EMBO reports

scientific report

Control of the activity of WW-HECT domain E3 ubiquitin ligases by NDFIP proteins

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HECT domain E3 ubiquitin ligases of the NEDD4 family control many cellular processes, but their regulation is poorly understood. They contain multiple WW domains that recognize PY elements. Here, we show that the small PY-containing membrane proteins, NDFIP1 and NDFIP2 (NEDD4 family-interacting proteins), activate the catalytic activity of ITCH and of several other HECT ligases by binding to them. This releases them from an autoinhibitory intramolecular interaction, which seems to be characteristic of these enzymes. Activation of ITCH requires multiple PY–WW interactions, but little else. Binding of NDFIP proteins is highly dynamic, potentially allowing activated ligases to access other PY-containing substrates. In agreement with this, NDFIP proteins promote ubiquitination *in vivo* both of Jun proteins, which have a PY motif, and of endophilin, which does not.

Keywords: HECT domain; ITCH; NDFIP1; NDFIP2; ubiquitin
EMBO reports (2009) **10**, 501–507. doi:10.1038/embor.2009.30

INTRODUCTION

Post-translational attachment of ubiquitin regulates various cellular processes. The protein ubiquitination process is catalysed by a three-component enzyme system: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase (E3) that determines substrate specificity. E3 ubiquitin ligases are classified into two main categories based on their ubiquitin transfer mechanisms. RING (really interesting new gene) finger and U-box E3 ubiquitin ligases act as scaffolding proteins that indirectly facilitate the transfer of ubiquitin by bringing E2 and substrate proteins into close proximity. Conversely, HECT domain E3 ligases transfer ubiquitin directly to substrate proteins by acting as catalytic intermediates through a strictly conserved cysteine residue (Ingham *et al*, 2004; Kerscher *et al*, 2006).

The NEDD4 family of HECT domain E3s shares a characteristic modular design, with an amino-terminal C2 domain, 2–4 WW domains and a catalytic HECT domain. The WW domains recognize proline-rich PY motifs, such as PPXY or PPLP, as well as phosphorylated serines and threonines (Lu *et al*, 1999; Ingham *et al*, 2004). There are nine of these ligases in humans, implicated in a wide variety of cellular processes, including differentiation (ITCH), endocytosis (NEDD4L), signalling (SMURF1/2 and ITCH) and transcription (NEDD4; Abriel *et al*, 1999; Zhu *et al*, 1999; Kavsak *et al*, 2000; Flores *et al*, 2003; Bai *et al*, 2004; Anindya *et al*, 2007; Liu, 2007).

In *Saccharomyces cerevisiae*, the small PY-motif containing membrane protein Bsd2 acts as an adapter for the HECT E3 ligase Rsp5, allowing modification of the metal transporter Smf1, as well as vacuolar enzymes (Hettema *et al*, 2004; Sullivan *et al*, 2007). The mammalian orthologues of Bsd2, the NEDD4 family-interacting proteins NDFIP1 (N4WBP5) and NDFIP2 (N4WBP5A), share three highly conserved transmembrane domains, have cytoplasmic PY motifs and are localized to the Golgi, endosomes and multivesicular bodies (Harvey *et al*, 2002; Konstas *et al*, 2002; Cristillo *et al*, 2003; Shearwin-Whyatt *et al*, 2004). NDFIP1 has recently been shown to bind to and promote the degradation of the divalent metal transporter DMT1, in a manner that is dependent on the HECT ligase WWP2 (Foot *et al*, 2008). In addition, mice lacking NDFIP1 develop severe inflammation; their T cells are hyper-activated and they induce T helper 2 cell-mediated inflammation. This phenotype strongly resembles that described for *Itchy* mice, and it has been shown that NDFIP1, which is induced in activated T cells, binds to ITCH and promotes degradation of JunB, consistent with a role in dampening activation (Perry *et al*, 1998; Fang *et al*, 2002; Oliver *et al*, 2006). NDFIP2 is 50% identical to NDFIP1 in sequence, and thus might perform a similar function.

The mechanisms regulating the catalytic activity of NEDD4-like E3 ligases are largely unknown, although at least some of them exist in an autoinhibited state. The catalytic activity of SMURF2 was shown to be inhibited by intramolecular binding of the HECT domain to the C2 domain, which can be prevented by the activator SMAD7 (Wiesner *et al*, 2007). A similar inhibitory mechanism was proposed for ITCH, in which it is thought that interaction of the HECT domain with a proline-rich region and the WW domains is responsible for its autoinhibition. In T cells, stress-activated protein kinase JNK1-mediated phosphorylation of the proline-rich region of ITCH is necessary and sufficient to relieve the autoinhibition (Gao *et al*, 2004; Gallagher *et al*, 2006).

Here, we show that NDFIP2 functions as both a recruiter and a strong activator of multiple NEDD4 family members *in vivo*, and

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Received 26 September 2008; revised 9 February 2009; accepted 9 February 2009; published online 3 April 2009

of ITCH and NEDD4 *in vitro*. We also present evidence that these E3 ligases assume an autoinhibited conformation that can be relieved by binding of the NDFIP PY motifs to their WW domains. Thus, in addition to their roles in localizing enzymes and recruiting substrates, the NDFIP proteins control ligase activity directly.

RESULTS

NDFIP2 induces autoubiquitination of E3 ligases

293T cells contain low levels of both NDFIP1 and NDFIP2, and we found that expression of either protein increased the total level of ubiquitinated proteins detectable in the cells (data not shown). This suggests that NDFIP proteins can increase the activity of HECT domain E3 ligases, which should bind to their PY elements. As the activity of these ligases is typically reflected in their autoubiquitination, we co-expressed individual haemagglutinin-tagged ligases with NDFIP2 and His-tagged ubiquitin, purified ubiquitinated proteins on Ni-NTA beads, and examined the modification state of the E3 proteins by immunoblotting with anti-haemagglutinin. We focused primarily on NDFIP2, as it has been less well characterized than NDFIP1. Fig 1A shows that NDFIP2 stimulated ubiquitination of all six ligases tested (lanes 2), whereas a variant of NDFIP2 in which all three PY elements were mutated did not (lanes 3). A catalytically inactive ITCH mutant (C830G) also failed to become modified, indicating that the ubiquitination was indeed self-induced.

Ubiquitination of other substrates

It has been shown previously that ITCH promotes ubiquitination of the c-Jun and JunB transcription factors (Fang *et al*, 2002). We found that NDFIP2 could stimulate ubiquitination of both these proteins when they were co-expressed, and again the PY-mutated form of NDFIP2 showed less activity (Fig 1B). We also examined another known ITCH substrate, the endosome-associated protein endophilin A1 (Angers *et al*, 2004). Fig 1C shows that endophilin also showed increased ubiquitination when it was co-expressed with wild-type NDFIP2. These results are consistent with stimulation of endogenous ITCH or other HECT E3 activity by NDFIP2. In agreement with this, we found that co-expression of either ITCH or NEDD4 with NDFIP2 greatly increased the ubiquitination of both JunB and endophilin (Fig 1D, right-hand panels), but efficient ubiquitination remained dependent on the presence of NDFIP2 (Fig 1D, left-hand panels).

NDFIP2 recruits NEDD4 family members to membranes

If the membrane protein NDFIP2 interacts directly with soluble E3 proteins, it should alter their localization. To test this, we co-expressed enhanced green fluorescent protein (EGFP)-tagged NEDD4 with NDFIP2 in COS-7 cells (Fig 2A). NDFIP2 was in punctate structures, which have previously been shown to be endosomes (Shearwin-Whyatt *et al*, 2004). Strikingly, significant amounts of NEDD4 were localized to the same structures. When all three PY elements of NDFIP2 were mutated, NEDD4 showed a diffuse distribution. Similar results were obtained with ITCH and SMURF2 (data not shown). This suggests that the E3 ligases indeed form complexes with the NDFIP proteins, and hence are recruited to, and activated on, endosomal membranes. In agreement with this, we found that when cells expressing ITCH were fractionated into a membrane-containing pellet and cytosol, the heavily ubiquitinated forms of ITCH were associated exclusively with the membrane pellet (Fig 2B). For this experiment, we used a version of ITCH that lacked its C2 domain, to reduce its intrinsic affinity for membranes.

To examine the stability of the NEDD4–NDFIP complexes *in vivo*, we used photobleaching of GFP-NEDD4. Surprisingly, at least 60% of the GFP-NEDD4 on individual punctate structures could be replaced within a few seconds of bleaching, indicating a rapid dynamic exchange of the ligase (Fig 2C,D). Taken together, these results imply that binding and activation of the ligases is a transient local phenomenon.

NDFIPs activate ITCH and NEDD4 *in vitro*

We performed *in vitro* ubiquitination assays using the cytoplasmic domains of the NDFIPs, and their respective PY motif mutants, together with bacterially expressed E3 proteins. The addition of NDFIPs resulted in a smear of polyubiquitinated material corresponding to modification of both the E3 ligases and the NDFIP proteins (Fig 3A). Immunoblotting confirmed that auto-ubiquitination of recombinant ITCH was markedly enhanced when it was incubated with the cytoplasmic domain of either NDFIP1 or NDFIP2, but not when the NDFIP PY motif mutants were used (Fig 3A). A similar effect was seen with NEDD4, although, in this case, a small proportion of the protein seemed to be constitutively active owing to a minor degradation product that had lost its autoinhibition (marked by X in Fig 3A). The NDFIP proteins were good substrates for ubiquitination only when their PY elements were intact. More detailed studies showed that the addition of increasing amounts of NDFIP protein resulted in progressively larger ubiquitinated species of ITCH, whereas the average size of ubiquitinated NDFIP decreased (supplementary Fig 1 online). This is consistent with the formation of transient NDFIP–ITCH complexes, with stochastic ubiquitination of both components when they interact.

We used methylated ubiquitin, which cannot be extended into ubiquitin chains, to see how many lysine residues were subject to modification. Fig 3B shows that NDFIP2 was modified at two sites, whereas ITCH received ubiquitins at around 10 lysines, as judged by the size of the modified protein.

Trimming of the NDFIP2 sequence showed that a minimal stretch of 52 amino acids encompassing just the three PY motifs retained the ability to activate ITCH (Fig 3C). However, mutation of any one of the PY elements reduced activity, with PY2 and PY3 being particularly important; mutants with only one PY had very low activity (Fig 3D). These data confirm that the PY elements are the main motifs responsible for enhancing E3 ubiquitin ligase activity, and suggest that the high avidity of multiple PY–WW interactions is important for this effect.

Mutation of the WW domains of ITCH confirmed that at least two are required for efficient interaction with NDFIP1. WW2 seemed to be the most effective, whereas WW1 made only a minor contribution (Fig 3E). This requirement for multiple PY–WW interactions raised the possibility that activation depended on the bridging of two ITCH molecules by a single NDFIP protein, with the ITCH proteins ubiquitinating each other. However, we were able to rule this out because the incubation of NDFIP1 with a mixture of catalytically active and inactive ITCH resulted in ubiquitination of only the active form (supplementary Fig 2 online).

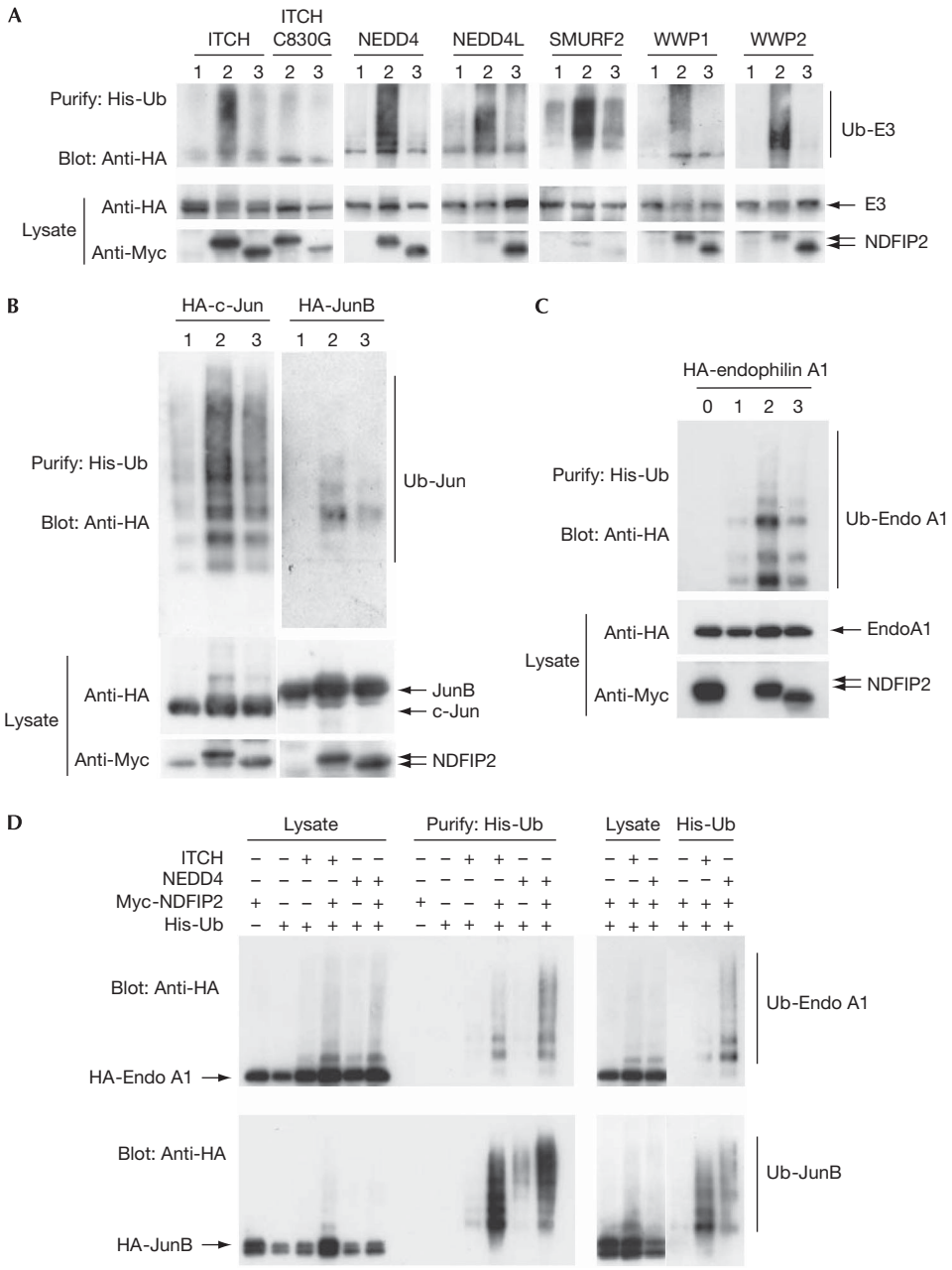

Fig 1 | NDFIP2 induces ubiquitination of HECT domain ligases and potential substrates. (**A**) HEK293T cells expressed His-tagged ubiquitin (His-Ub), HA-tagged E3 ligases as indicated and an empty vector control (lane 1), Myc–NDFIP2 (lane 2) or Myc–NDFIP2 with triple PY motif mutations (lane 3). (**B**) Cells expressed His-tagged ubiquitin, HA-tagged c-Jun or JunB and either an empty vector (1), Myc-tagged NDFIP2 (2) or the PY mutant NDFIP2 (3). (**C**) As in (**B**) but with HA–endophilin A1 instead of Jun proteins. Lane 0 is a control in which His–ubiquitin is omitted. (**D**) As in (**B**,**C**), with indicated proteins expressed. All samples are from the same experiment, but the panels to the right are from a separate gel. HA, hemagglutinin; HECT, homologous to E6-AP carboxy terminus; HEK, human embryonic kidney; NDFIP, NEDD4 family-interacting protein.

Distinct domains contribute to ligase autoinhibition

The ability of NDFIPs to activate various NEDD4-like E3 ligases suggests that these enzymes commonly exist in an autoinhibited state. To test this, we made deletion mutants of both NEDD4 and ITCH. Removal of the C2 domain (ΔN140) from NEDD4 greatly increased its activity *in vitro*, as judged by its self-ubiquitination, and it could not be stimulated further by NDFIP1 (Fig 4A); by contrast, ITCH lacking its C2 domain (ΔN120) was fully regulated (Fig 3; we used this form routinely for *in vitro* experiments because it was well expressed in bacteria). Further deletion of the proline-rich region from ITCH (ΔN225) did not remove the autoinhibition, but deletion of an additional 59 amino-acid residues up to the first WW domain (ΔN284) resulted in a huge increase in ubiquitination (Fig 4A). Thus, autoinhibition is a common feature

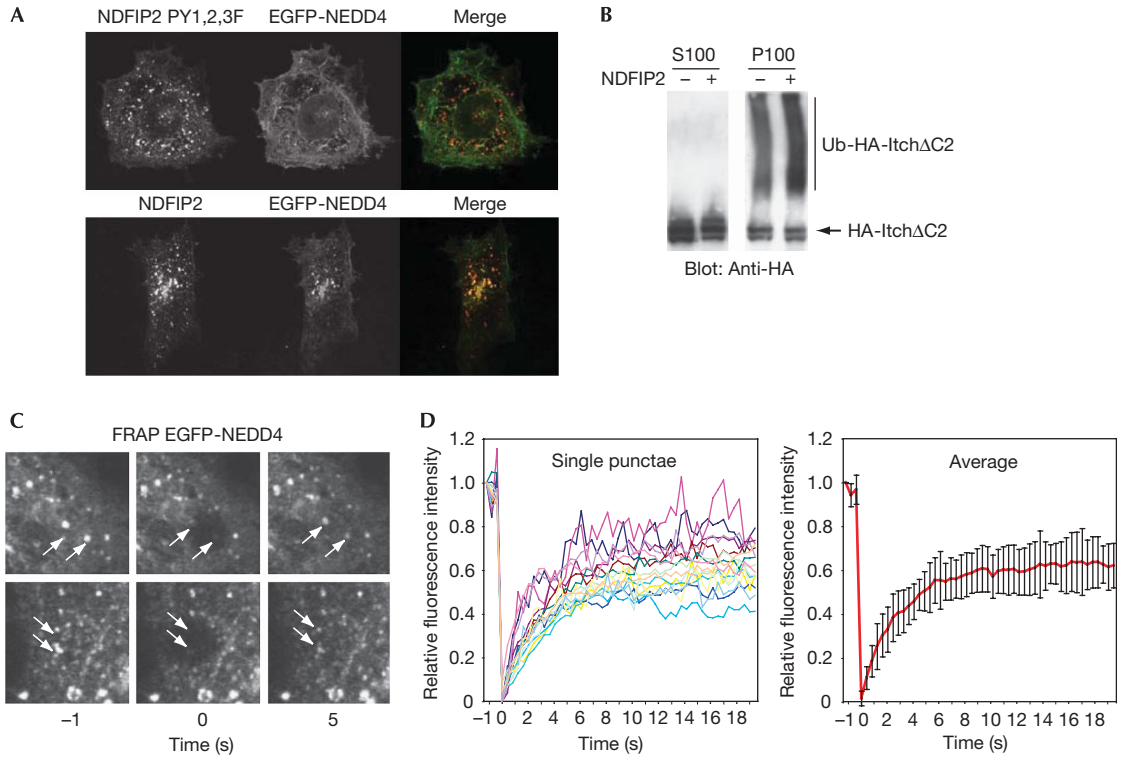

Fig 2 | Recruitment of NEDD4 by NDFIP2. (**A**) COS-7 cells expressing both GFP-tagged NEDD4 and either Myc-NDFIP2 or the PY mutant form of this were imaged. (**B**) Cells expressing HA-ITCHΔC2 with or without NDFIP2 were lysed and centrifuged, and the supernatant (S100) and pellet (P100) were blotted. (**C**) Examples of FRAP experiments; time 0 is immediately after bleaching. (**D**) Quantitation of FRAP experiments. Each curve represents one bright dot in the bleached region. FRAP, fluorescence recovery after photobleaching; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; NDFIP, NEDD4 family-interacting protein.

of NEDD4-like E3 ligases, but the details of the inhibitory interactions differ. Fig 4B presents a possible model to account for activation by the NDFIPs.

Activation of substrate modification in trans

Although NDFIPs activate ubiquitination of the ligases themselves and are also substrates for modification, *in vivo* they are able to stimulate ubiquitination of other substrates. We tested endophilin, which lacks a PY motif and instead is recognized through its SH3 domain, which binds to the proline-rich region of ITCH (Angers *et al*, 2004). We found that NDFIP2, but not the PY mutant, was not only a substrate *in vitro* but also stimulated the ITCH-dependent ubiquitination of endophilin (Fig 5).

DISCUSSION

HECT domain ubiquitin ligases are subject to tight controls. Here, we have shown that multiple members of the ITCH-like E3 family are normally self-inhibited, but can be activated by the binding of the NDFIP proteins, through multiple PY elements, to their WW domains. The requirement for multiple interactions means that this is a specific characteristic of the NDFIPs; typical substrates containing only one or two PY motifs will not be efficient activators.

The structures of HECT domains reveal a conformationally flexible two-lobed structure, and mutational analysis suggests that rotation about the hinge region between the lobes is essential for catalytic activity (Verdecia *et al*, 2003; Ogunjimi *et al*, 2005). It has been shown that the N-terminal portion of ITCH, which includes the WW domains, can bind to the HECT domain and inhibit activity, probably by limiting this flexibility (Gallagher *et al*, 2006). The simplest interpretation of our results is that NDFIP binding to the WW domains introduces a conformational constraint that disrupts the intramolecular interaction, thus freeing the HECT domain to perform its function (Fig 4B).

This simple model probably applies to all of the E3 ligases that we tested, namely ITCH, NEDD4, NEDD4L, SMURF2, WWP1 and WWP2. However, the precise nature of the inhibitory intramolecular interactions varies: inhibition of NEDD4, SMURF2 and WWP2 requires the C2 domain (Wiesner *et al*, 2007; this study), whereas for ITCH we have shown that it depends on a short sequence between the proline-rich domain and the first WW domain. Previously, it has been shown that autoinhibition of ITCH activity can be relieved by JNK-dependent phosphorylation at any of the three sites, presumably by disrupting the inhibited conformation (Gallagher *et al*, 2006). One of these sites (Ser 232) lies in the region that we have found to be crucial for inhibition but, surprisingly, the other two lie outside this region, in the proline-rich domain. Phosphorylation at these sites might create a new interaction that rearranges the structure.

We have shown that NDFIP2 can stimulate the activity of ITCH on a heterologous substrate, namely endophilin, in a simple *in vitro* reaction, as well as *in vivo*. However, the ability of the

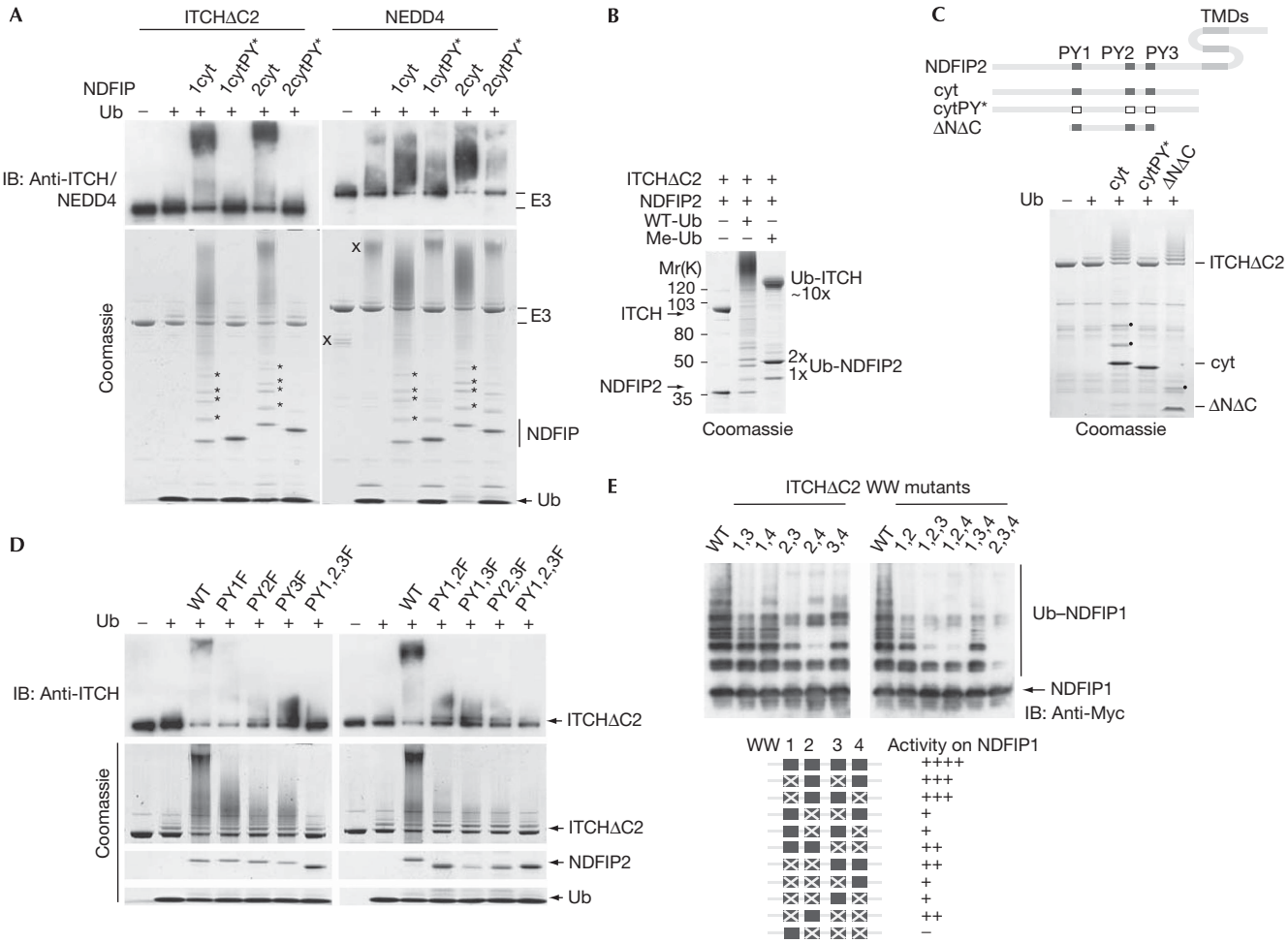

Fig 3 | NDFIPs are substrates that increase autoubiquitination of E3 ligases *in vitro*. (**A**) Coomassie-stained gels and immunoblots (IB) of assays containing the indicated proteins and the cytoplasmic domains of NDFIP1 (1cyt) or NDFIP2 (2cyt) or the same with all three PY motifs mutated (PY*). Asterisks indicate the smaller ubiquitinated forms of the NDFIPs; X is a constitutively active fragment of NEDD4. (**B**) Assay with methylated ubiquitin. (**C**) Assays with NDFIP2 fragments. This experiment used His–ubiquitin, which reduces modification and allows a clear separation of the modified forms of NDFIP and ITCH. (**D**) Assays with Y to F changes in individual PY motifs of NDFIP2. Note that polyubiquitinated proteins sometimes transfer poorly to the blot. (**E**) Modification of NDFIP1 by various ITCH WW mutants. The schematic diagram summarizes the results. NDFIP, NEDD4 family-interacting protein; Ub, ubiquitin; WT, wild type.

NDFIP proteins to promote ubiquitination of JunB is more puzzling: JunB has a PY element that is essential for its interaction with ITCH (Fang *et al*, 2002), and the NDFIPs would be expected to compete with this substrate for the same WW domain-binding sites. In theory, NDFIPs could bind to three of the four WW domains in ITCH, whereas the fourth interacts with a substrate. However, we have not been able to reproduce NDFIP-dependent Jun ubiquitination using the soluble *in vitro* system. An alternative explanation comes from our findings that NDFIP–NEDD4 and NDFIP–ITCH complexes rapidly dissociate. On release, the ligases should be in an active state, and might be able to bind to and modify nearby substrates before the inactive conformation is re-established. This would require the substrates to be positioned close to the NDFIP proteins. Indeed, although Jun is predominately nuclear, it has been shown that ubiquitinated Jun is located on ITCH-positive endosomes and ends up in lysosomes (Fang & Kerppola, 2004). Whether unmodified Jun is specifically targeted to endosomes, and thus to the vicinity of activated ITCH, is as yet unknown.

Together with previous work, our results suggest that the NDFIP proteins can stimulate ubiquitination in two ways. First, they bind to substrates and recruit ligases to them, as shown for the metal transporter DMT1 (Foot *et al*, 2008) and the yeast NDFIP homologue Bsd2 (Sullivan *et al*, 2007). Second, they activate the catalytic activity of the ligases, allowing them to modify proteins that are in the immediate vicinity.

METHODS

Plasmids and antibodies. ITCH and NEDD4 open reading frames (ORFs) were obtained from COS-7 cDNA by PCR. The ITCH ORF is isoform-2 (orthologous to Q96J02-2; UniProtKB), and the NEDD4 ORF is orthologous to A7MD29. These and the ORFs of

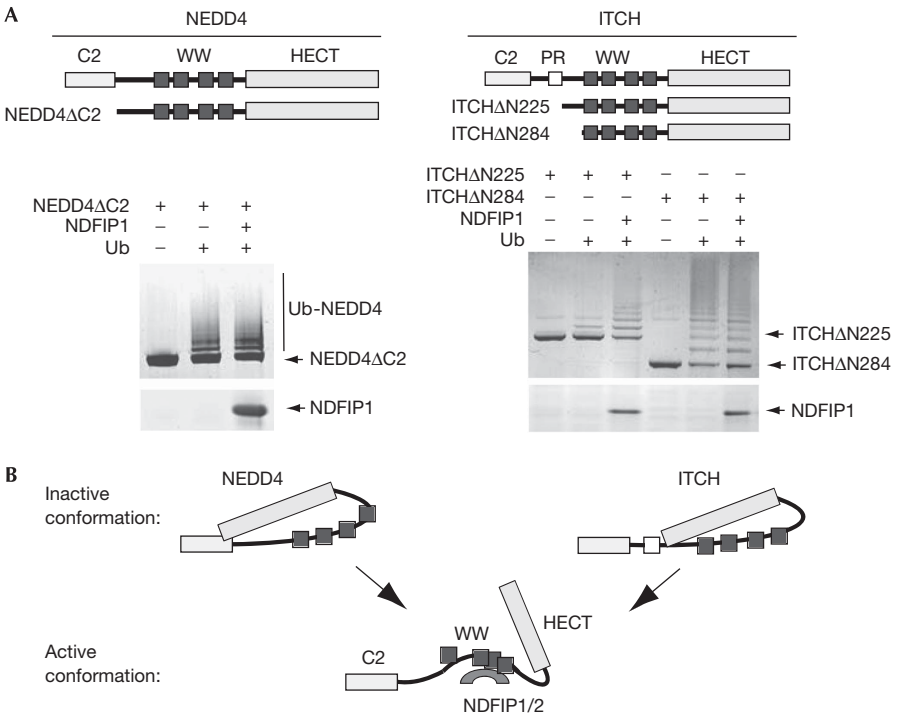

Fig 4 | Sequences required for autoinhibition of NEDD4 and ITCH. (**A**) Ubiquitination assays using His-ubiquitin were Coomassie stained. PR indicates the proline-rich region in ITCH. (**B**) Cartoon indicating proposed model; NDFIP, NEDD4 family-interacting protein.

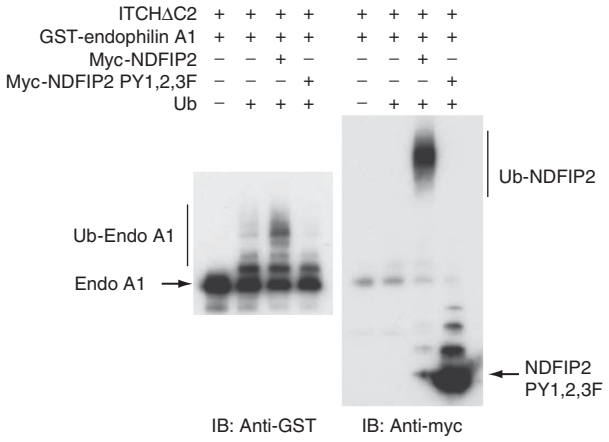

Fig 5 | NDFIP2 promotes the modification of endophilin by ITCH *in vitro*. Ubiquitination assays containing the indicated proteins were analysed by immunoblotting. GST, glutathione S-transferase; NDFIP, NEDD4 family-interacting protein; Ub, ubiquitin.

NEDD4L (Q96PU5-2), WWP1 (Q9H0M0), WWP2 (O00308) and SMURF2 (Q9HAU4) were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) with a triple N-terminal haemagglutinin tag or an N-terminal EGFP. For recombinant protein expression, all ITCH and NEDD4 constructs were inserted into pGEX6P-2 (GE Healthcare, Piscataway, NJ, USA). The following N-terminal deletion mutants were used: ITCH, ΔC2 (aa121–862), ΔN191 (aa192–862), ΔN225 (aa226–862), ΔN284 (aa285–862); NEDD4, ΔC2/ΔN140 (aa141–900). The ORFs of NDFIP1 (Q8R0W6) and NDFIP2 (A6NF21) were cloned into pcDNA3.1 with a single N-terminal Myc tag. Cytoplasmic fragments of NDFIP1 (aa1–113) and NDFIP2 (aa1–134) or the minimal NDFIP2 construct ΔNΔC (aa47–98) were inserted with or without Myc tag into pET28a (Novagen, Madison, WI, USA) to provide an N-terminal His6 tag. The ORFs of rat endophilin A1 (O35179), human c-Jun (P05412) and human JunB (P17275) were cloned into pcDNA3.1 with a triple N-terminal haemagglutinin tag or bacterial expression vectors. Point mutations were: catalytic inactive ITCH C830G, ITCH WW domain mutants (WW1m GW290, 291VC, WW2m GW322,323VC, WW3m GW402,403VC, WW4m GW442,443VC), and the PY motif mutants in the NDFIP ORFs: (L,P)PXY mutated to (L,P)PAG (NDFIP1) or (L,P)PXF (NDFIP2). Antibodies were from Abcam (Cambridge, UK; ITCH, NEDD4) or Sigma (St Louis, MO, USA).

Ubiquitination assays. *In vitro* ubiquitination assays had a total volume of 20 μl consisting of 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.5 mM ATP, 100 ng of human E1 (BIOMOL, Plymouth Meeting, PA, USA), 500 ng of the human E2 UbcH7 (BIOMOL), 5 μg of ubiquitin or, in some experiments, His-ubiquitin (BIOMOL) and 1–2 μg of recombinant bacterially purified glutathione *S*-transferase–E3 (GST-E3; ITCH and NEDD4) and His-NDFIP constructs as indicated. Reactions were incubated for 30–60 min at 30 °C before the addition of 20 μl of 2 × SDS sample buffer. Proteins were separated on 4–12% NuPAGE gels (Invitrogen).

Other methods. Cell culture, immunoprecipitation, immunofluorescence and protein expression in bacteria were performed

using standard procedures, and are described in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

ACKNOWLEDGEMENTS

We thank Jim Sullivan and Marc Fiedler for advice and reagents, Manfred Frick and Ben Nichols for help with confocal microscopy, Harvey McMahon for the endophilin clone, and Elina Nikko and Mike Lewis for helpful comments on the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Abriel H, Loffing J, Rebhun JF, Pratt JH, Schild L, Horisberger JD, Rotin D, Staub O (1999) Defective regulation of the epithelial Na^+ channel by Nedd4 in Liddle's syndrome. *J Clin Invest* **103:** 667–673

Angers A, Ramjaun AR, McPherson PS (2004) The HECT domain ligase itch ubiquitinates endophilin and localizes to the trans-Golgi network and endosomal system. *J Biol Chem* **279:** 11471–11479

Anindya R, Aygun O, Svejstrup JQ (2007) Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1. *Mol Cell* **28:** 386–397

Bai Y, Yang C, Hu K, Elly C, Liu YC (2004) Itch E3 ligase-mediated regulation of TGF-β signaling by modulating smad2 phosphorylation. *Mol Cell* **15:** 825–831

Cristillo AD, Nie L, Macri MJ, Bierer BE (2003) Cloning and characterization of N4WBP5A, an inducible, cyclosporine-sensitive, Nedd4-binding protein in human T lymphocytes. *J Biol Chem* **278:** 34587–34597

Fang D, Kerppola TK (2004) Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. *Proc Natl Acad Sci USA* **101:** 14782–14787

Fang D, Elly C, Gao B, Fang N, Altman Y, Joazeiro C, Hunter T, Copeland N, Jenkins N, Liu YC (2002) Dysregulation of T lymphocyte function in itchy mice: a role for Itch in TH2 differentiation. *Nat Immunol* **3:** 281–287

Flores SY, Debonneville C, Staub O (2003) The role of Nedd4/Nedd4-like dependant ubiquitylation in epithelial transport processes. *Pflugers Arch* **446:** 334–338

Foot NJ, Dalton HE, Shearwin-Whyatt LM, Dorstyn L, Tan SS, Yang B, Kumar S (2008) Regulation of the divalent metal ion transporter DMT1 and iron homeostasis by a ubiquitin-dependent mechanism involving Ndfips and WWP2. *Blood* **112:** 4268–4275

Gallagher E, Gao M, Liu YC, Karin M (2006) Activation of the E3 ubiquitin ligase Itch through a phosphorylation-induced conformational change. *Proc Natl Acad Sci USA* **103:** 1717–1722

Gao M, Labuda T, Xia Y, Gallagher E, Fang D, Liu YC, Karin M (2004) Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase Itch. *Science* **306:** 271–275

Harvey KF, Shearwin-Whyatt LM, Fotia A, Parton RG, Kumar S (2002) N4WBP5, a potential target for ubiquitination by the Nedd4 family of proteins, is a novel Golgi-associated protein. *J Biol Chem* **277:** 9307–9317

Hettema EH, Valdez-Taubas J, Pelham HR (2004) Bsd2 binds the ubiquitin ligase Rsp5 and mediates the ubiquitination of transmembrane proteins. *EMBO J* **23:** 1279–1288

Ingham RJ, Gish G, Pawson T (2004) The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. *Oncogene* **23:** 1972–1984

Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL (2000) Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF β receptor for degradation. *Mol Cell* **6:** 1365–1375

Kerscher O, Felberbaum R, Hochstrasser M (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* **22:** 159–180

Konstas AA, Shearwin-Whyatt LM, Fotia AB, Degger B, Riccardi D, Cook DI, Korbmacher C, Kumar S (2002) Regulation of the epithelial sodium channel by N4WBP5A, a novel Nedd4/Nedd4-2-interacting protein. *J Biol Chem* **277:** 29406–29416

Liu YC (2007) The E3 ubiquitin ligase Itch in T cell activation, differentiation, and tolerance. *Semin Immunol* **19:** 197–205

Lu PJ, Zhou XZ, Shen M, Lu KP (1999) Function of WW domains as phosphoserine- or phosphothreonine-binding modules. *Science* **283:** 1325–1328

Ogunjimi AA, Briant DJ, Pece-Barbara N, Le Roy C, Di Guglielmo GM, Kavsak P, Rasmussen RK, Seet BT, Sicheri F, Wrana JL (2005) Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. *Mol Cell* **19:** 297–308

Oliver PM *et al* (2006) Ndfip1 protein promotes the function of itch ubiquitin ligase to prevent T cell activation and T helper 2 cell-mediated inflammation. *Immunity* **25:** 929–940

Perry WL, Hustad CM, Swing DA, O'Sullivan TN, Jenkins NA, Copeland NG (1998) The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. *Nat Genet* **18:** 143–146

Shearwin-Whyatt LM, Brown DL, Wylie FG, Stow JL, Kumar S (2004) N4WBP5A (Ndfip2), a Nedd4-interacting protein, localizes to multivesicular bodies and the Golgi, and has a potential role in protein trafficking. *J Cell Sci* **117:** 3679–3689

Sullivan JA, Lewis MJ, Nikko E, Pelham HR (2007) Multiple interactions drive adaptor-mediated recruitment of the ubiquitin ligase rsp5 to membrane proteins *in vivo* and *in vitro*. *Mol Biol Cell* **18:** 2429–2440

Verdecia MA, Joazeiro CA, Wells NJ, Ferrer JL, Bowman ME, Hunter T, Noel JP (2003) Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. *Mol Cell* **11:** 249–259

Wiesner S, Ogunjimi AA, Wang HR, Rotin D, Sicheri F, Wrana JL, Forman-Kay JD (2007) Autoinhibition of the HECT-type ubiquitin ligase Smurf2 through its C2 domain. *Cell* **130:** 651–662

Zhu H, Kavsak P, Abdollah S, Wrana JL, Thomsen GH (1999) A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* **400:** 687–693