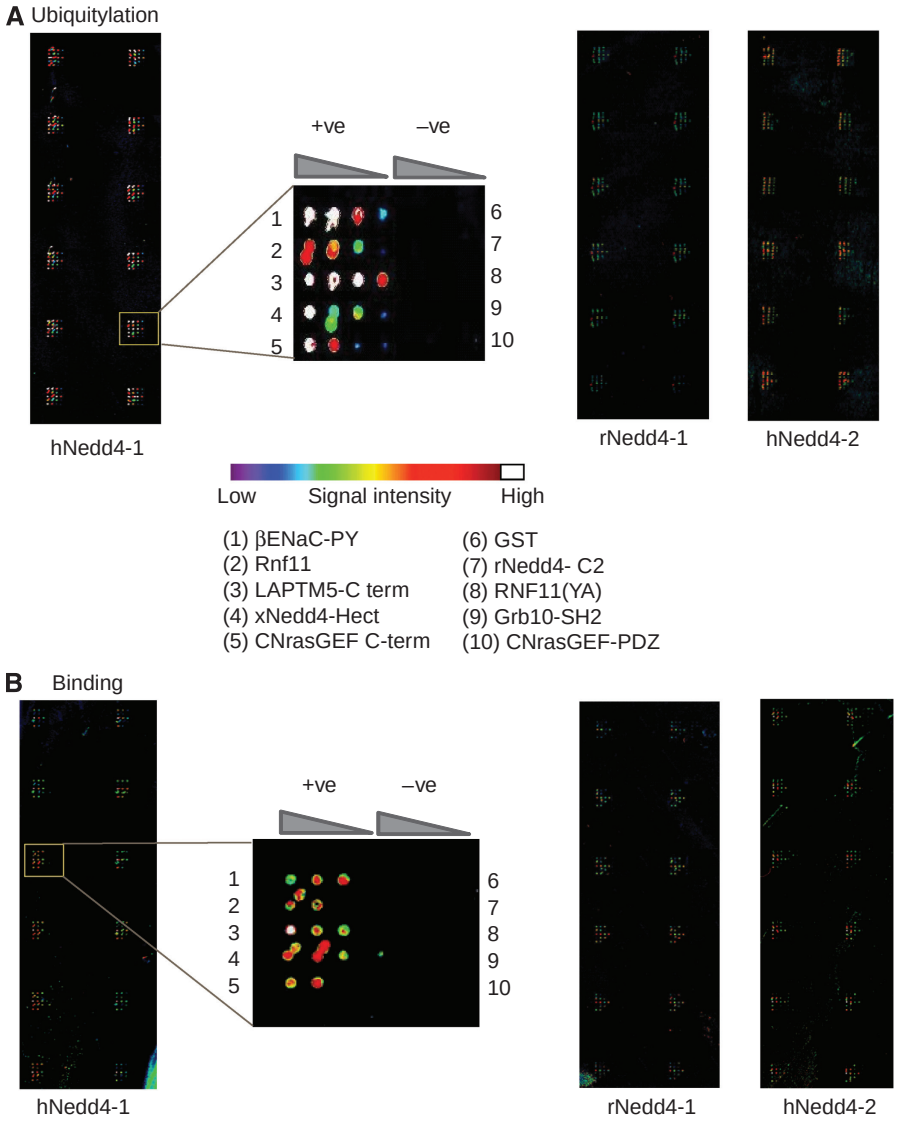

Figure 2 Control protein arrays tested for ubiquitylation and binding of known substrates of Nedd4 proteins. (**A**) Decreasing concentration of the indicated proteins (1:5, 1:10, 1:50, i.e. 0.8–0.016 ng) were spotted multiple times on control PATH slides and the slides incubated with the indicated Nedd4 proteins (E3), E2 (UbcH5b), E1, FITC–Ub and Mg–ATP to test for substrate ubiquitylation (see Materials and methods section). An example of one sub-array incubated with hNedd4-1 is enlarged. (**B**) Parallel control protein arrays incubated with Alexa647–Nedd4 proteins to test for their binding to the spotted test proteins. Positive control proteins (all containing PY motifs and previously shown to be ubiquitylated by Nedd4 proteins): (1) a region within βENaC C terminus; (2) Rnf11; (3) the C terminus of LAPTM5; (4) the *Xenopus* xNedd4-HECT domain; (5) The C terminus of CNrasGEF. Negative controls (which lack PY motifs): (6) GST; (7) the C2 domain of rNedd4-1; (8) RNF11 with mutated PY motif; (9) the SH2 domain of Grb10 (which was previously shown to bind the C2 domain of rNedd4-1 but not to be ubiquitylated by it (Morrione *et al*, 1999)); (10) the PDZ domain of CNrasGEF.

(KD) cells for at least 2 h, along with enhanced signalling via Akt (pAkt) and Erk (pErk). As expected, cells in which Nedd4-1 was not knocked down exhibited transient activation of the receptor (as well as Akt and Erk), which started to diminish within 30 min and was undetectable by 2 h. Moreover, a similar FGF-mediated sustained activation of Akt and Erk was observed in the Nedd4-1$^{-/-}$ MEFs, but not in WT MEFs (Figure 7B). Such sustained activation of Erk was also obtained after EGF stimulation (Supplementary Figure S5). These results suggest that Nedd4-1 negatively regulates signalling via FGFR and EGFR (and possibly other RTKs).

A summary of the interaction/signalling networks in which our substrates participate is depicted in Figure 8 and discussed in the Discussion section below.

Binding of Nedd4 proteins to proteome arrays

To compare the ubiquitylation results with the ability of Nedd4 proteins to bind their substrates, we tested binding of Alexa647-conjugated Nedd4 proteins to parallel human protoarrays tested in duplicates (Figure 3B and Supplementary Figure S2) after initially establishing the binding assay on test protein arrays (Figure 2B). As listed in Supplementary Table SI,

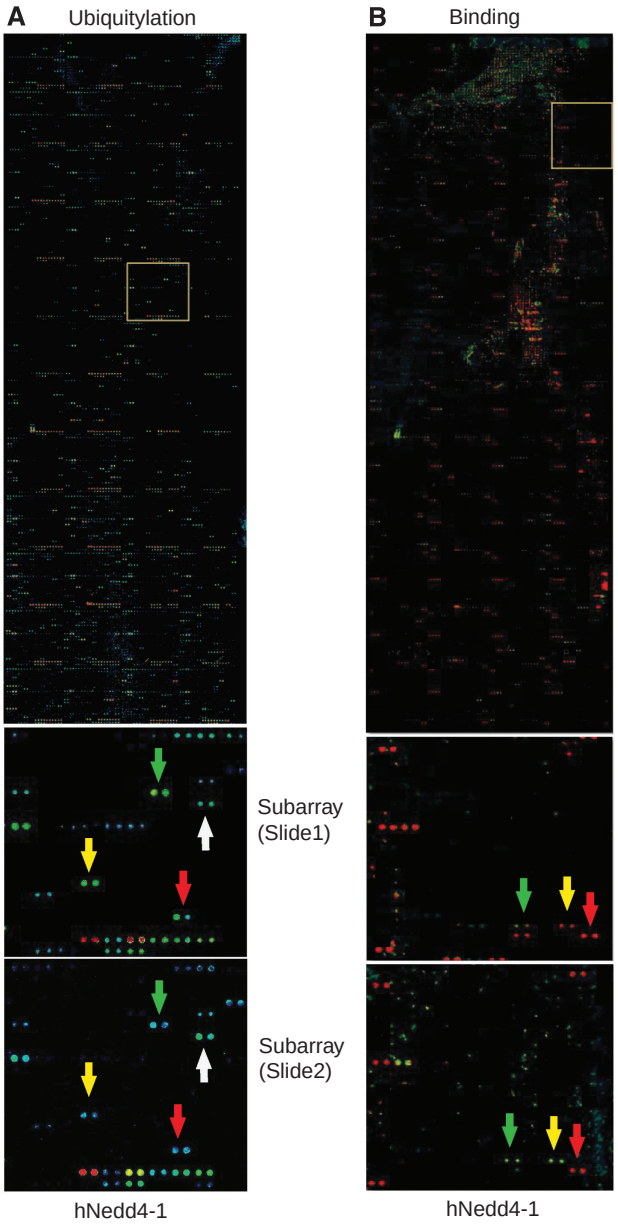

Figure 3 Nedd4 proteins-mediated ubiquitylation of the human proteome. (**A**) Ubiquitylation of proteins spotted on the human protoarray by hNedd4-1, with an enlargement of one sub-array from this array/slide and from a parallel array/slide. Arrows point to several hits, which appear to be identical between the two parallel arrays/slides. Similar analysis of ubiquitylation of the protoarrays by rNedd4-1 and hNedd4-2 is depicted in Supplementary Figure S1. (**B**) As in (A), analysis of binding of hNedd4-1 to proteins spotted on the human protoarray. Similar analysis of binding by rNedd4-1 and hNedd4-2 is depicted in Supplementary Figure S2.

85, 81 and 69 proteins met the cutoff criteria for being hits for hNedd4-1, hNedd4-2 and rNedd4-1 binding, respectively, with the top ~50 hits listed in Table IV. As seen for the ubiquitylation screen, most of the top hits contain a PY motif. Moreover, many of the hits (binding partners) were also hits in the ubiquitylation screen (Table IV). Much like our earlier observations with Rsp5 (Gupta *et al*, 2007), the binding data set appear smaller than the ubiquitylation data set, suggesting that detection of binding is less sensitive than that of ubiquitylation.

Although many of the top hits identified in our ubiquitylation and binding screens possess PY motifs, some do not, suggesting they interact with Nedd4 proteins by other means. In an effort to identify alternative recognition sequences in the substrates, we analysed bioinformatically all the hits of our screens for common sequences other than the PY motif. Our analysis identified a short sequence, RxxQE that was the next most common motif after the PY motif (present in ~16–18% of the hits). This sequence, however, did not appear to cluster (i.e. be more prevalent) among the top hits. Moreover, mutation of the Arg, Gln and Glu residues of this motif to Ala in two of our validated hits that do not possess PY motifs, CACNB1 and ANXA9, did not prevent binding to, or ubiquitylation by, Nedd4 proteins (Supplementary Figure S3). Thus, other yet unknown sequence(s) must be involved in substrate recognition in the absence of a PY motif.

Discussion

The screens we describe here to identify substrates for the closely related mammalian Nedd4 family members, Nedd4-1 and Nedd4-2, not only allow the identification of their new substrates, but also the comparison of their substrate specificity. The validation of some of these substrates also confirmed this specificity. For example, whereas the RNA Pol II subunit RTF1 and the Tyr kinase receptor FGFR1 were substrates for Nedd4-1 proteins, the Ca^{2+} channel CACNB1 and the activin receptor ACVR1B were unique substrates for Nedd4-2. Overall, while 25 of the top hits were shared between hNedd4-1 and hNedd4-2, 19 were unique to hNedd4-1 and 13 to hNedd4-2. Many of the top substrates (17) identified for rNedd4-1 were also substrates for hNedd4-1, as expected. However, hNedd4-1 had additional 26 substrates that were not found in the rNedd4-1 screen, suggesting that its added WW3 domain might have contributed to the recognition of these additional substrates.

Earlier and recent studies identified RNA Pol II as substrate for the Rsp5 in yeast and Nedd4-1 in mammals (Huibregtse *et al*, 1997; Anindya *et al*, 2007; Somesh *et al*, 2007), in accord with its identification here as a substrate for Nedd4-1. Interestingly, although in our current screen we identified numerous Ser/Thr kinases as substrates for both Nedd4-1 and Nedd4-2 proteins, we noticed a significant number of Tyr kinases that are substrates for Nedd4-1 proteins only (Table II and Figure 8). These include the PDGFR(A,B), EGFR, FGFR1 and 2, Flt1, Fyn, TEC, BLK and BTK, with some of these possessing PY motifs (e.g. the PDGFR-A, Flt1 and TEC). We also validated the ability of Nedd4-1 to bind and ubiquitylate FGFR1 in mammalian cells (Figure 6), in line with our observation of sustained signalling via FGF in Nedd4$^{-/-}$ MEFs or after knockdown of Nedd4-1 in HEK293T cells (Figure 7), and supporting the idea that Nedd4-1 is a negative regulator of the FGFR. The EGFR, which was identified here as both a binding partner and a substrate for Nedd4-1, was previously shown to be downregulated in a process that involves Nedd4-1 (Katz *et al*, 2002), an observation supported

Table I Top hits (substrates) of ubiquitylation screen

Rat Nedd4-1 substrates		Human Nedd4-1 substrates		Human Nedd4-2 substrates	
Protein name	PY Motif	Protein name	PY Motif	Protein name	PY Motif
FOXJ2	PPLY	UBOX5, tv.2	PPVY	KCNB1	LPYY
PLSCR3	PPPY	NTRK1, tv.3	PPVY	MAPKAPK3	PPFY
WBP2	PPGY, PPPY, PPYY	RANBP10	PPFY	CLIC2	PPRY
ABL1, tv.a	PPFY	PDGFRA	LPQY	EFEMP2	PPGY
FGF12		CAMK1D, tv.1	PPFY	HGS	PPEY
FKBP3	PPKY	ABL2, tv.a	PPFY	NDFIP2	PPPY, PPPY, LPTY
ANXA9		MAPKAPK3	PPFY	MAP3K3	PPGY
EIF5	PPTY	YY1	PPDY	VDAC2	PPSY
MAPKAPK5, tv.1	PPFY	WBP2	PPGY, PPPY, PPPY	ZDHHC3	PPPY
YY1	PPDY	RTF1	PPNY	CAMK1D, tv.1	PPFY
MAP2, tv.2	PPSY	EGFR, tv.1		RANBP10	PPFY
PKC	LPEY	ANKRD13D		CDK5	LPDY
ZAK	PPNY	TRIM52	PPPY	ABL1, tv. A	PPFY
SLC23A2	PPWY,LPIY	TRIM44		MAGEB1, tv.1	PPRY
BEAN	PPGY, PPPY, LPPY	BAIAP2, tv.2	PPDY	EPB49	PPIY, LPAY
AKT3	LPFY	PLK2		AICDA	LPLY
AQP9	LPFY	CUEDC1	PPVY, PPAY	MARK2, tv.3	LPDY
MAPKAPK3	PPFY	FLT1	PPDY	KCNAB1, tv.1	
FLT1	PPDY	RAD51AP1		FKBP3	PPKY
BRAF		SAMSN1		DAPK3	
TIMM10	PPHY	LUC7L2		BRAF	
SERPIND1		CLIC2	PPRY	MAP3K5	PPFY
CUEDC1	PPVY, PPAY	SMAD3	PPGY	DDX54	
CAMK1D, tv.2	PPFY	CALCOCO1	LPPY	ZAK	PPNY
SGK2	PPFY	FYN, tv.1		KCNAB3	LPEY
MAP3K3	PPGY	MAP3K3	PPGY	NTRK1, tv.3	PPVY
TEC	PPEY	ABL1, tv. a	PPFY	CUEDC1	PPVY, PPAY
CDK5	LPDY	CDK5	LPDY	RPS6KB2	LPPY
AKT1	LPFY	STK31, tv.1		ABL2, tv.a	PPFY
PDGFRA	LPQY	AKT1	LPFY	CACNB1, tv.1	
ACAN	LPNY	NDFIP2	PPPY, PPPY	YY1	PPDY
MAP3K8		MARK2, TV.3	LPDY	MAP3K2	PPGY
TRPT1, tv.2	LPKY	KIAA1189, tv.2	LPHY	BBX	LPQY
BLK		SGK2, tv.2	PPFY	PRKX	PPVY
PKCD		AKT1, tv.3	LPFY	ACVR1B, tv.1	LPYY
FYN, tv.1		PKX	PPVY	ADAP2	
MAP2K1		MAP3K5	PPFY	BMPR1A	LPYY
BMPR1B		MAP3K2	PPGY	AKT3, tv.1	LPFY
C14orf104		TOM1L2, tv.1	PPPY	MINK1, tv.4	
MAPK12		RPS6KB1	LPPY	AURKC, tv.1	
RGS3, tv.4		C11orf63, tv.2		CCDC55, tv.1	
KLC4, tv.1		TOM1		WIBG	
NTRK2, tv.b		MARKL1		RPS6KB2, tv.2	LPPY
BTK		SGK	PPFY	SGK2, tv.2	PPFY
UCHL5IPn		RPS6KA3	LPQY	UBADC1	LPSY
EGFR, tv.1		EGFR, tv.1		RPL30	
POLR3D		PDGFRB		MERTK	
CSNK2A1		FGFR1, tv.7		SRMS	
STK16, tv.2		SPANXN3		SERPINF1	
ADRBK1		GBA	LPLY	DYRK3, tv.2	
		AKT3	LPFY		
		DEAD			
		FGFR2, tv.1	LPQY		
		GRK7			

PY motif; tv: transcript variant.

by our finding of enhanced EGF-mediated signalling in Nedd4-1$^{-/-}$ MEFs (Supplementary Figure S5). On the basis of the known role of tyrosine kinases in the regulation of cell proliferation, the identification of Nedd4-1 as a regulator of cellular and animal growth on the basis of mouse knockout studies (Cao *et al*, 2008; Fouladkou *et al*, 2008), and the results shown here (Figures 6 and 7), it is likely that some cellular tyrosine kinases may be substrates for Nedd4-1. Indeed, the IGF-1R was previously shown to be endocytosed in a Nedd4-1-dependent manner, by the adaptor protein Grb10 that binds the C2 domain of Nedd4-1 (Morrione *et al*, 1999; Vecchione *et al*, 2003). However, recent study using Nedd4-1 KO mice suggested that Nedd4-1 positively regulates cell surface stability of the IGF-1R, opposite to the above studies (Cao *et al*, 2008). Further studies are needed to resolve this discrepancy.

As indicated above, our screen with hNedd4-2 identified numerous ion channels as its *in vitro* substrates (top hits, see

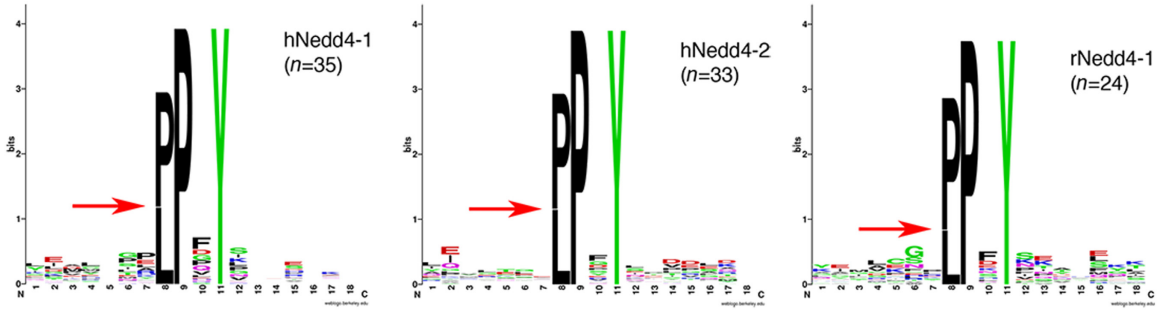

Figure 4 Sequence logos for the PY motif region. Sequence logos depicting the region around the L/PPxY motifs in hits containing one such motif from screens for ubiquitylation substrates of hNedd4-1, hNedd4-2 and rNedd4-1. Red arrows indicate the border between the Pro and Leu in the first position of the L/PPxY motif, and show that a Leu in this position is relatively more abundant in substrates for hNedd4-1 and hNedd4-2 than in substrates for rNedd4-1. The number of total sequences (*n*) used to generate the logo is indicated in each image.

Table II and Figure 6), including the voltage-gated K^+ channels KCNB1, KCNAB1, KCNAB3; the voltage-gated anion channel VDAC2; the Ca^{2+} channel CACNB1; and the Cl^- channel CLIC2, with most of these (KCNB1, KCNAB3, VDAC and CLIC2) containing PY motifs (CLIC2 was also a substrate for Nedd4-1). Nedd4-2 was previously shown to regulate cell surface levels of ENaC (Abriel *et al*, 1999; Kamynina *et al*, 2001a; Lu *et al*, 2007). Subsequent studies also documented similar regulation of other ion channels and transporters by Nedd4-2, including the cardiac voltage-gated Na^+ channels; Nav1.5/SCN5a (Abriel *et al*, 2000; van Bemmelen *et al*, 2004); the neuronal voltage-gated Na^+ channel (Fotia *et al*, 2004); the voltage-gated K^+ channels KCNQ1, KCNQ2/3, KCNQ3/5 (Ekberg *et al*, 2007; Jespersen *et al*, 2007), Kv1.3/KCNA3, Kv1.5/KCNA5 (Henke *et al*, 2004; Boehmer *et al*, 2008), Kv4.3/KCND3; the Cl^- channels ClC2, ClC5 (Hryciw *et al*, 2004; Palmada *et al*, 2004a), ClC-Ka/barttin (Embark *et al*, 2004) and the Tweety family TTYH2 and TTYH3 (He *et al*, 2008); the phosphate transporter NaPi-IIb (Palmada *et al*, 2004b); the excitatory glutamate transporters EAAT1, EAAT2 and EAAT4 (Boehmer *et al*, 2003, 2006; Rajamanickam *et al*, 2007) and others. Unfortunately, these channels and transporters (including ENaC) were not present on the protoarray.

Overall, several substrates that we identified in our screens were previously reported to bind to or serve as substrates for Nedd4 proteins. These include Rtf1 (RNA Pol-II subunit), Sgk 1 and 2, some Akt isoforms, the EGFR, the neurotrophin receptor Trk, Nfip2, WWBP2, Bean, the IGF-1R and insulin receptor (the latter two present in our relaxed data set). Thus, the ubiquitylation-on-chip we described here and previously (Gupta *et al*, 2007) is likely a good predictive tool for the identification of *in vivo* substrates for E3 ligases. Indeed, several recent studies published simultaneously or subsequent to our screen with Rsp5 confirmed our findings. For example, of our top hits, Sna3 was found to associate with Rsp5 *in vivo*, which was required for its MVB sorting (McNatt *et al*, 2007; Oestreich *et al*, 2007; Stawiecka-Mirota *et al*, 2007; Watson and Bonifacino, 2007), and Ear1p (YMR171C), itself ubiquitylated by Rsp5 (which targets it to the vacuole), was shown to serve as an adaptor for Rsp5-mediated sorting of cargo to MVBs (Leon *et al*, 2008). Moreover, recent studies identified nine arrestin-related yeast proteins (ARTs) that serve as adaptors for Rsp5-mediated ubiquitylation and endocytosis of numerous plasma membrane proteins (Lin *et al*, 2008); Five of these proteins (Ygr068c, Rod1, Rog3, Aly1, Aly2 (i.e. Art5, Art4, Art7, Art6 and Art3)), which are also ubiquitylated by Rsp5 (a process required for their function), were previously identified in our Rsp5 substrate screen (Gupta *et al*, 2007). Although all these proteins were ubiquitylated by Rsp5, some serve an adaptor function, raising the possibility that the screen can also uncovers adaptors for Nedd4 family proteins, which themselves become ubiquitylated.

Many of the substrates for mammalian Nedd4 proteins that we identified in this screen are new. Bioinformatic analysis of the binding partners for our top substrate hits reveals some interesting patterns (Figure 8). A large cluster of signalling proteins connecting to tyrosine kinases that were substrates for Nedd4-1 (e.g. EGFR, FGFR1, PDGFR, TEC and Fyn) supports the idea that this E3 ligase is involved in regulating cellular proliferation and growth, as suggested earlier (Cao *et al*, 2008; Fouladkou *et al*, 2008). On the basis of our screen and our demonstration of enhanced signalling downstream of some of these receptors (e.g. FGFR, EGFR) after knockdown of Nedd4-1 in HEK293T cells or its knockout in MEFs (Figure 7), we propose that Nedd4-1 inhibits cellular signalling via at least some tyrosine kinase receptors. Interestingly, the tyrosine kinase network (Figure 8) also connects to Baiap2 (IRSp53), a PY-motif containing substrate for Nedd4-1 observed in our screen, which is known to interact with Cdc42 and the actin cytoskeleton, and to regulate lamellipodia and filopodia formation. This observation is consistent with our findings of abnormal cytoskeleton organization in our Nedd4-1 knockout MEFs (P Alberts and D Rotin, unpublished data). It is also apparent that both Nedd4-1 and Nedd4-2 proteins can ubiquitylate (and possibly regulate) components of the TGFβ/BMP/activin pathways, previously shown to be regulated by Smurf proteins (Attisano and Wrana, 2002). As Smurf1 and 2 are Nedd4 family relatives, substrate specificity of these family members needs to be analysed in detail, preferably *in vivo* as well, to properly delineate possible redundancy with Nedd4-1 and Nedd4-2. We also found several MAP kinases that seem to be substrates for both Nedd4-1 and

Table II Common and unique top substrates for hNedd4-1 and hNedd4-2

Unique hits		Similar hits
hNedd4-1	hNedd4-2	
U-box domain containing 5 (UBOX5)	Potassium voltage-gated channel, Shab-related subfamily, 1 (KCNB1)	Mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3)
Platelet-derived growth factor receptor, alpha polypeptide (PDGFRA)	EGF-containing fibulin-like extracellular matrix protein 2 (EFEMP2)	Nedd4 family-interacting protein 2 (NDFIP2)
Rtf1, Paf1/RNA polymerase II complex component (RTF1)	Hepatocyte growth factor-regulated tyrosine kinase substrate (HGS)	Mitogen-activated protein kinase kinase kinase 3 (MAP3K3)
BAI1-associated protein 2 (BAIAP2)	Voltage-dependent anion channel 2 (VDAC2)	RAN-binding protein 10 (RANBP10)
fms-related tyrosine kinase 1 (FLT1)	Zinc finger, DHHC domain containing 3 (ZDHHC3)	Cyclin-dependent kinase 5 (CDK5)
Glucosidase, beta; acid (includes glucosylceramidase) (GBA)	Erythrocyte membrane protein band 4.9 (EPB49)	v-abl Abelson murine leukaemia viral oncogene homologue 1 (ABL1), tv. a
SMAD family member 3 (SMAD3)	Activin A receptor, type IB (ACVR1B)	MAP/microtubule affinity-regulating kinase 2 (MARK2)
KIAA1189 (KIAA1189)	Potassium voltage-gated channel, shaker-related subfamily, beta 3 (KCNAB3)	Mitogen-activated protein kinase kinase kinase 5 (MAP3K5)
v-akt murine thymoma viral oncogene homologue 1 (AKT1), tv. 3	Bobby sox homologue (*Drosophila*) (BBX)	Mitogen-activated protein kinase kinase kinase 2 (MAP3K2)
Ribosomal protein S6 kinase, 90 kDa, polypeptide 3 (RPS6KA3)	Bone morphogenetic protein receptor, type IA (BMPR1A)	Protein kinase, X-linked (PKX)
Fibroblast growth factor receptor 2 (FGFR2)	Melanoma antigen family B (MAGEB1)	v-Akt murine thymoma viral oncogene homologue 3 (AKT3), tv. 1
Calcium binding and coiled-coil domain 1 (CALCOCO1)	Potassium voltage-gated channel, shaker-related subfamily, beta 1 (KCNAB1)	Chloride intracellular channel 2 (CLIC2)
Serum/glucocorticoid-regulated kinase (SGK)	Calcium channel, voltage-dependent, beta 1 subunit (CACNB1)	WW domain-binding protein 2 (WBP2)
Minichromosome maintenance complex component 7 (MCM7)		Tripartite motif-containing 52 (TRIM52)
Target of myb1-like 2 (chicken) (TOM1L2)		Serum/glucocorticoid regulated kinase 2 (SGK2)
Tripartite motif-containing 52 (TRIM52)		Calcium/calmodulin-dependent protein kinase ID (CAMK1D)
Epidermal growth factor receptor (EGFR)		Ubiquitin-associated domain containing 1 (UBADC1)
Platelet-derived growth factor receptor, beta polypeptide (PDGFRB)		YY1 transcription factor (YY1)
Fibroblast growth factor receptor 1 (FGFR1)		CUE domain-containing 1 (CUEDC1)
		Neurotrophic tyrosine kinase, receptor, type 1 (NTRK1), tv. 3
		v-abl Abelson murine leukaemia viral oncogene homologue 2 (ABL2), tv.a
		Ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (RPS6KB1) / polypeptide 2(RPS6KB2)
		Sterile alpha motif and leucine zipper-containing kinase AZK (ZAK)*
		v-Raf murine sarcoma viral oncogene homologue B1 (BRAF)*
		FK506-binding protein 3, 25 kDa (FKBP3)*

*Also substrates for rNedd4-1.
PY motif.

Nedd4-2 proteins (Figure 8). Interestingly, a component of the MAPK pathway, MEKK2, was previously found to be regulated *in vivo* by Smurf1 (Yamashita *et al*, 2005). In addition to the ability of Nedd4-2 to regulate ubiquitylation and cell surface stability of ion channels and transporters, which is also corroborated by our current screen, this E3 ligase has been demonstrated to regulate sorting of cargo proteins. Thus, it is interesting that one of its hits in our screen was Hrs/Hgs, a critical regulator of protein sorting in sorting endosomes that are known to recruit Nedd4 (e.g., see Katz *et al* (2002).

Most of the top hits in our ubiquitylation and binding screens contain PY motifs, as expected. We notice that while hNedd4-1 and hNedd4-2 (both possessing WW3 domain) recognized PPxY and LPxY sequences with equal frequency, rNedd4-1 (which lacks WW3 domain) exhibited preference towards PPxY. This may be explained by our previous findings that demonstrated strong ability of WW3 domain to bind LPxY, a sequence is poorly recognized by WW4 (Kanelis *et al*, 2006). Some of our identified substrates do not possess a PY motif, suggesting that other, yet unidentified motif(s) must be present in those PY-deficient substrates that recognize Nedd4 proteins.

In our previous study (Gupta *et al*, 2007), we applied an approach similar to that used here to identify substrates of

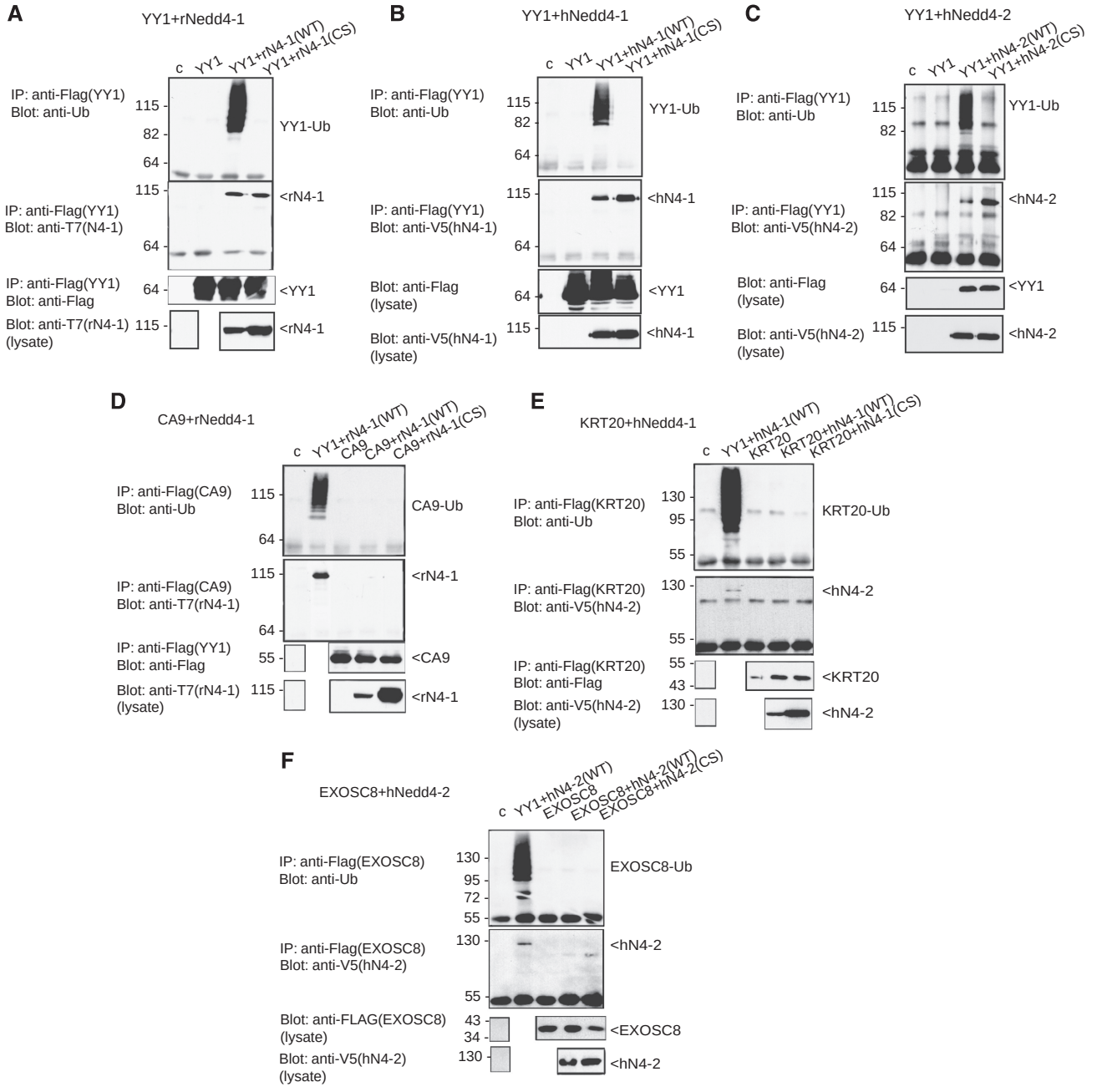

Figure 5 Validation of hits common to the Nedd4 proteins. HEK293T cells were transfected (or not) with the indicated cDNA for hit protein (Flag tagged) alone or with co-transfected Nedd4 protein (either WT or a catalytically inactive CS mutant). The Nedd4 protein was N-terminally tagged with either T7 (rNedd4-1) or V5 (hNedd4-1 and hNedd4-2). After transfections, half of the lysate was boiled in SDS, SDS diluted out, the hit protein immunoprecipitated and immunoblotted with anti-ubiquitin (Ub) antibodies to test for substrates ubiquitylation (upper panels). The other half of the lysate (not boiled) was used for co-IP analysis to determine interactions between the hit protein and the indicated Nedd4s (middle panels). Lower panels are controls for protein loading. (**A**–**C**) Example of a hit protein, Yang-Yang1 (YY1), that was common to all three Nedd4s tested, as indicated. (**D**–**F**) Negative controls: examples of proteins that were not hits in the screens: (D) carbonic anhydrase 9 (CA9); (E) keratin 20 (KRT20); and (F) exosome component 8 (EXOCSC8). A full list of all validation assays performed in provided in Table III.

Rsp5, the only Nedd4 family member found in *S. cerevisiae*. Of the 78 yeast genes with orthology to a human Nedd4 substrate, only 14 (18%) have been identified as Rsp5 interactors (derived from the BioGRID resource (Breitkreutz *et al*, 2008), which includes our previous protoarray data for Rsp5 (Gupta *et al*, 2007)). These include genes for: five ubiquitin-conjugating enzymes; three related cAMP-dependent protein kinases; three related casein kinases; an aurora kinase involved in microtubule organization during mitosis; and a serine/arginine-rich protein-specific kinase involved in regulating splicing. The limited overlap of substrates previously identified in our Rsp5 screen with those identified here is not completely unexpected. In our previous study, we identified fewer orthologous Rsp5 substrates with increasing evolutionary

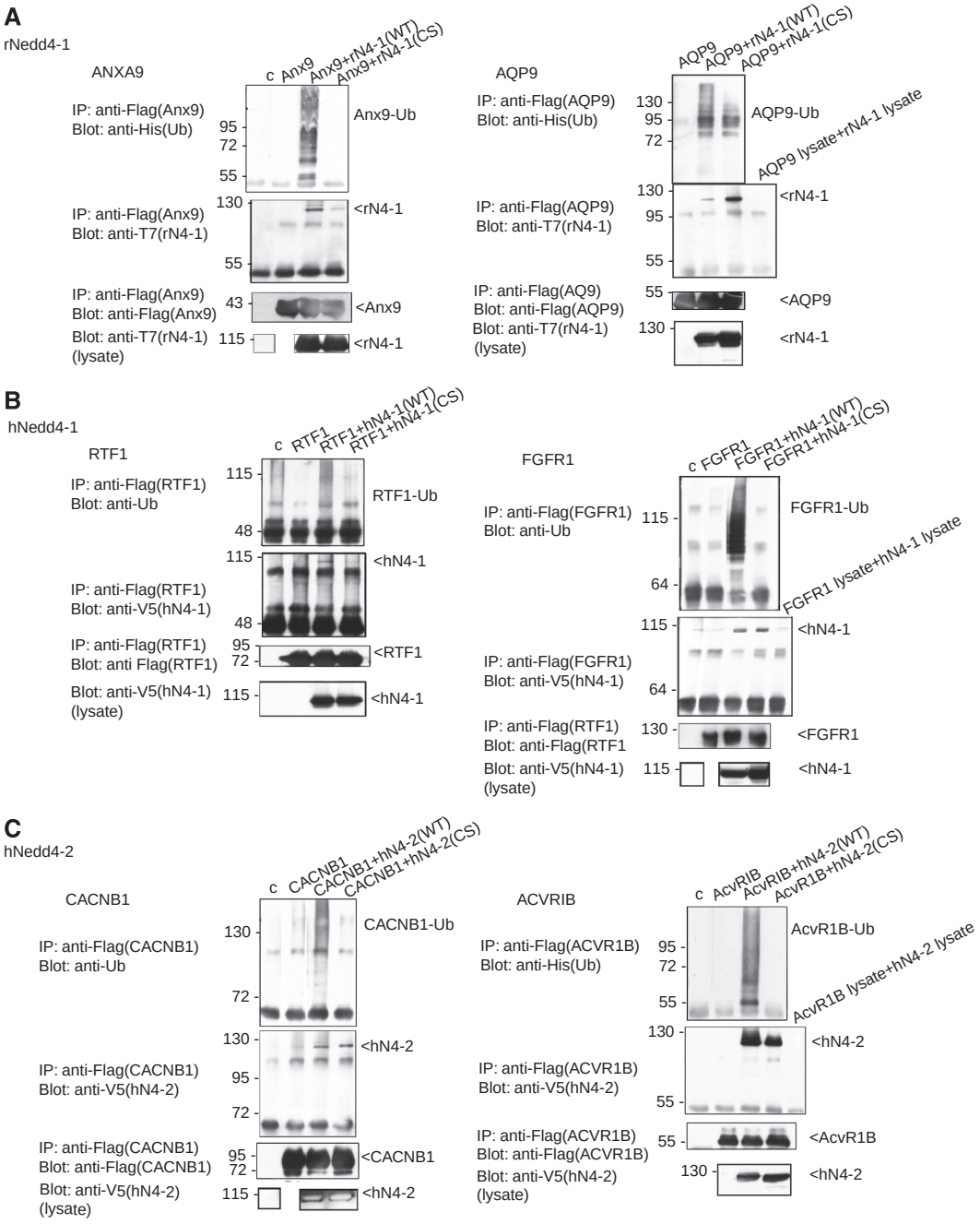

Figure 6 Validation of hits unique to each Nedd4 protein. HEK293T cells were co-transfected (or not) with the indicated hit cDNA and Nedd4 protein (WT or catalytically inactive CS mutant) and ubiquitylation and binding assays performed as described in the legend to Figure 5 and in the Materials and methods. (**A**) Ubiquitylation and binding by rNedd4-1 to annexin A9 (ANXA9, left panel) or aquaporin9 (AQP9, right panel). (**B**) Ubiquitylation and binding by hNedd4-1 to the large subunit of RNA Pol-II (RTF1, left panel) or the FGF receptor 1(FGFR1, right panel). (**C**) Ubiquitylation and binding by hNedd4-2 to the voltage-gated Ca^{2+} channel CACNB1 (left panel) or the activin receptor 1B (ACVR1B, right panel). A full list of all validation assays performed is provided in Table III. To ensure that binding between the Nedd4 proteins and their targets did not occur after cell lysis, independent transfections were also carried out on parallel plates and the lysates from these were mixed after lysis to test for co-IP. No co-IP was detected in any of them (see right-hand panels in A–C, middle blots).

distance. For example, only 17 of the top 49 Rsp5 substrates identified in our previous screen had orthologs in *Schizosaccharomyces pombe* with a PY motif. Strikingly, within our set of Nedd4s substrates with orthologues on the yeast protoarray, 11 had canonical PY motifs, but only two of their orthologues on the yeast protoarray also had PY motifs: Ypk1—the orthologue of SGK1 and SGK2; and Por1—an orthologue of VDAC2. These findings suggest that Ypk1 and Por1 may indeed be substrates of Rsp5 but were missed in the original screen. So far, nine members of the Nedd4 family have been identified in humans.

Table III RESULT SUMMARY: Validation of hits from ubiquitylation microarray screens

Proteins	rat Nedd4-1		human Nedd4-1		human Nedd4-2	
	Ubiquitination	Binding	Ubiquitination	Binding	Ubiquitination	Binding
CA9						
D2LIC						
KRT20						
CPNE3						
EXOSC8						
WBP2	*		*			
YY1	*		*		*	
MAP2	*					
ANXA9	*					
AQP9	*					
SGK2	*		*			
RTF1			*			
NDFIP2			*			
FGFR1			*			
CACNB1					*	
ACVR1B					*	
BMPR1A					*	
ZAK					*	

(–)ve controls (not a hit in screen, no PY motif)
* Hit in protein array screen
Possess PY motif
Negative
Positive
Not tested

Typically such family expansions occur through gene duplication followed by subsequent sequence divergence often associated with gene products gaining specialized and/or additional roles (subfunctionalization/neofunctionalization (Force *et al*, 1999)). Our results suggest that although the Rsp5/Nedd4 family of proteins is well conserved, each family member has evolved its own (albeit potentially overlapping) range of substrates.

To explore the conservation of Nedd4 substrates in greater depth, we performed a comparative analysis to identify orthologues in 111 eukaryotes (Supplementary Figure S4). In general, Nedd4 substrates tend to be less conserved (found in fewer genomes) than proteins involved in translation, but were more conserved than other proteins involved in signal transduction (Supplementary Figure S4A). By generating and clustering phylogenetic profiles for each Nedd4 substrate (Supplementary Figure S4B), we identified large groups of genes that are largely specific to either deuterostomes (SERPINF1 and AICDA) or Metazoa (FLT1 and SAMSN1). Furthermore, with the exception of SPANXN3 and TRIM52, such specificity is not restricted to a few species, but rather surprisingly, orthologues of these substrates appear to be consistently conserved throughout these taxa. Interestingly, we also identified orthologues of Nedd4s' substrates in taxa that apparently lack Nedd4 homologues (e.g. plants). One possibility is that these taxa do indeed possess a remote homologue for Nedd4. Alternatively, their homologues for the human Nedd4 substrates may perform unrelated functions and/or be regulated through different mechanisms. Together, these results suggest that although some of these substrates may have orthologues in a limited number of non-metazoan species, their regulation through Nedd4 family members is a relatively recent development that has nonetheless arisen to perform core multicellular or deuterostome-specific functions, such as regulation of developmental pathways and cell–cell communication.

Materials and methods

Purification of the E2 enzyme UbcH5b

The mammalian E2 gene *UbcH5b* (6 × His tagged) was expressed in *Escherichia coli* strain BL21 (DE3) from pT7-7 plasmids. Transformed cells were grown at 37°C to an absorbance of A_{590} of 0.6 in 2 l of Luria broth and expression was induced by addition of 1 mM isopropyl-β-1-thio-D-galactopyranoside (IPTG). After 3 h of induction at 30°C, cells were lysed by sonication in binding buffer (137 mM NaCl, 2.7 mM KCl, 10 mM KH_2PO_4, 0.5 mM $Na_2HPO_4.7H_2O$ (pH 7.4), 0.1 M PMSF and 100 μg/ml each of leupeptin, aprotinin and pepstatin), Triton X-100 was added (10%) and lysate was clarified by centrifugation at 10 000 r.p.m. for 30 min at 4°C. His-tagged proteins were purified from the clarified lysate by incubating with a nickel–agarose bead slurry at 4°C for 1.5 h. Bound proteins were washed twice with binding buffer containing 20 mM imidazole and eluted with binding buffer containing 250 mM imidazole.

Sub-cloning of human hNedd4-2, Nedd4-1 and rNedd4-1

Full-length human Nedd4-2 (hNedd4-2), hNedd4-1 and (rat) rNedd4-1 cDNA were cloned into the GST (glutathione *S*-transferase) expression plasmid, pDEST15-nGST, using the Gateway system (Invitrogen) for bacterial expression. The pDEST15 expression vector was engineered

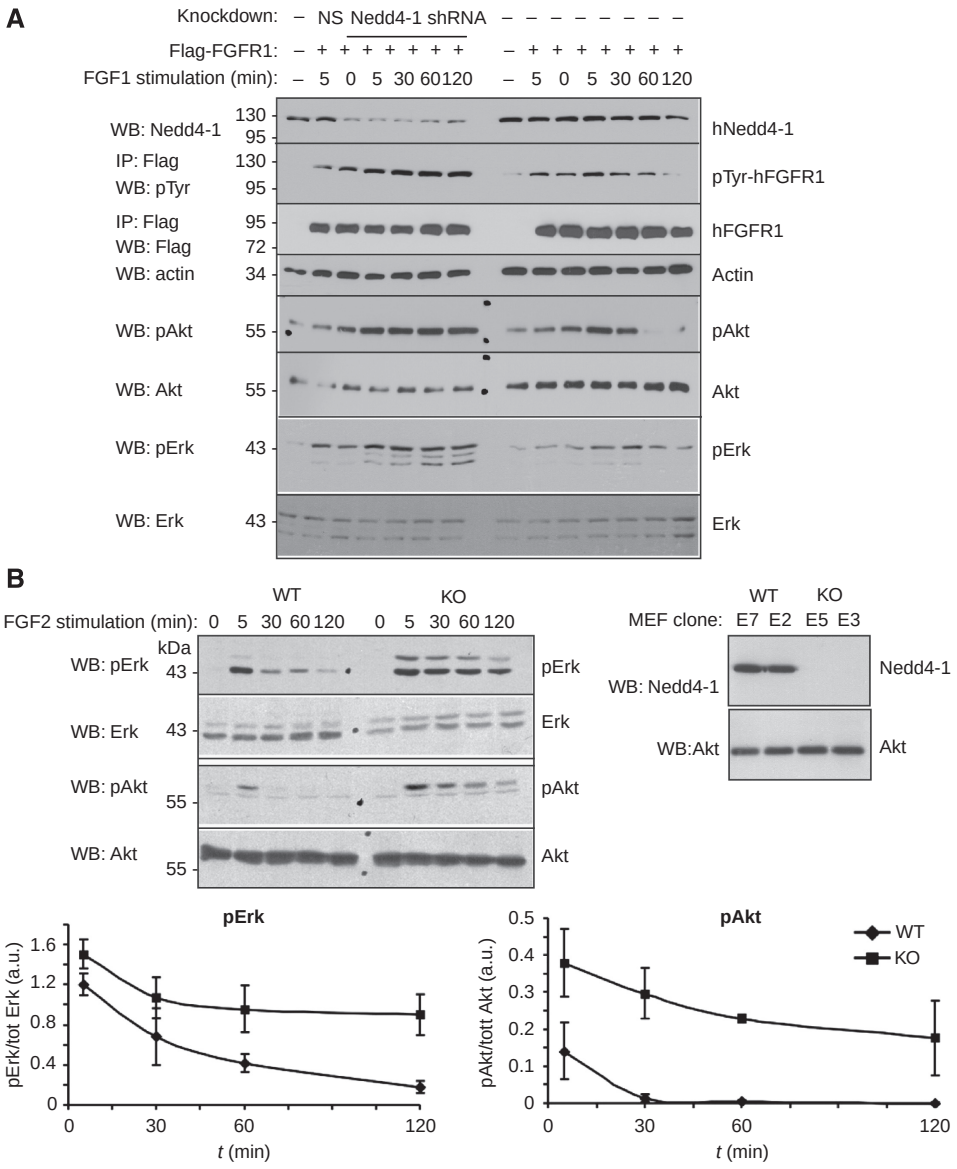

Figure 7 Sustained FGF-mediated signalling by knockdown or knockout (KO) of Nedd4-1 in cells. (**A**) HEK293T cells were either not transfected (−) or transfected with non-specific (NS) shRNA or a mixture of two shRNAs that target Nedd4-1, as described earlier (Fouladkou *et al*, 2008). Where indicated, cells were also co-transfected with human Flag-tagged FGFR1 (Flag–hFGFR1). At 1 day after transfections, cells were serum-starved (or not (−)) for 36 h and then treated with human FGF1 (100 ng/ml) plus 10 μg/ml heparin for the indicated times. Cells were then lysed, Flag–hFGFR1 immunoprecipitated with anti-Flag antibodies and immunoblotted (WB) either with anti-pTyr antibodies to detect activated FGFR1, or with anti-Flag antibodies to determine total FGFR1 protein. Aliquots of the lysates were also blotted for hNedd4-1 to prove knockdown, for phospho-Akt (pAkt) and phospho-Erk1/2 (pErk) to determine activation of Akt and Erk, respectively, as well as for total Akt, Erk and β-actin, used as controls. (**B**) Wild-type (WT) and Nedd4-1$^{-/-}$ knockout (KO) MEFs were incubated without (starved- time 0) or with mouse FGF2 plus heparin for 5, 30, 60 or 120 min. FGF-dependent signalling was tested by immunoblotting with phospho-specific antibodies against Erk1/2 (pErk) and Akt (pAkt) and compared with total expression of Akt and Erk1/2. Lower panels: quantification of kinetics of Erk and Akt activation (pErk and pAkt) normalized to total Erk and Akt expression. Two independently derived MEF cell lines for each genotype (E2 and E7 for WT and E3 and E5 for KO—see upper right panel) were used in three independent experiments. Error bars represent s.d. values of pooled data.

with a PreScission protease (GE Healthcare) cleavage site by standard PCR and restriction enzyme cloning that would allow separation of the expressed proteins from the GST tag.

Expression and purification of hNedd4-2, hNedd4-1 and rNedd4-1

Transformed cells were grown at 37°C to an absorbance of A_{590} of 0.9 in 2 l of Luria broth and expression was induced by addition of 1 mM IPTG. After 1.5 h induction at 30°C, cells were collected and lysed by sonication in binding buffer, Triton X-100 (10%) was added and lysate was clarified by centrifugation at 10 000 r.p.m. for 30 min at 4°C. The GST-tagged proteins were purified from the clarified lysate by incubation with a glutathione–Sepharose resin (Amersham Biosciences) at 4°C for 5 min. Bound proteins were washed twice with binding buffer followed by a wash with 5 ml of PreScission cleavage buffer (PCB: 50 mM Tris–HCl (pH 7.0), 150 mM NaCl, 1 mM TCEP and 10% glycerol). Expressed proteins were proteolytically cleaved from the GST moiety by incubating the resin for 4 h with 1 ml of PCB

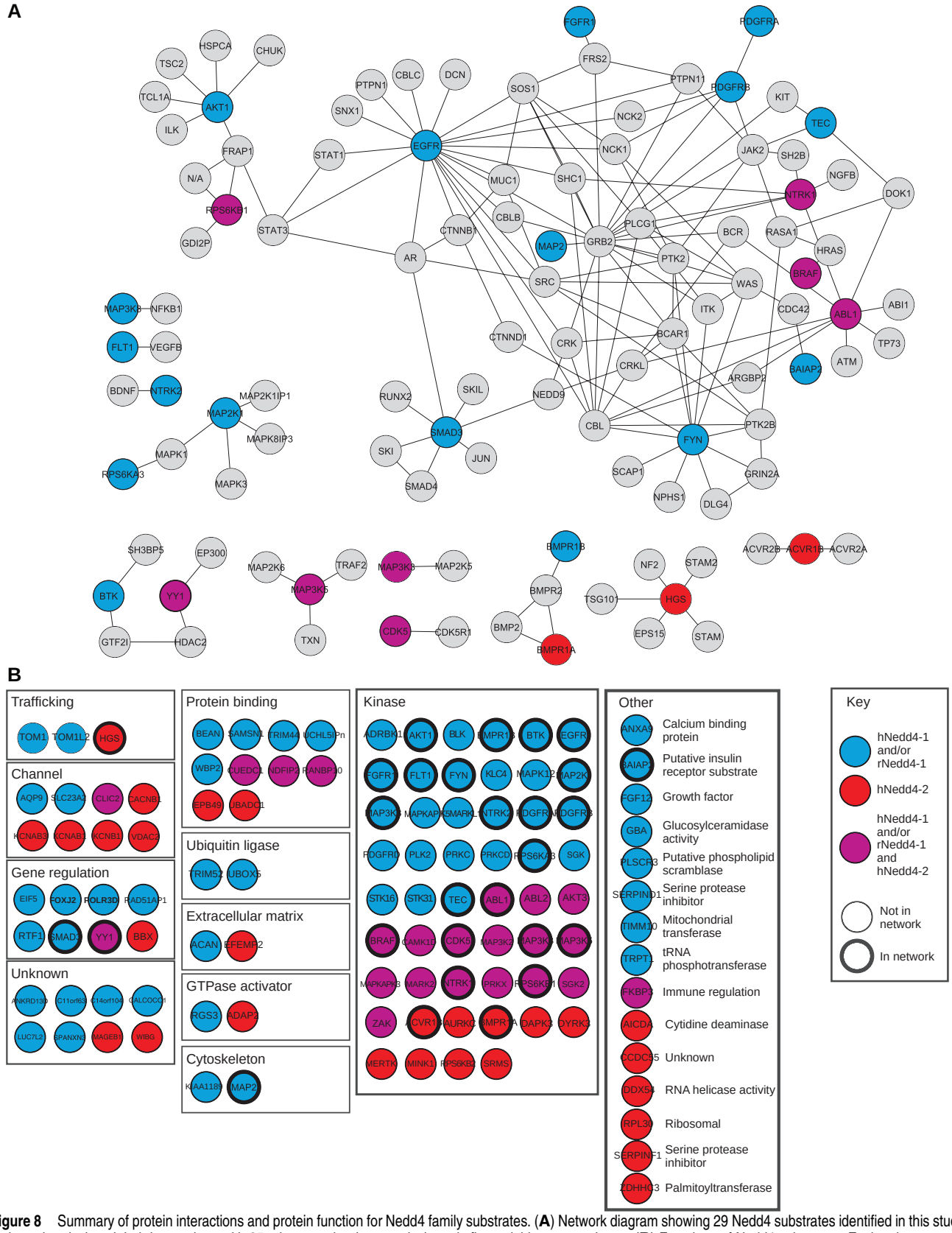

Figure 8 Summary of protein interactions and protein function for Nedd4 family substrates. (**A**) Network diagram showing 29 Nedd4 substrates identified in this study (coloured nodes) and their interactions with 85 other proteins (grey nodes)—only first neighbours are shown. (**B**) Functions of Nedd4 substrates. Each substrate was manually assigned to one of 11 categories on the basis of their GO categories. For both (A) and (B), nodes (proteins) are coloured according to their enzyme specificity. Source data is available for this figure at www.nature.com/msb.

Table IV Top hits of binding screen

Rat Nedd4-1 binding partners		Human Nedd4-1 binding partners		Human Nedd4-2 binding partners	
Protein name	PY Motif	Protein name	PY Motif	Protein name	PY Motif
UBOX5 tv.2	PPVY	TEAD2	LPGY	FKBP3	PPKY*
CSNK1E, tv.2	LPPY	RFT1	PPNY*	DDX54	*
PRRG1	PPTY, PPEY	UBOX5, tv.2	PPVY*	MRPL19	LPEY
FKBP3	PPKY*	CUEDC1	PPVY*	CLIC2	PPRY*
FLT1	PPDY*	NTRK1, tv.3	PPVY*	ZAK	PPNY*
FOXJ2	PPLY*	AKT3, tv.3	LPFY*	MAP3K2	PPGY*
CUEDC1	PPVY*	NDFIP2	PPPY, PPPY*	CUEDC1	PPVY*
BMPR1A	LPYY	MRPL19	LPEY	TGFB1I1, tv.2	PPSY
PPARD		MAP3K2	PPGY*	TMEM55A	PPPY
BRAF		CAMK4	LPEY	ACVR1B, tv.1	LPYY*
NTRK1, tv.3	PPVY	PARP16	PPKY	KCNB1	LPPY*
PKC	LPEY*	MAP3K3	PPGY*	BMPR1A	LPYY*
MAPKAPK5, tv.1	PPFY*	FYN, tv.1	*	VDAC2	PPSY*
MAPKAPK2, tv.1	PPFY	TCP11L1	PPAY	PRRG1	PPTY, PPEY
ABL1, tv.a	PPFY*	WBP2	PPGY, PPPY, PPPY*	MAP3K5	PPFY*
EIF5	PPTY*	CDK5	LPDY*	PKX	PPVY*
ZAK	PPNY*	AKT3	LPFY*	BRAF	
TIMM10	PPHY*	EGFR, tv.1	*	H1F0	
AKT3, tv.3	LPEY*	FGFR2, tv.1	LPQY*	CDK3	LPDY
H1F0		PRRG1	PPTY, PPEY	NTRK1, tv.3	PPVY*
STAC		FKBP3	PPKY	UBADC1	LPSY*
EGFR, tv.1	*	FLT1	PPDY*	KCNAB1, tv.3	
PKG2		RANBP10	PPFY*	MOBKL2A	
DDX54	LPGY	UBE2M		RANBP10	PPFY*
MGC11257		BMPR1A	LPYY	TARBP2, tv.1	LPEY
MAP2, tv.2	PPSY*	SLC23A2	PPWY, LPIY	MAP2, tv.2	PPSY
CDK5	LPDY*	SAAL1	LPFY	AKT3, tv.1	LPFY*
PLK1		SULF1	LPQY	ROBO3	
TRIM41		C3orf37, tv.1		TRIM41	
IKBKB		PDGFRB	*	RPL30	*
BEAN	PPGY, PPPY, LPPY*	TBC1D7		MGC11257	*
PPID		CAMK1D, tv.1	PPFY*	C4orf43	*
SYK		SIVA, tv.2	*	RASL10B	
TTK		MOBKL2A		DECR2	LPAY
CENTA2		FGF21		CENTA2	*
FGF12	*	RBCK1	*	SENP2	
ZADH2		PRR16		YES1	
DYRK4		KCNAB2, tv.1		KCNAB2, tv.1	*
TEK		RPS6KA4, tv.2		PLK1	
DTRK3, tv.2		AMPD2, tv.2	LPEY	SYK	*
KCNAB1, tv.3		KCNAB1, tv.1		APEX2	*
STK24, tv.2		YES1	*	ZADH2	*
RASL11B		MST4, tv.3		CCDC55, tv.1	*
C3orf37, tv.1		FES	*	STAC	
SIVA1, tv.2		PLK1		NUAK1	
FGF21		IRS1		TBC1D7	
KCNAB2, tv.1		TBK1		PKG2	*
NUDT16L1		SYK		TBK1	
CLK3, tv.phclk3	*	TRIM52	PPPY*	KIT	*
NOLA2, tv.1		DDX54	*	PRR16	
		FGF12			
		CLK3	*		
		WEE1	*		
		RNF7			

*Also identified in the ubiquitylation screen using respective E3 ligase.
PY motif; tv: transcript variant.

containing 40 U of PreScission protease (Amersham Biosciences). Desired proteins were further purified by size exclusion chromatography. Final yield of proteins varied between 150–200 μg.

Labelling of Nedd4 proteins

Purified human hNedd4-1, hNedd4-2 and rNedd4-1 proteins (1 mg/ml) were labelled with AlexaFluor 647 using the Microscale Protein Labeling kit (Molecular Probes) according to manufacturers' instructions. A total of 50 μg of Nedd4 protein was used for the reaction. The final concentration of AlexaFluor 647-labelled proteins was 0.1 mg/ml in a volume of 150 μl.

Purification of GST-tagged control substrates

The GST-tagged proteins were used as control substrates. The PY motif-containing proteins already known to be ubiquitylated by Nedd4 proteins (and verified again in this study (data not shown)), were used as positive controls. These included: the βP2 region of ENaC (in pGEX-KG), mouse Rnf11 (in pGEX-6P), mouse LAPTM5 C terminus (pGEX-6P), *Xenopus* Nedd4-HECT(CS) domain (in pGEX-6P) and mouse

CNrasGEF C terminus (in pGEX-2T) (Staub *et al*, 1996; Abriel *et al*, 1999; Pham and Rotin, 2001; Pak *et al*, 2006; Bruce *et al*, 2008). Negative controls included: GST alone (in pGEX-6P), mouse Rnf11(YA) bearing a mutation in its PY motif (in pGEX-6P), rNedd4-C2 domain (in pGEX-6P), mouse Grb10-SH2 domain (in pGEX-2TK) and the PDZ domain of human CNrasGEF (in pGEX-2TK).

These constructs were expressed in *E. coli* strain BL21 (RIL). Culture and expression of these recombinant GST proteins were conducted as described above for UbcH5b. Proteins were purified from 100 ml of growth medium using glutathione–Sepharose resin and eluted in 100 μl of binding buffer containing 40 mM glutathione (Amersham Biosciences). The final yield of purified proteins varied from 100 to 400 μg. All purified proteins were resolved on a 10% SDS–polyacrylamide gels and visualized by Coomassie Blue staining (Sigma).

Production of control protein microarrays

Control microarray slides were printed to verify and optimize conditions for both ubiquitylation and binding assays. Purified GST-tagged control proteins were spotted onto PATH™ nitrocellulose slides (Gentel Biosciences) at increasing concentrations using a Piezoarray™ (Perkin-Elmer) platform. After printing, slides were stored at −20°C.

Ubiquitylation assay using protein microarrays

Invitrogen Human ProtoArray® Microarray (V4.0) slides were used for these experiments, in which 8222 human GST fusion proteins (+1326 controls) generated in insect cells are arrayed onto PATH slides in duplicates. Slides were rinsed with 0.5% PBST, blocked for 1 h at room temperature in ubiquitylation-blocking buffer (50 mM HEPES (pH 7.5), 200 mM NaCl, 0.08% Triton X-100, 25% glycerol, 20 mM glutathione, 1.0 mM DTT, 10 M NaOH and 1% BSA), rinsed for 5 min with ubiquitylation reaction buffer after which 500 μl of ubiquitylation reaction mixture (25 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1 μM dithiothreitol, 4 mM $MgCl_2$, 0.303 μM E1, 1.96 μM E2, 0.49 μM E3, 5 μg FITC–Ub (Boston Biochem) and 8 mM ATP in 300 μl) was gently pipetted onto the surface of the slide. The reaction mixture on the slide was kept humid using wet filter paper and the reaction was allowed to proceed for 2.5 h in the dark. After the reaction, the slide was washed three times with 0.5% PBST for 10 min and dried by centrifugation (4 min at 1000 *g* at 4°C).

Spot fluorescence was visualized by a fluorescent laser scanning at 20-μm resolution using a 488-nm laser on a ProScan Array HT scanner (Perkin-Elmer).

Binding assay using protein microarrays

Slides were rinsed with PBS, followed by a rinse in 0.5% PBST, and then blocked for 1.5 h in ubiquitylation-blocking buffer. Probe solution (0.026 μM Alexa647-conjugated hNedd4-1, hNedd4-2 or rNedd4-1 in 300 μl of ubiquitylation reaction buffer) was carefully pipetted over the entire area of the slide, kept humid with a wet filter paper and then incubated for 1.5 h in the dark. The slide was washed twice in 0.1% PBST for 5 min and once in 0.1% PBST for 10 min and dried by centrifugation (2 min at 200 *g*, 4°C). The slide was then scanned at 20-μm resolution using a 633-nm laser on a ProScan Array HT™ (Perkin-Elmer) scanner. Printed slides containing fluorescent proteins and dyes were kept in the dark for the duration of the experiment.

Quantification and data analysis

Spot fluorescence intensity from the microarray screens were quantified using ProScan Array HT (Perkin-Elmer) software. Duplicate screens were compared using Protein Prospector Analyzer (Invitrogen) software to align similar protein spots on both slides. Spots on which 50% of the pixels produced a signal greater than 2 s.d. values above background were identified as 'hits'. These proteins were included in the substrate and interaction data sets only if both duplicate spots in the two replicated slides (i.e. all four spots) met the criteria. Once the data sets were generated, spots were normalized and ranked according to their signal intensity per unit protein spotted on slide; (signal intensity=mean signal on the spot−background)/RFU (relative fluorescent units) of the protein spotted). Once the lists were generated, the top ~50 hit proteins (54 for hNedd4-1 and 50 for hNedd4-2) were chosen as the 'high-confidence' data sets, based on an approximately five-fold difference in normalized signal between the top and bottom hits of the high-confidence data sets for hNedd4-1 and hNedd4-2. For the sake of comparison, the top 50 hits for rNedd4-1 were also considered 'high confidence'.

In vivo ubiquitylation and binding assays

HEK293T cells, maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, were transiently transfected (using the calcium phosphate precipitation method) with a total of 20 μg of DNA per 10-cm diametre dish. They were co-transfected with Flag-tagged substrate of interest and either V5-tagged hNedd4-1, V5-hNedd4-1(CS) (Cys-to-Ser mutation in the catalytic Cys of the HECT domain) or T7-rNedd4-1, and His-tagged ubiquitin (His-Ub) where indicated. For the ubiquitylation assays, cells were treated with 20 μM MG132 (Boston Biochem) for 3 h before lysis and lysed 48 h post-transfection in 1 ml of lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1.0 mM EGTA, 10 mg/ml leupeptin, 10 mg/ml aprotinin and 1 mM PMSF) supplemented with 50 μM LLnL (*N*-acetyl-Leu-Leu-norleucinal; Sigma) and 0.4 mM chloroquine (Sigma) and cleared by centrifugation at 14 000 r.p.m. for 10 min. To ensure ubiquitylation of Flag-tagged substrate, and not of associated proteins, 1 mg of cleared cell lysates was treated with 1% SDS and boiled for 5 min. The boiled lysates were then diluted 11 times with lysis buffer (to dilute the SDS) before immunoprecipitation. Lysates were then incubated with 10 μl of Anti-Flag M2 affinity agarose (Sigma) at 4°C for 2 h. Bound proteins were washed (thrice) with HNTG (50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol) and ubiquitylation of substrate detected by immunoblotting with anti-His, or anti-ubiquitin (Ub), where indicated.

For binding (co-immunoprecipitation) experiments, cells were transfected and lysed as above. A total of 1 mg of cleared lysate was incubated with 10 μl of Anti-Flag M2 affinity agarose (Sigma) at 4°C for 2 h. Bound proteins were washed with HNTG (thrice) and co-immunoprecipitated Nedd4 proteins were detected by immunoblotting with anti-V5 (hNedd4-1 and hNedd4-2) or anti-T7 (rNedd4-1) antibodies.

For experiments testing the role of the RxxQE motif of CACNB1 and ANXA9 in binding to Nedd4 proteins and ubiquitylation by them, the Arg, Gln and Glu residues in the motifs were mutated to Ala using site-directed mutagenesis, and the binding and ubiquitylation assays carried out as described above.

The antibodies used for immunoblotting were: anti-ubiquitin antibody (1:1000 dilution; Covance), anti-Flag antibody (1:10 000 dilution, Sigma), anti-V5 antibody (1:5000 dilution; Invitrogen), anti-His antibody (1:1000; Qiagen) and anti-T7 (1:10 000; Novagen). Incubation with primary antibodies was followed by either horseradish-peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (both at 1:20 000 dilution; Jackson ImmunoResearch) and detected by ECL® (enhanced chemiluminescence; GE Healthcare).

FGFR signalling

For knockdown of endogenous hNedd4-1, HEK293T cells were transfected (where indicated) with two different shRNAmir directed against the human Nedd4-1, purchased from OpenBiosystems (V2LHS_254872 and V2LHS_72553, all in pGIPZ) or a non-specific (NS) control plasmid in pGIPZ. Cells were also co-transfected with Flag-tagged hFGFR1 (where indicated) using the calcium phosphate method. These cells were serum starved (where indicated) one day after transfection in Dulbecco's modified Eagle medium for 36 h and then treated with 100 ng/ml hFGF1 (Upstate) supplemented with 10 μg/ml heparin (Sigma) for the indicated times. Cells were then

lysed, Flag–hFGFR1 immunoprecipitated with Flag antibodies (M2 agarose) and immunoblotted with either anti-pTyr (Y653) antibodies to detect activated hFGFR1, or with anti-Flag antibodies to determine total hFGFR1 protein. Aliquots of the lysates were also immunoblotted for hNedd4-1 (Cell Signaling Technology) to prove knockdown, for phospho-Akt (pAkt-S473) and phospho-Erk (pErk1/2, pTEpY) to determine the activation of Akt and Erk, as well as for total Akt, Erk and β-actin, used as controls.

For FGFR signalling in MEFs: Nedd4 WT or (−/−) (knockout) MEFs were seeded onto six-well plates at a density 300 000 cells per well in starvation medium (DMEM + 0.1% FCS) over night. Next day, cells were stimulated with 100 ng/ml mouse FGF2 (Ebioscience) plus 10 μg/ml heparin (Sigma), or with EGF–biotin (100 ng/ml, Invitrogen) for the time points indicated. Total cell lysates were separated by SDS–PAGE and analysed by immunoblotting with anti-pErk1/2 (pTEpY) and anti-total Erk (Promega) antibodies, or with anti-pAkt (Ser 473) and anti-Akt (Cell Signaling Technology) antibodies. Nedd4-1 expression in MEFs was determined with anti-mouse mAb against mNedd4-1 (Becton-Dickinson).

Sequence logos and searches for new Nedd4-binding motifs on substrates

Sequence logos were created for our hits that contain PY motifs (LPxY and PPxY). Only hits with one such motif were considered (not those with multiple L/PPxY motifs). To avoid over-representation of motifs from hits with near-identical sequences (isoforms or mutants of other hits), a non-redundant set of local sequences was used as input alignment. Sequence logos were created with Weblogo (http://weblogo.berkeley.edu).

For searches of new Nedd4-binding motif(s), a data set of substrates sharing no more than 90% sequence identity (i.e. non-redundant) was assembled from the binding and ubiquitation hit lists. The over-represented motifs were identified with SlimFinder (http://www.plosone.org/article/info:doi/10.1371/journal.pone.0000967). The motif length range was set from three to six and default options used for all other parameters. An initial search led to PPxY and LPxY being identified as the two highest-scoring motifs. Proteins containing these motifs were removed from subsequent searches of the data set.

In silico evolution analyses

For details of the sources of protein complements for the 111 genomes used in this study see supplementary Table SIII. Ordering of the genomes for Supplementary Figure S4 is provided in the table and reflects current understanding of phylogenetic relationships. Orthologous relationships between human proteins and those in all 111 genomes were determined on a pairwise basis with the InParanoid algorithm (Remm *et al*, 2001) using default settings. It should be noted that the inability of the InParanoid algorithm to detect an orthologue in a genome does not necessarily imply its absence, but may instead reflect a high degree of sequence divergence. Protein–protein interaction data for Nedd4 substrates were obtained from the BioGRID resource (Breitkreutz *et al*, 2008). Only interactions supported by two independent publications were included. The network was visualized using Cytoscape version 2.6.2 (Shannon *et al*, 2003). Gene Ontology data for categorizing and describing protein function were downloaded from the GO repository (http://www.geneontology.org) on Feb 21st 2009.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Conflict of interest

The authors declare that they have no conflict of interest.

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