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The RING Finger Protein RNF8 Recruits UBC13 for Lysine 63-Based Self Polyubiquitylation

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Abstract The heterodimeric ubiquitin conjugating enzyme (E2) UBC13-UEV mediates polyubiquitylation through lysine 63 of ubiquitin (K63), rather than lysine 48 (K48). This modification does not target proteins for proteasome-dependent degradation. Searching for potential regulators of this variant polyubiquitylation we have identified four proteins, namely RNF8, KIA00675, KF1, and ZNRF2, that interact with UBC13 through their RING finger domains. These domains can recruit, in addition to UBC13, other E2's that mediate canonical (K48) polyubiquitylation. None of these RING finger proteins were known previously to recruit UBC13. For one of these proteins, RNF8, we show its activity as a ubiquitin ligase that elongates chains through either K48 or K63 of ubiquitin, and its nuclear co-localization with UBC13. Thus, our screening reveals new potential regulators of non-canonical polyubiquitylation. J. Cell. Biochem. 97: 572–582, 2006.

Key words: polyubiquitylation; RING finger; ubiquitin ligase; protein–protein interaction

Ubiquitylation occurs in distinct steps that use three types of enzymatic activities [Hershko and Ciechanover, 1998; Pickart, 2001]: ubiquitin-activating enzymes or E1, ubiquitin-conjugating enzymes or E2, and ubiquitin-protein ligases or E3. An ubiquitin polypeptide bound to a substrate protein can be conjugated to additional ubiquitin moieties to form polyubiquitin chains, through the formation of isopeptide bounds between the carboxy terminal glycine residue on the donor ubiquitin moiety and one of seven lysine residues present on the acceptor ubiquitin moiety [Sloper-Mould et al., 2001]. Polyubiquitylation through lysine 48 of ubiquitin (K48), with a minimum of four ubiquitin moieties in the chain [Thrower et al., 2000], entails the recognition of the conjugated protein by the proteasome [Lam et al., 2002] and subsequent proteolytic degradation of the targeted protein. Polyubiquitylation through lysine 63 (K63) does not appear to involve recognition by the proteasome or to the proteolytic degradation of the proteins carrying this modification [Deng et al., 2000; Spence et al., 2000]. Less is known about the consequences of polyubiquitylation through the use of lysines at positions other than 48 or 63 on the ubiquitin polypeptide, such as K29 [Arnason and Ellison, 1994; Mastrandrea et al., 1999; You and Pickart, 2001; Russell and Wilkinson, 2004] or other lysines [Peng et al., 2003; Wu-Baer et al., 2003; Nishikawa et al., 2004].

While polyubiquitylation through K48 can be catalyzed by many E2 enzymes, polyubiquitylation through K63 appears to be mediated exclusively by the heterodimer formed by UBC13 and UEV/Mms2 [Hofmann and Pickart, 1999, 2001], in which UBC13 bears the catalytic site containing the reactive cysteine residue, whereas UEV, structurally a member of the E2

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superfamily [Sancho et al., 1998; Moraes et al., 2001; VanDemark et al., 2001], lacks a catalytic center [Sancho et al., 1998]. UBC13 has been shown to interact with a number of proteins bearing RING finger domains, which act as ubiquitin ligases or E3 that tether substrates for the K63 polyubiquitylation by UBC13/UEV [Deng et al., 2000; Ulrich and Jentsch, 2000], including the RING finger domain-containing E3's themselves [Deng et al., 2000; Ulrich and Jentsch, 2000; Wang et al., 2001; Didier et al., 2003]. Polyubiquitylation through K63 regulates key cellular processes, such as postreplicative DNA repair [Ulrich and Jentsch, 2000; Hoege et al., 2002; Haracska et al., 2004], mitotic progression [Bothos et al., 2003], signal transduction [Deng et al., 2000; Wang et al., 2001], vacuolar protein targeting [Galan and Haguenauer-Tsapis, 1997], or cytoskeleton dynamics [Didier et al., 2003].

In a search of potential substrates of K63 polyubiquitylation and to identify cellular processes regulated by this post-translational modification, we have performed yeast two-hybrid screenings of human proteins that interact with UBC13. With the exception of UEV, all the UBC13-interacting proteins identified in our screenings contain RING finger domains, and interact with UBC13 through this motif. We also show that one of the UBC13/UEV-interacting RING finger proteins identified in our screening, RNF8, previously described as a E3 ubiquitin ligase [Ito et al., 2001] and an interaction partner for the nuclear receptor regulator RXRα [Takano et al., 2004], can accommodate on its RING finger domain several E2's, including UBC13, and functions as an E3 enzyme onto itself with the conjugation of more than one class of polyubiquitin chains, namely those that use K29, K48, and K63.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening

Full-length cDNA for human UBC13 was generated by RT-PCR from the cell line HepG2, and cloned in frame with the Gal4 DNA-binding domain in pBD (Stratagene, La Jolla, CA). pBD-UBC13 was co-transfected into the *S. cerevisiae* strain AH109 together with either a human fetal brain cDNA library or a prostate cancer library cloned in pACT2 (Clontech, Palo Alto, CA). Shuttle plasmids were isolated by phenol–chloroform extraction, rescued in *E. coli* and retransformed into AH109. Inserts from confirmed interaction positives were analyzed by sequencing. Full-length cDNA's for human UBCH7, UBE2E2, CDC34, and UBCH6 were generated by RT-PCR, and cloned in pAS2, as described [Ito et al., 2001]. cDNA fragments containing the RING finger domains of CHFR (Acc. NM_018223), NY-REN-43 (RNF12, Acc. NM_183353), GOLIATH (RNF24, Acc. NM_007219), RNF139 (Acc. NM_007218), and BMI1 (RNF51, Acc. NM_005180) were amplified by PCR from a human fetal brain or a prostate cancer cDNA libraries (Clontech) and cloned in pACT2 (Clontech). All cDNA fragments for the selected RING finger proteins were designed such that they contained, in addition to the sequences coding for the RING finger domain, sequences coding for predicted alpha-helical or coiled-coil regions located at either end of the RING finger domain. Colorimetric assays for β-galactosidase activity were performed on yeast cells co-transformed with constructions that yielded positive interactions, and liquid cultures grown overnight, lysed by freeze-thaw, and the lysates assayed for enzyme activity using ONPG as a substrate. One unit of β-galactosidase activity was defined as the amount that hydrolyzes 1 mmol of ONPG per minute per cell. To assess the expression of RING finger domains, lysates from transfected yeast cells were subjected to Western blotting with anti-HA antibodies (Roche, Barcelona, Spain).

Real-Time RT-PCR

RT-PCR products were quantitated by SYBR Green incorporation on an ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA). Collections of human cDNAs were purchased from Clontech. Reaction conditions were 95°C for 15 min, 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. All determinations were performed in triplicate. Levels of ribosomal S14r amplification were used as an endogenous reference to normalize each sample value of C_t (threshold cycle) and the tissue which showed the lowest expression of the studied gene was used as calibrator for the rest of the tissues. The final results, expressed as n-fold differences in target gene expression were calculated as follows:

$$n_{target} = 2^{-[(C_t\ target - C_t\ reference)\ tissue - (C_t\ target - C_t\ reference)\ lowest\ tissue]}$$

Expression Plasmid Constructs, Site-Directed Mutagenesis, and Cell Transfection

pFLAG-UBC13 was generated by subcloning full-length UBC13 cDNA into pFLAG-CMV-6c (Sigma, Madrid, Spain). pHA-RNF8 was generated by inserting full-length RNF8 into pACT2. The resulting HA-tagged RNF8 was excised with *Bgl* II and *Xho* I, and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). To generate pHA-Ub, the cDNA for ubiquitin was amplified from Cos7-derived cDNA and cloned into pCMV-HA (Clontech). Construct pHis$_6$-RNF8 was generated by inserting full-length RNF8 cDNA into the *Eco* RI and *Xho* I sites of pcDNA3.1HisC (Invitrogen).

For site-directed mutagenesis, the QuickChange procedure (Stratagene) was used. Cysteine 403 of RNF8 was mutagenized to serine, using as templates pHA-RNF8 or pHis$_6$-RNF8. For the generation of single and multiple-site ubiquitin mutants in which lysine residues at positions 29, 48, or 63 were mutagenized to arginine, pHA-Ub was mutagenized at one of the positions, and subsequently mutagenized to a second position. To obtain a dominant negative variant of UBC13 [Luftig et al., 2003], cysteine at position 87 was mutagenized to alanine to yield pUBC13C87A.

For transfections, Cos-7 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories, Linz, Austria) supplemented with 10% fetal bovine serum and antibiotics in 5% CO_2. Cells were seeded on petri dishes or sterile glass coverslips at 60%–80% confluence and transfected the following day with cationic liposomes (lipofectamine, Invitrogen).

Co-Immunoprecipitation

Transfected cells were washed twice with PBS and lysed with lysis buffer (50 mM Tris HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, and protease inhibitors). Lysates were precleared for 30 min with Sepharose and incubated with matrix-bound anti-FLAG antibody (Sigma) or Sepharose for 3 h at 4°C. The matrix was washed three times with lysis buffer and the immune complexes eluted by boiling in Laemmli sample buffer. Samples were electrophoresed by SDS–PAGE, and processed for Western blotting with mouse anti-FLAG M2 monoclonal antibody (Sigma) and goat anti-mouse IgG-peroxidase (Dako, Glostrup, Denmark), or rat anti-HA monoclonal antibody (Roche, Mannheim, Germany) and goat anti-rat IgG-peroxidase (Dako), followed by ECL detection (Amersham, Buckinghamshire, England).

Generation of Antibodies

To generate anti-RNF8, rabbits were immunized with the synthetic peptide KNKELRTKRKC conjugated to KLH. The specificities and reactivities of the sera were assessed by ELISA and Western blotting, and the antibodies were purified by affinity chromatography on peptides immobilized on SulfoLink columns (Pierce, Rockford, IL).

In Vivo Ubiquitylation

Transfected cells were washed twice with PBS and scraped in 6 M guanidine buffer at room temperature. Lysates were batch-incubated with ProBond matrix (Invitrogen) for 1 h at room temperature, transferred to a column and washed twice with denaturing binding buffer at pH 7.8, twice with denaturing wash buffer at pH 6.0 and twice with the same buffer at pH 5.3. Columns were eluted with 25 mM Tris-HCl, pH 7.5, 200 mM imidazol. Eluted samples were boiled with Laemmli sample buffer, separated electrophoretically on SDS–PAGE, and transferred to PDVF membranes. Membranes were processed as described earlier, blotted with anti-HA, and reactions were detected with ECL chemiluminescent assays (Amersham).

RESULTS AND DISCUSSION

A Yeast Two-Hybrid Screening for Human Proteins That Interact With UBC13

We used UBC13 as a bait in two yeast two-hybrid screenings against cDNA libraries from

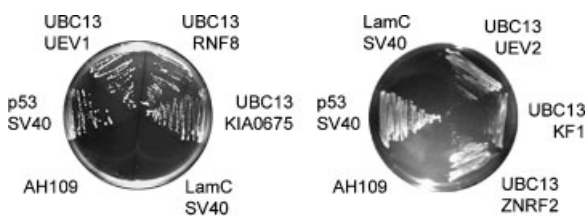

Fig. 1. UBC13 interactors retrieved from yeast two-hybrid screenings with cDNA libraries from human fetal brain (**left**) and prostate cancer (**right**). Negative controls included the untransfected yeast strain AH109 and cells co-transfected with SV40 large T and lamin C. As a positive control for interaction, cells were co-transfected with SV40 large T and p53.

human fetal brain and prostate cancer (Clontech). Out of 3×10^6 independent clones obtained from both screenings, we confirmed interactions with UBC13 for clones bearing inserts corresponding to six distinct proteins (Fig. 1): UEV1 and UEV2, whose interaction with UBC13 provided a good control of the specificity of the interactions yielded by the screening; a fragment of RNF8, a protein containing a RING finger domain, and previously shown by others to interact with other E2's [Ito et al., 2001], that included a predicted coiled-coil region, the RING finger domain, and the carboxy terminal tail of the protein; a fragment of the KIAA0675 protein, also known as hRUL138 and DZIP3 [Kreft and Nassal, 2003; Moore et al., 2003], containing a predicted coiled-coil region, the RING finger domain, and the carboxy terminal tail; a fragment of KF1, a RING finger protein with a HTH motif and putative transmembrane domains [Yasojima et al., 1997], containing the RING finger domain and the carboxy terminal tail of the protein; and a fragment of ZNRF2, with one Zn finger motif in close association with its carboxy terminal RING finger domain [Araki and Milbrandt, 2003], containing the RING finger domain and the carboxy terminal end of the protein.

A BLAST search for human RING finger domains similar to those isolated in our screenings human yielded two distinct groups of RING finger domains (Fig. 2). The RING finger domains most similar to that of RNF8 show a C3HC4 pattern of cysteine and histidine residues, and those most similar to that of KIAA0675 are of the C3H2C3 type. Ten of the these RING finger domains were isolated and used in yeast two-hybrid assays to determine their capacity to interact with E2 proteins, including UBC13, UBE2E2, UbcH6, UbcH7, and Cdc34. The protein fragments selected for this analysis were constructed such that they contained the corresponding RING finger domains and additional flanking sequences of 30–70 residues of predicted alpha helical or coiled-coil regions at either end of the RING finger domains (see "Materials and Methods"). Several of the selected RING finger domains indeed interacted with UBC13 (Fig. 3), including CHFR, previously reported to recruit UBC13 [Bothos et al., 2003], and also proteins not previously known to interact with UBC13, such as ZNRF2. The RING finger domains that interact with UBC13 interact also with at least one of the other E2's used in the assay. Specifically, the RING finger domains from RNF8 and CHFR interacted with UBC13 and UBE2E2, and the RING finger domain from KIAA0675 interacted with UBC13, UBE2E2, and UbcH6. None of the RING finger domains studied in this analysis interacted with Cdc34 (Fig. 1C,D). Several of the selected RING finger domains did not interact with any of the E2's included in this study. Therefore, not unexpectedly, these experiments show that a given E2 can interact with multiple RING finger domains, and, conversely, that a given RING finger domain has the capacity to interact with more than one E2.

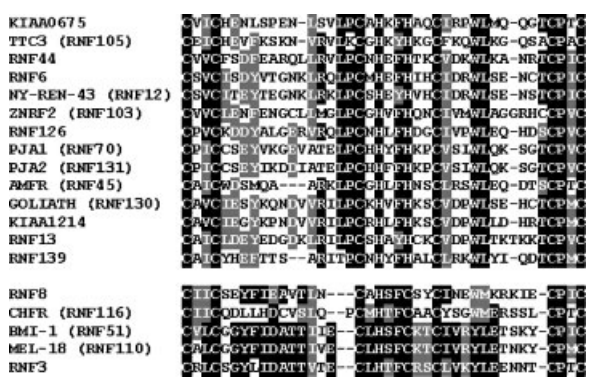

Fig. 2. Sequence alignment of RING finger domains with the highest similarities to the RING finger domains of proteins found to interact with UBC13 in the yeast two-hybrid screening as shown in Figure 1.

The occurrence of these cross-interactions between different E2's with a variety of RING finger domains is well documented [Ito et al., 2001; Ulrich, 2003; Winkler et al., 2004], and reflects the fact that a given RING finger domain can recruit more than one type of E2. In most cases the different E2's thus recruited may have interchangeable functions in the ubiquitylation of substrates simultaneously recruited to the E3, and the choice of E2 to be tethered to the complex may depend solely on its availability in a particular cell type or process, or coincident subcellular localizations. The recruitment of UBC13 and other E2's to the RING finger domains studied here implies that the same E3 can function as a ligase in more than one class of polyubiquitylation, and therefore the specific E2 recruited determines the function of the E3 in the final fates of the modified substrate proteins. This is likely to be the case for CHFR in K48 and K63 polyubiquitylations [Scolnick and Halazonetis, 2000;

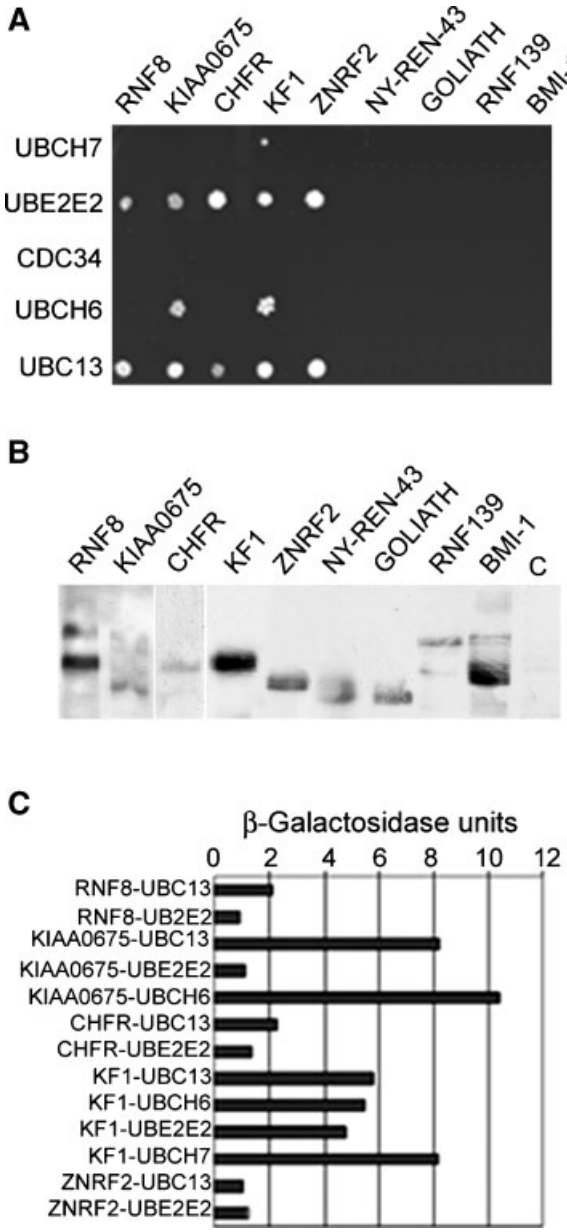

Fig. 3. Specificity of interaction of RING finger domains with different E2 enzymes. **A**: Growth of yeast cells co-transfected with the shown combinations of proteins. **B**: Western blotting showing the expression of the indicated RING finger domains in the co-transfections shown in (A), detected with anti-HA antibody. **C**: Normalized β-galactosidase activities of AH109 yeast cells co-transfected with the indicated combinations of E2 proteins and RING finger domains.

Chaturvedi et al., 2002; Kang et al., 2002; Bothos et al., 2003], and in this sense would be similar to the recruitment of Ubc9 or UbcH7 for the alternative functions of Mdm2 and other E3's in either sumoylation or K48 polyubiquitylation of substrates [Buschmann et al., 2000].

Domain Features of RING Finger Proteins Interacting With UBC13

Several proteins identified in our screening were not previously known to interact with UBC13. RNF8 has been described as a E3 ubiquitin ligase that recruits several possible E2's [Ito et al., 2001; Takano et al., 2004], but its interaction with UBC13 has not been previously reported. In addition to its RING finger domain at its carboxy terminus, RNF8 contains a coiled-coiled region adjacent to the RING finger domain and a forkhead-associated (FHA) domain near its amino terminus (Fig. 4). FHA domains are structural motifs that recognize phosphopeptides in particular sequence contexts [Liao et al., 1999; Durocher et al., 2000]. In addition to RNF8, only three other proteins contain a combination of a RING finger domain and a FHA domain (Fig. 4): In metazoans, CHFR, a mitotic checkpoint regulator [Kang et al., 2002; Seong et al., 2002]; in fission yeast, Dma1p, a regulator of the septation initiation network [Murone and Simanis, 1996; Guertin et al., 2002], and its orthologues in budding yeast, Dma1p and Dma2p, that function in the spindle position checkpoint and the mitotic exit network [Fraschini et al., 2004]. CHFR has recently been shown to interact with UBC13, and to function as a E3 ligase for the K63 polyubiquitylation of substrates, including itself [Bothos et al., 2003]. To our knowledge, the yeast proteins Dma1p and Dma2p have not been explored with regards to their possible interaction with UBC13.

KIAA0675 is also known as hRUL138 [Kreft and Nassal, 2003] or DZIP3 [Moore et al., 2003]. This large protein has been reported to be cytoplasmically located and to bind to hepatitis B viral RNA [Kreft and Nassal, 2003] and, independently, to bind to DAZ, a protein that regulates germ cell development [Moore et al., 2003]. Our analysis shows that KIAA0675 bears, in addition to a RING finger domain located near its carboxy terminus, a region with a predicted coiled-coil structure, and several tetratricopeptide repeats, loosely conserved motifs that engage in protein–protein interactions [Goebl and Yanagida, 1991; Lamb et al., 1995; Das et al., 1998]. The motif composition and architecture of KIAA0675 is very similar to that of a second protein known as TTC3 [Tsukahara et al., 1996] or DCRR1 [Eki et al., 1997], that bears tricopeptide repeats, coiled-

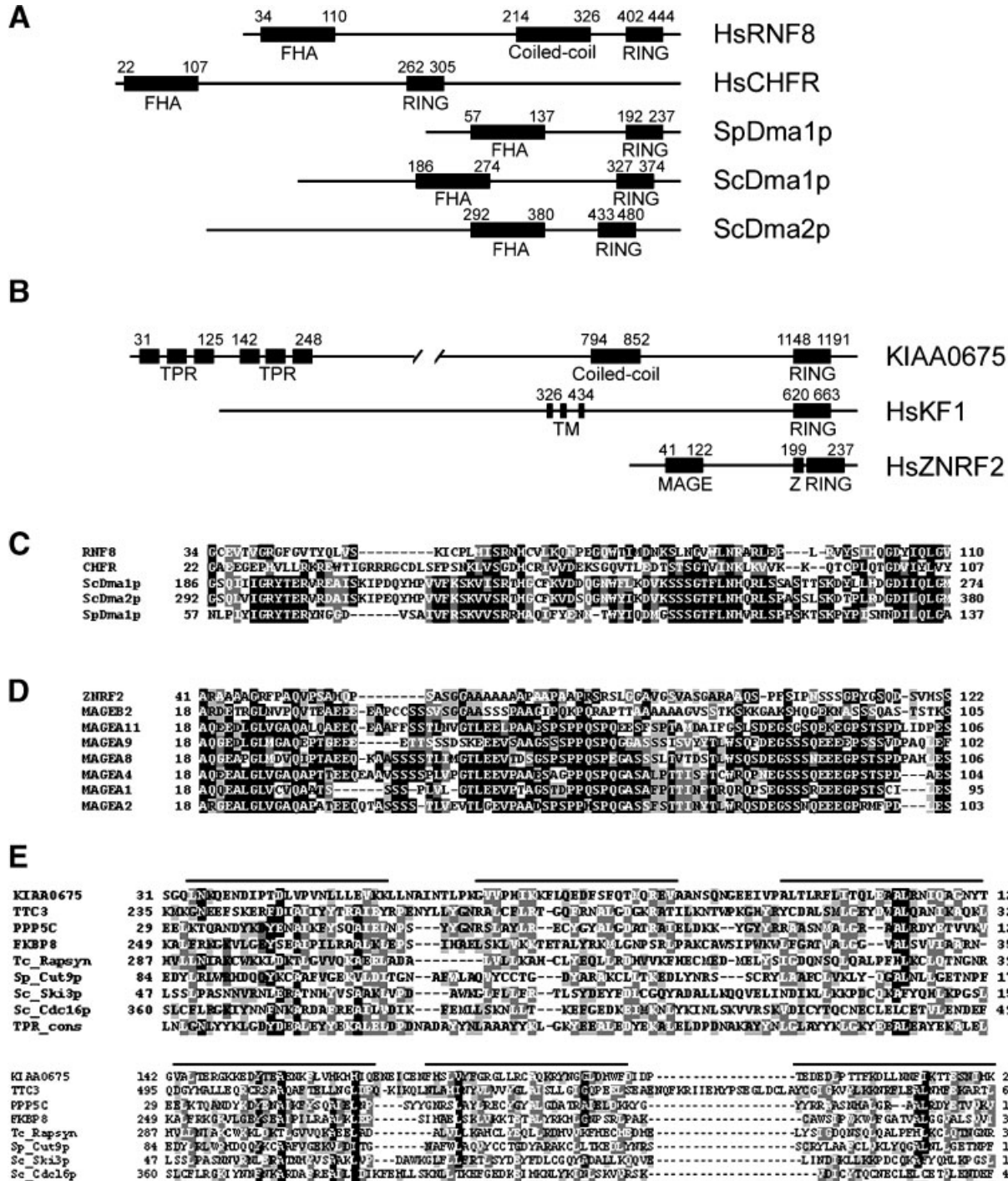

Fig. 4. **A**: Schematic depiction of the domain architecture of proteins interacting with UBC13. RNF8 bears one forkhead-associated (FHA) domain and a RING finger domain (R). CHFR in metazoans, Dma1 in *Schizosaccharomyces pombe* and Dma1p and Dma2p in *Saccharomyces cerevisiae* share this domain architecture. RNF8 also contains a predicted coiled-coil region at the amino end of its RING finger domain. KIAA0675 bears a carboxy terminally placed RING finger domain, several tetratricopeptide repeats (TRP) and a predicted coiled-coil region. KF-1 contains a RING finger domain at its carboxy end, a predicted helix-turn-helix (HTH) motif, and several potential transmembrane sequences. ZNFR2 contains a special domain that combines a RING finger and a C_2H_2-type zinc finger at its carboxy terminus. **B**–**D**: Alignments of the FHA, TRP and MAGE domains of RNF8, KIAA0675, and ZNRF2 with sets of reference domains in the Pfam database.

coiled regions and a carboxy terminal RING finger domain in the same order as KIAA0675. The gene for TTC3 is located at chromosome 21q22.2, at the Down syndrome critical region 1. The gene for KIAA0657 is located at 3q13.13.

KF1 contains a helix-turn-helix motif and several hydrophobic stretches predicted as potential transmembrane domains (Fig. 4). This protein, expressed in the cerebellum and the hippocampus [Yasojima et al., 1997], has not been studied functionally so far. ZNRF2 contains one Zn finger motif in close association with its RING finger domain at its carboxy terminus (Fig. 4). ZNRF2 is a paralogue of ZNRF1, also called PNIP or peripheral nerve injury protein, a protein identified in the course of a search for proteins upregulated, and with potential functions, in response to chemical and excitotoxic nervous damage [Araki et al., 2001]. ZNRF1 and ZNRF2 are associated with presynaptic vesicles and the presynaptic plasma membrane, respectively, and both have been shown to inhibit Ca^{2+}-dependent exocytosis dependent on their ubiquitin-ligase activities [Araki and Milbrandt, 2003].

Real-time RT-PCR was performed to assess the levels of expression of in human tissues of two of these genes, *RNF8* and *KIAA0675*, which

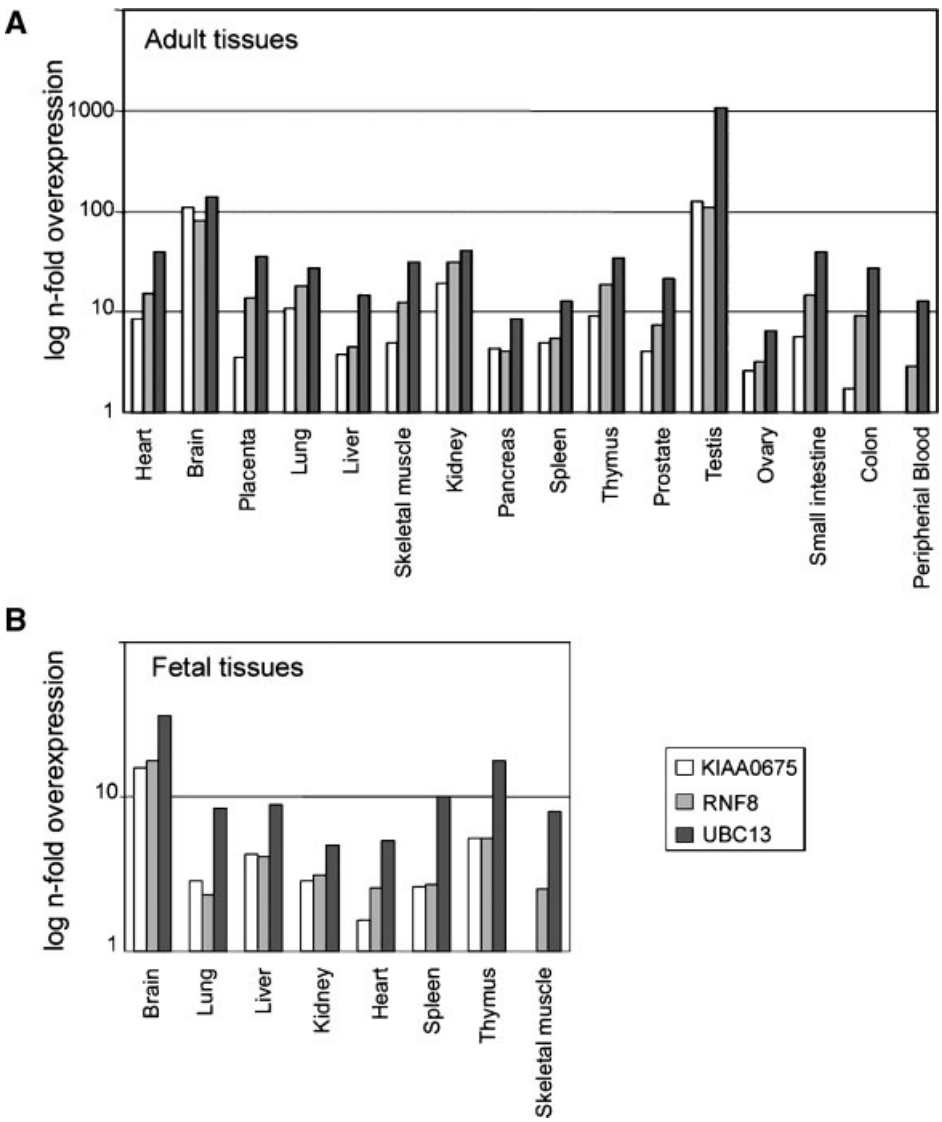

Fig. 5. Relative expression levels of the genes for UBC13 and the UBC13-interacting proteins RNF8 and KIAA0675 in normal human adult (**A**) and fetal (**B**) tissues, determined by real-time RT-PCR. cDNA collections from adult (A) or fetal (B) tissues (Clontech) were used as templates. C_t values were normalized in each case against values obtained for the reference gene S14r, and then further normalized against the tissue with the lowest expression levels in each set, adult or fetal.

were the first to be obtained in our screening for UBC13-interacting proteins. In human fetal tissues, expression of RNF8 transcripts is highest in brain, thymus, and liver, while in adult tissues, expression is very high in brain, and testis, and low in peripheral blood (Fig. 5). As reported by others [Kreft and Nassal, 2003], expression levels of KIAA0675 is generally low in all tissues tested, showing a tissue distribution that is similar to that of RNF8, with the highest levels in brain and thymus in fetal tissues, and in brain and testis in adult tissues (Fig. 5). The expression levels of UBC13 were highest in brain, thymus, skeletal muscle, and spleen in fetal tissues, and in testis and brain in adult tissues (Fig. 5). Therefore, all three genes are co-expressed at significant levels in several tissues, notably brain and thymus in fetal tissues, and brain and testis in adult tissues.

RNF8 Is a Ubiquitin Ligase In Vivo for K48 and K63 Polyubiquitin Chains

Of the UBC13-interacting RING finger proteins found in our screenings, RNF8 is the protein for which more functional data are available, and thus we studied its capacity to elongate non-canonical polyubiquitin chains in vivo. First, we determined that RNF8 associates with UBC13 in mammalian cells in vivo, as shown by co-immunoprecipitation experiments in transfected Cos-7 cells (Fig. 4A). This interaction required the integrity of the RING finger domain of RNF8, since a point mutation to alanine of the cysteine at position 403, that coordinates with Zn atoms in the RING finger domain, resulted in a complete loss of the ability of RNF8 to bind to UBC13 (Fig. 6A). Both HA-tagged wild-type and endogenous RNF8 were detected exclusively in the cell nucleus under standard culture conditions (Fig. 6B). In contrast, the RING finger mutant form of RNF8 ($RNF8^{C403S}$) was also seen in a cytoplasmic localization that excluded the nucleus in a variable proportion of transfected cells (Fig. 6B). UBC13 was detected in several subcellular localizations, that included the cell nucleus, cell membrane, and the cytoplasm (Fig. 6B,C). Upon co-transfection with HA-RNF8, the signal for nuclear localization of UBC13 was enhanced, with a corresponding decrease in the intensity of other subcellular localizations (Fig. 6C). This increase in the relative intensity of nuclear UBC13 was not seen when the co-transfection was done with the

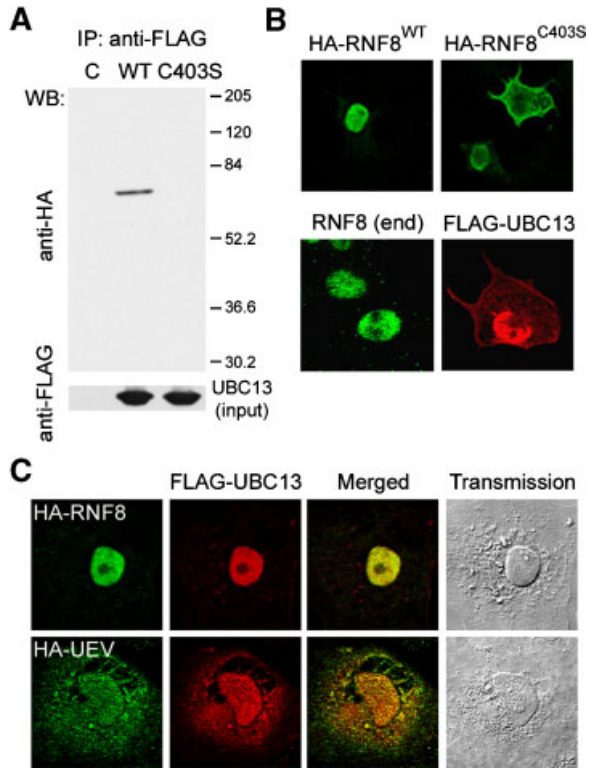

Fig. 6. Interaction between RNF8 and UBC13 in mammalian cells. **A**: UBC13 pulls down wild-type RNF8, but not a variant bearing a mutation in its RING finger domain ($RNF8^{C403S}$). Extracts of Cos-7 cells co-transfected with FLAG-UBC13 and either wild-type (WT) or mutant (C403S) HA-tagged RNF8 were immunoprecipitated with anti-FLAG and blotted with either anti-HA for the detection of HA-RNF8, or anti-FLAG to allow for comparison of input co-transfected FLAG-UBC13 (**bottom**). **B**: Subcellular localizations of wild-type and mutant (C403S) HA-RNF8 and of FLAG-UBC13 in transfected Cos-7 cells stained with anti-HA or anti-FLAG, and endogenous RNF8 stained with affinity-purified rabbit anti-RNF8. **C**: Immunolocalization of co-transfected HA-RNF8 and FLAG-UBC13 (**top panel**) and HA-UEV and FLAG-UBC13 (**bottom panel**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mutant $RNF8^{C403S}$ (not shown), or in co-transfections of FLAG-UBC13 with HA-UEV (Fig. 6C). These observations suggest the occurrence of a nuclear retention of UBC13 in association with high levels of RNF8, that is dependent on its interaction with the RING finger domain of the latter protein.

To test the possible function of RNF8 as a ubiquitin ligase (E3) in vivo, and to analyze the class of polyubiquitin chains supported by this activity, constructs for the expression of HA-tagged wild type and mutant ubiquitin were generated, namely UbK48,63R, UbK29,63R, and UbK29,48R, each carrying two Lys → Arg

mutations at the indicated positions. Co-transfection of these constructs with pHis$_6$-RNF8^{WT} showed that wild-type RNF8 can be modified by all three mutant ubiquitins, implying the formation of polyubiquitin chains that use predominantly the lysines at positions 29, 48, and 63, although the latter with less efficiency (Fig. 7A). In contrast, the RING-dead mutant RNF8^{C403S} fails to be modified by UbK29,63R and UbK29,48R but is still modified by UbK48,63R (Fig. 7A). To further confirm that polyubiquitylation of RNF8 through K63-based chains is dependent on UBC13, a dominant negative variant was generated and co-transfected together with RNF8 and UbK29,48R. As shown in Figure 7B, overexpression of the UBC13C87A variant significantly inhibited the K63-based polyubiquitylation of RNF8.

We conclude that RNF8 functions as a self ubiquitin ligase for polyubiquitylation through lysines 48 and 63 of ubiquitin. We show that the latter modification is mediated by UBC13. Our experiments also show that polyubiquitylation of RNF8 with K29-type polyubiquitin chains does not require its own functional RING finger domain, suggesting that this modification of RNF8 is mediated by a different E3, rather than by itself. Finally, the lack of detectable polyubiquitylation of the RING-dead mutant of RNF8 by the ubiquitin variants with lysines mutated at residues 48 and 63 would argue that lysines on the ubiquitin molecule other than those at positions 29, 48, or 63 are not likely to be

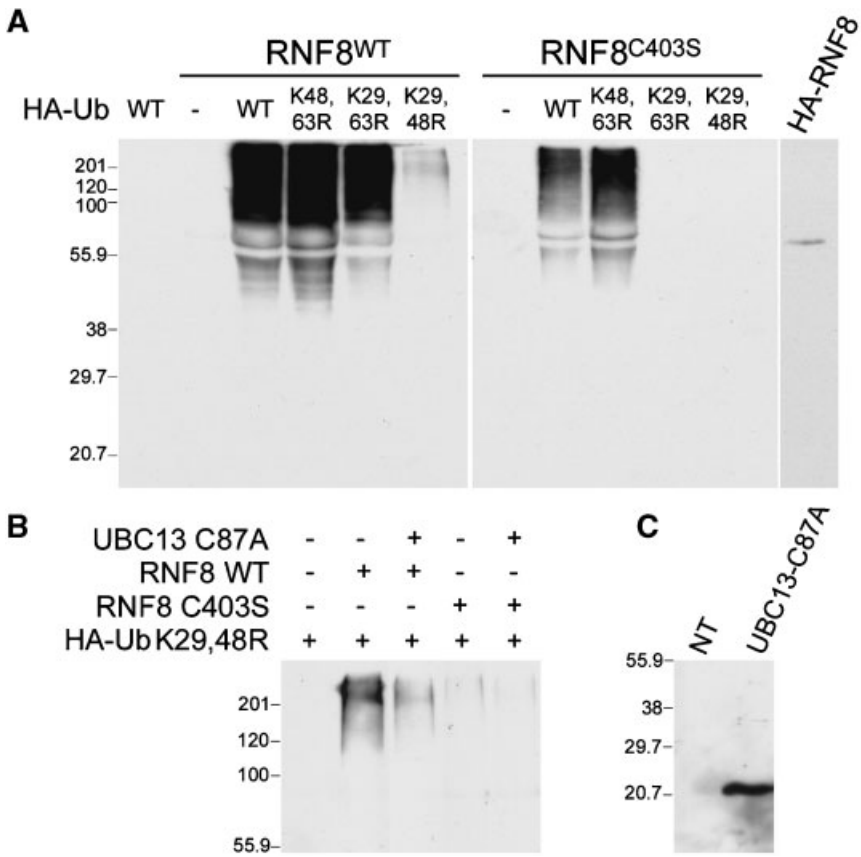

Fig. 7. **A**: In vivo ubiquitin-ligase activity of RNF8 with specificity for different classes of polyubiquitin chains. Extracts of HeLa cells co-transfected with wild-type or RING finger mutant His$_6$-RNF8 and HA-tagged ubiquitin variants were enriched for polyhistidine-containing proteins by Ni affinity chromatography, and analyzed for ubiquitylation of RNF8 by Western blotting with anti-HA antibodies. One lane (HA-RNF8) was run in parallel and blotted with anti-HA, to show the size of the unmodified transfected HA-tagged RNF8. It can be seen in the RNF8 is heavily ubiquitylated by the Ub variants with available lysine residues at positions 29 and 48, and less quantitatively by Ub with lysine available at position 63. **B**: K63-based polyubiquitylation of RNF8 is dependent on UBC13. Wild-type or RING-dead His$_6$-RNF8 and the ubiquitin variant UbK29,48R were co-transfected into HeLa cells with the dominant negative variant UBC13C87A, and processed as in (A). A significant inhibition of K63-based ubiquitylation is seen by co-expression with UBC13C87A. **C**: Western blotting showing the prototypical expression of the dominant negative mutant UBC13C87A in transfected HeLa cells.

used to a significant degree for the polyubiquitylation of RNF8.

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