The RING-H2 protein RNF11 is overexpressed in breast cancer and is a target of Smurf2 E3 ligase

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Breast malignancy at the molecular level is partly due to genetic mutations and deletions that result in the alteration of expression and function of various cellular genes. Although progress has been made in characterising some of these genes, the specific molecular mechanisms underlying many of the cellular pathways remain to be elucidated. Once identified, such differentially expressed genes will be useful in monitoring disease progression and understanding the molecular mechanisms of tumour development. In addition, the molecular definition of new genes involved in breast tumours will yield novel targets for new therapeutic strategies.

The T2A10 gene was originally cloned as a partial cDNA from a library enriched for breast tumour messenger ribonucleic acids. Our survey of 125 microarrayed primary tumour tissues using affinity purified polyclonal antibodies has revealed that corresponding protein is overexpressed in invasive breast cancer and is weakly expressed in kidney and prostate tumours. Now known as RNF11, the gene encodes a RING-H2 domain and a PY motif, both of which mediate protein–protein interactions. In particular, the PPPPY sequence of RNF11 PY motif is identical to that of Smad7, which has been shown to bind to WW domains of Smurf2, an E3 ubiquitin ligase that mediates the ubiquitination and degradation of the TGFβ receptor complex. Using various mutants of RNF11 in GST pulldown and immunoprecipitation assays, we found that RNF11 interacts with Smurf2 through the PY motif, leading to ubiquitination of both proteins. Smurf2 plays an active role in the repression of TGFβ signalling, and our data indicate that overexpression of RNF11, through its interaction with Smurf2, can restore TGFβ responsiveness in transfected cells.

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Antibodies

C14N polyclonal antibody was raised in rabbits against a synthetic hexadecapeptide sequence derived from the RNF11 C-terminal region conjugated to keyhole limpet haemocyanin (KLH). Rabbit

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IgG polyclonal antibodies were purified on a peptide affinity column. FLAG (M2), β-actin and gluthathione-S-transferase (GST) antibodies were purchased from Sigma. Haemagglutinin (HA) antibody (12CA5), which recognises a nonapeptide sequence (YPYDVPDYA), derived from the influenza haemagglutinin protein, was from Roche. Anti-polyhistidine (HIS-tag) antibody was from R&D systems. Protein A-sepharose beads were from Sigma and glutathione sepharose beads were from Amersham Pharmacia.

**Tumour tissue microarray (TMA) and immunohistochemistry (IHC)**

Tumour cases were selected from the archives of the Department of Anatomic Pathology, Sunnybrook and Women’s College Health Sciences Centre. Following selection of tumour blocks and verification of the diagnosis, tissue was anonymised by stripping all identifiers from each case. Areas of cancer and normal tissue elements were marked on haematoxylin and eosin (H&E) sections by a pathologist (JZ). A multi TMA was assembled consisting of 20 breast, 15 prostate, 17 colon, 11 head and neck, seven lung, 17 renal and 22 pancreas carcinomas by punching 0.6 mm tissue cores from the donor block and transferring them to a recipient block. A total of 125 cases were arrays in duplicates using two blocks for each case. The array was essentially constructed and transferred onto slides following the TMA methodology as described (Kononen et al, 1998).

**Tumour-specific RNF11 expression was monitored by IHC using the Histostain SP rabbit kit with DAB substrate from Zymed (San Francisco, CA, USA). The primary RNF11 antibody (0.4 mg ml–1, 1:125 dilution) was used to probe TMA sections processed and evaluated as reported previously (Landberg et al, 2001). For negative controls, RNF11 antibody was preincubated with the RNF11 peptide (10:1 ratio with antibody) used for immunisation 1 h prior to the reaction with the TMA. Results are representative of three independent experiments.**

**Vectors**

Human Flag-Smurf2 and HA-tagged deletion mutants of Smurfs, HA-tagged Smad7, p3TP-Lux, polyhistidine (HIS) tagged Ubc3, Ubc11sa, Ubc11sb, Ubc15, and HA-tagged Ub are described by Kavak et al (2000) and Bonni et al (2001). RNF11 and the engineered mutants of RNF11 were derived from the RT–PCR cloned ORF of RNF11 in vector plasmid pCMV-T2C using the Quikchange Site Directed Mutagenesis Kit (Stratagene). For the RNF11-mtPY construct, the tyrosine at position 40 was substituted with alanine, and in the RNF11-mtRING construct the cysteines at positions 89 and 93 were mutated to serine 89 and serine 93. The RNF11-ntDouble mutant has both the PY and the Ring mutation.

**RESULTS**

**RNF11 protein is overexpressed in breast tumours**

RNF11 protein expression in primary tumours was monitored by IHC using affinity purified anti-human RNF11 polyclonal antibody on a TMA composed of duplicate punches from 125 primary tumours representing eight different histologies. IHC results for the TMA are summarised in Table 1, and indicate that RNF11 is predominantly expressed in the cytoplasm of cancer cells (Figure 1A, B, Table 1). Staining of primary breast tissues with anti-RNF11 antibody showed that the corresponding protein is high in cancer cells, but low in normal ductal structures and absent in surrounding stroma (Figure 1B). Strong cytoplasmic RNF11 staining was found in >90% of the invasive breast cancer cases, in adenocarcinomas of the pancreas (78%), colon cancer (47%) and in bladder tumours (31%) (Table 1, Figure 1). Table 2 summarises the RNF11 staining with clinicopathologic features such as oestrogen and progesterone receptor status (ER and PR), Bloom–Richardson grading (BR grade), lymph node (LN) status and histology.

**Immunoprecipitation and Western blots**

Cell lysates were immunoprecipitated (IP) with mouse anti-FLAG, anti-GST or anti-HA antibody, and Western blots were performed as described (Winberg et al, 2000). Briefly, 42–48 h post-transfection, cells were lysed in 900 μl of lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.1% NP-40) containing protease inhibitors. Cell lysates were incubated with antibodies for 1 h at 4°C. Protein A-sepharose beads were added to the lysates, which were then incubated for 30 min at 4°C, and the resulting immunoprecipitates were washed with lysis buffer three times. Immunoprecipitates and aliquots of total cell lysates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a Hybond–P membrane (Amersham). Antibody reactive proteins were detected using horse radish peroxidase (HRP)-conjugated secondary antibodies and visualised by chemiluminescence (Amersham). Representative Western blots from one of at least three similar experiments are shown. C14N antibody competition with the immunising peptide was carried out on duplicate Western blots using a 10:1 ratio of peptide to antibody.

**Transcriptional response assay**

HepG2 is one of the tumour lines that retain TGFβ responsiveness in culture and is therefore used here (Di Guglielmo et al, 2003). For TGFβ inducible luciferase reporter assays, HepG2 cells were transiently transfected with the reporter plasmid (3TP-Lux), pCMV-β-gal and the indicated constructs or with the empty vector alone. To induce the luciferase reporter, cells were treated overnight with 100 pM of TGFβ1 as described (Hayashi et al, 1997). Luciferase activity in cell lysates was measured using the luciferase assay system in a Berthold Lumat LB 9501 luminometer (Promega).
nuclear RNF11 accumulation was observed in lung, renal, and head and neck cancers (Figure 1B).

RNF11 interacts with Smurf2 through the PY motif

In order to test RNF11 interaction with Smurf2, we transiently transfected the HEK293T cell-line with GST-tagged RNF11 and three mutant RNF11 constructs together with a FLAG-tagged Smurf2 expression vector (Figure 2). The GST-tagged RNF11 mutants included RNF11-PY in which the tyrosine of the PY motif was mutated to alanine, RNF11-RING, where the first two cysteines in the RING-finger domain were replaced by two serines and the RNF11-Double having both mutations. GST pulldown of lysates from cultures cotransfected with Flag-Smurf2 and wild-type GST-RNF11 isolated a protein corresponding to Flag-tagged Smurf2 (as shown by anti-Flag Western blot), but not from lysates of cells cotransfected with Flag-Smurf2 and the GST-vector alone (Figure 2A). This result was confirmed by anti-Flag immunoprecipitation and GST immunoblot of lysates from HEK293T cells

Table 1 Immunohistochemical staining with RNF11 antibody in 125 microarrayed tumour tissue sections; duplicate micropunches of each tumour were remounted and sectioned to produce the microarray

| Tumour type | No. evaluated | Positive (%) | IHC staining intensity |
|-------------|---------------|--------------|-----------------------|
| Breast      | 20            | 90           | +++                   |
| Bladder     | 17            | 31           | ++                    |
| Colon       | 16            | 47           | ++                    |
| Head and neck | 11         | 63           | +                     |
| Kidney      | 17            | 11           | +                     |
| Lung        | 7             | 57           | +                     |
| Pancreas    | 22            | 78           | ++                    |
| Prostate    | 15            | 10           | +                     |

+++ = weak; ++ = intermediate; +++ = strong to very strong immunoperoxidase/DAB intensity (brown).

Table 2 RNF11 Expression in relation to clinicopathological parameters in breast cancers

| Histology | # | RNF11 | BR grade | ER | PR | LN |
|-----------|---|-------|----------|----|----|----|
| INV, ductal NOS | 18 | 2.26 ±0.35 | II–III | 14+/4– | 7+/11– | 7+/8– | 3NK |
| INV, ductal papillary | 2 | 2 ±1 | II–III | 1+/1– | 2– | 2– |

INV = invasive; # = number of cases; BR grade = Bloom–Richardson grading system; ER = oestrogen receptor status; PR = progesterone receptor status; LN = lymph node status; NK = not known; RNF11 = mean staining intensity ± s.d. of IHC score.
interactions of GST-RNF11 and HA-Smurf2 proteins (Figure 3A, lanes 1, 3 and 5).

Endogenous RNF11 interaction with Smurfl WW domains was examined by anti-RNF11 Western blot after HA pulldown of the WW2&3-Smurfl2 construct from transfected cells (Figure 3B). The C14N anti-RNF11 antibody recognises an 18 kDa band that is not visible in the presence of the immunising peptide.

RNFl1 interacts with UbcH5s but not with Ubc3

Most ubiquitination complexes require an E2 conjugating protein to provide the Ub moiety for the E3 ligases or multisubunit E3 ligase complexes (Fremont, 2000; Joazeiro and AM Weissman, 2000; Thrower et al, 2000; Zheng et al, 2002). We found that Smurfl E3 ligase interacts with RNFl1 through its WW domains (Figures 2 and 3). RNFl1 also contains a RING-H2 domain, some examples of which are known to bind ubiquitin conjugating (Ubc) proteins involved in ubiquitination (Fremont, 2000; Thrower et al, 2000; Zheng et al, 2002). This suggested to us that one or more Ubc proteins might interact with RNFl1, and this was tested by cotransfection of HEK293T cells with epitope tagged expression vectors for Ubc3 and UbcH5 a, b and c. GST pulldown was used to extract and concentrate proteins associated with GST-RNF11, and an anti-GST Western blot shows that this protein was present in all the lysates. Ubc3 and three variants of UbcH5 were also found in lysates transfected with His-tagged protein expression vectors for each, as revealed by an anti-His tag Western blot of whole-cell lysates (Figure 4). Interestingly, the His-tagged UbcH5 proteins, but not the His-Ubc3 protein, are seen on the Western blot made after GST pulldown of GST-RNF11. This indicates that RNFl1 interacts with UbcH5s but not with Ubc3.

Ubiquitination of RNFl1 by Smurfl requires the PY motif

Smurfl is a HECT-type E3 Ub ligase which suggests to us that RNFl1 may be one of its targets for ubiquitination. This was tested

Smurfl interaction with RNFl1 requires WW domains 2 and 3

In order to identify which of three WW domains in Smurfl could mediate RNFl1 binding, HEK293T cells were cotransfected with expression vectors for GST-RNF11 and each of three HA-tagged Smurfl deletion mutants (Figure 3A). The HECT-Smurfl2 mutant contains no WW domains, and GST pulldown followed by anti-HA Western blot did not resolve a band corresponding to HECT-Smurfl2, indicating that in the absence of WW domains, Smurfl2 does not interact with RNFl1 (Figure 3A, lane 6). No HA-tagged Smurfl2 band is seen in HA blot of GST pulldowns from cells cotransfected with GST-RNF11 and WW1-Smurfl2, indicating that WW domain 1 is not sufficient for Smurfl2–RNFl1 interaction (Figure 3A, lane 2). However, when Smurfl2 deletion mutant containing WW2 and WW3 domains was tested, a band corresponding to WW2&3-Smurfl2 protein can be seen on the HA Western blot after GST pulldown using GST-RNF11 protein (Figure 3A, lane 4). In all three cotransfections, the anti-HA Western blot of whole-cell lysates shows that mutant HA-tagged Smurfl2 proteins were available for pulldown mediated by
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by cotransfection of FLAG-Smurf2 and GST-RNF11 with HA-tagged Ubc and Ubc3 or UbcH5 constructs followed by immunoprecipitation of ubiquitinated proteins with anti-HA or anti-Flag antibody and anti-GST or anti-HA Western blotting (Figure 5). Anti-HA immunoblotting showed ubiquitination of Smurf2 and associated proteins immunoprecipitated by anti-Flag antibody when RNF11 and UbcH5a were cotransfected with Smurf2 (Figure 5). Weak bands resulting from cotransfection with the PY mutant of RNF11 suggest that this mutation severely reduces ubiquitination of Smurf2 (Figure 5, lane 1). An anti-GST Western blot of the same lysates also revealed bands corresponding to ubiquitinated GST-RNF11 in lysates of cells cotransfected with Flag-Smurf2 and UbcH5a (Figure 5, lane 5). In the absence of Ubc5a, Flag-Smurf2 protein was not sufficient to allow immunoprecipitation of either ubiquitinated RNF11 or ubiquitinated Smurf2 (Figure 5, lane 3). Similarly, no ubiquitinated proteins were seen on either blot in lysates transfected with Ubc3 (Figure 5, lanes 2 and 3). As expected, anti-FLAG and anti-GST reactive proteins were seen in whole-cell lysates transfected with constructs for FLAG-Smurf2 and GST-RNF11.

RNF11 relieves Smurf2-mediated transcriptional inhibition of a TGFβ responsive promoter

Cooperative interaction between Smurfl2 and Smad7 results in inhibition of TGFβ signalling by degradation of the TGFβ receptor 1 (Kavsak et al., 2000). The inhibitory activity of Smurf2 is dependent on interaction of its WW domains with the PY motif of Smad7 (Kavsak et al., 2000). We have shown that the WW2 and WW3 domains of Smurf2 are necessary for interaction with RNF11 (Figure 3). RNF11 protein might therefore inhibit interactions between Smad7 and Smurf2, thereby affecting TGFβ signal transduction. The effect of RNF11 on TGFβ-dependent transcription activation was investigated by transient cotransfection of RNF11 and 3TP Lux promoter–reporter plasmids (Figure 6). The 3TP Lux reporter vector contains a TGFβ responsive portion of the plasminogen activator inhibitor (PAI) promoter linked to the coding region of the firefly luciferase gene. The PAI promoter is highly active in TGFβ-treated cells and inactive in the absence of TGFβ (Figure 6). We found that its reporter gene activity is reduced by more than seven-fold by cotransfection with the Smurf2 expression vector even in the presence of exogenous TGFβ (Figure 6). Interestingly, reporter gene activity is restored when RNF11 is included in cotransfection with Smurf2. This relief of inhibition depends upon the wild-type PY motif in RNF11, as the RNF11 mtPY does not produce this effect when cotransfected with the Smurf2 vector in TGFβ-treated HepG2 human hepatoma cells.

DISCUSSION

In this study, we chose the RNF11 gene for characterisation of its protein expression and mechanisms of action in normal and cancer cells because it was isolated from a tumour cell-enriched cDNA library, is unique, and contains modular domains and motifs known to interact with other proteins involved in oncogenesis. TMA technology allowed for a rapid and simultaneous analysis of RNF11 protein expression in 125 primary tumours microarrayed in duplicate by immunohistochemical staining using anti-RNF11 antibody (Kononen et al., 1998; Landberg et al., 2001). Comparison of RNF11 expression between breast, prostate, head and neck, kidney, colon and lung cancers revealed that the majority of our tumour array specimens produced clear immunostaining without any detectable background, and the morphology of the tissues was well preserved (Figure 1). To facilitate comparison of our results with published data, we employed the commonly used semiquantitative evaluation of protein expression based on distinct differences between strong, moderate and lack of staining in tumour specimens developed with immunoperoxidase methods (Kononen et al., 1998; Landberg et al., 2001). RNF11 was markedly overexpressed in breast and to a somewhat lesser extent in pancreatic as well as colon cancer samples, whereas it was only weakly expressed in prostate and renal cell carcinomas (Table 1, Figure 1).

The RNF11 gene was originally cloned as T2A10, a cDNA fragment from our library enriched for breast tumour-specific mRNAs. We found it to be identical to the human RNF11 protein, and it encodes modular domains and motifs known to interact with other proteins involved in oncogenesis (Kitching et al., 2003). Chief among these is the RING-H2-finger domain that could facilitate protein–protein interaction(s) leading to the degradation of specific substrate(s) involved in oncogenesis and the PY motif...
that could bind to WW domain proteins, including several HECT-type E3 ligases such as NEDD4, AIP4 and Smurf2. The PPPPY motif sequence of RNF11 is identical to that of Smads 2, 3 and 7, which have been shown to bind WW domains of Smurfl Ub ligase and mediate the ubiquitination and degradation of various target proteins (Coopman et al, 2000; Longnecker et al, 2000). Smurfl plays a key role in the ubiquitination and proteasomal degradation of receptor-activated Smad2 and Smad3, and corepressor SnoN (Bonnii et al, 2001; Mizuide et al, 2003). The Smurfl and Smad7 complex ubiquititates TjRI, a key event resulting in degradation of the receptor and TGF\beta resistance in cancer cells (Kavsak et al, 2000).

Through site-directed mutagenesis, we showed that the tyrosine residue of the RNF11 PY motif is essential for binding to Smurfl (Figure 2). Indeed, mutation of the PY motif, but not the RING-H2, eliminates the interaction with Smurfl (Figure 2). We also investigated which of the Smurfl WW domains might be necessary for interaction with the PY domain of RNF11 (Figure 3). Only wild-type Smurfl and the mutant that contains the WW2 and WW3 domains were able to interact with RNF11, indicating that interaction of RNF11 with Smurfl does not require the WW1 domain (Figure 3).

RNF11 binding to Smurfl in mammalian cells suggests that, similar to Smads 2 and 3, it may also recruit targets for destruction by Smurfl E3 ligase. RNF11 is a small RING-finger protein, similar to ROC1 and APC11, which are components of the SCF and the anaphase promoting complexes respectively (Chen et al, 2000; Jackson et al, 2000; Maeda et al, 2001). These two ubiquitin ligase complexes regulate Ub-mediated protein degradation during G1/S and anaphase. The importance of the small ring-finger proteins ROC1 and APC11 in these complexes suggests that RNF11 may also have a role in multi-subunit E3 complexes.

In general, ubiquitin moieties are transferred from an E2 to a catalysing ubiquitin ligase E3 and then on to the substrate protein. However, the E2 enzyme for the HECT-type ligase Smurfl is unknown. Our results suggest that interaction of RNF11 with Smurfl allows ubiquitination of Smurfl, and this is dependent upon the presence of Ubch5a (Figure 5). This suggests that RNF11 is required for the ubiquitination of Smurfl that is essential to its ligase activity (Yagi et al, 1999). Interaction between Ubch5a and RNF11 was confirmed in a similar but separate experiment showing that Ubch5a, but not Ub3c, interacts with RNF11 in the absence of Smurfl (Figure 4). RNF11 is therefore a PY motif containing RING-finger protein that may mediate activation of the HECT-type E3 ligase Smurfl by the E2 conjugating Ubch5 enzymes (Figure 5).

Smurfl2 also interacts with the PY motif of Smad7, forming a complex essential to the ubiquitination and degradation of TjRI. It has been reported that the Smad7 protein suppresses TGF\beta dependent induction of the 3TP-Lux reporter (Hayashi et al, 1997). Transactivation and association of Smurfl2 with Smad7 further reduces this transcription activity due to degradation of the TGF\beta R1 (Hayashi et al, 1997; Kavsak et al, 2000). We hypothesised that the PY motif of RNF11 could affect interactions with Smad7. This was tested by us in a transcription – transactivation assay similar to that used to illustrate Smad7/Smurfl2 effects on TGF\beta responsive gene transcription (Hayashi et al, 1997; Kavsak et al, 2000). As expected, TGF\beta treatment induced 3TP-Lux reporter activity in transfected cells, and this transcriptional activity was inhibited by Smurfl2 but not by RNF11 (Figure 6). However, the transcriptional activity of the reporter gene was restored when increasing amounts of RNF11 were used in conjunction with Smurfl2 (Figure 6). This suggests that RNF11 may interfere with the binding of Smad7 to Smurfl2, thus inhibiting the degradation of TjRI protein resulting in restoration of transcription of reporter gene activity. Moreover, the PY mutant of RNF11 which does not bind to Smurfl2 is unable to restore the activation of the 3TP reporter. Thus, our data strongly support a role for RNF11 affecting the Smurfl2/Smad7 proteo – protein interactions involved in TGF\beta signalling. High-level expression of Smurfl2 has been found in oesophageal squamous cell carcinoma (SCC) and seems to correlate with poor prognosis in these patients (Fukuchi et al, 2002). The expression pattern of RNF11 in human primary tumours is interesting when compared to Smurfl2 in that head and neck tumours including oesophageal SCC samples expressed RNF11 in 63% of the surveyed cases. This is particularly relevant in the light of the observed overexpression of RNF11 in invasive breast cancers and its effects on TGF\beta responsive gene activation (Figures 1 and 6).

Although Smad proteins inhibit cell proliferation, they can also induce tumour invasion and metastasis. Similarly, in animal models it has been reported that TGF\beta can inhibit early stages of tumour development but stimulate later tumour progression and metastasis. The interaction of RNF11 and Smurfl2 could be important here because it restores TGF\beta signalling and RNF11 could have multiple functions similar to Smad proteins. Perhaps, RNF11 blocks some of the inhibitory effects of Smad7 on TGF\beta signalling on cell proliferation and apoptosis, but leaves intact the positive effects of this pathway on malignant progression.

An abundance of regulatory proteins, including growth factors and their receptors, adaptor proteins involved in signal transduction, transcription factors and cell cycle regulating proteins, is coordinated by balancing their synthesis and degradation. As shown here, RNF11 interacts with ubiquitin E2 conjugating enzymes and E3 ligases and thus could potentially facilitate the degradation of specific substrate(s) involved in oncogenesis. The ongoing identification of target proteins that are ubiquitinilated in the presence of RNF11 will allow us to determine the significance of its role in tumorigenesis (Li and Seth, 2003). In addition, we are investigating how its gene product can be used as a target for molecular diagnosis and therapeutic intervention of breast and other cancers.

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REFERENCES

Attisani L, Wrana JL (2002) Signal transduction by the TGF-beta superfamily. Science 296: 1646 – 1647

Bonnii S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Luo K, Wrana JL (2001) TGF-beta induces assembly of a Smad2 – Smurfl2 ubiquitin ligase complex that targets SnoN for degradation. Nat Cell Biol 3: 587 – 589

Burger A, Li H, Zhang XK, Pienikowska M, Venanzoni M, Vournakis J, Papas T, Seth A (1998) Breast cancer genome anatomy: correlation of morphological changes in breast carcinomas with expression of the novel gene product Di12. Oncogene 16: 327 – 333

Chen A, Wu K, Fuchs SY, Tan P, Gomez C, Pan ZQ (2000) The conserved RING-H2 finger of ROC1 is required for ubiquitin ligation. J Biol Chem 275: 15432 – 15439

Conway RC, Bower CS, Conway JW (2002) Emerging roles of ubiquitin in transcription regulation. Science 296: 1254 – 1258

Coopman PJ, Do MT, Barth M, Bowden ET, Hayes AJ, BasuYK, Elblanco J, Jeza PR, McLeskey SW, Mangeat PH, Mueller SC (2000) The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. Nature 406: 742 – 747
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Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL (2003) Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* 5: 410–421

Freemont PS (2000) RING for destruction? *Curr Biol* 10: R84–7

Fukuchi M, Fukui Y, Masuda N, Miyazaki T, Nakajima M, Sohda M, Manda R, Tsukada K, Kato H, Kuwano H (2002) High-level expression of the Smad ubiquitin ligase Smurf2 correlates with poor prognosis in patients with esophageal squamous cell carcinoma. *Cancer Res* 62: 7162 – 7165

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone Jr MA, Wrana JL, Falb D (1997) The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 89: 1165 – 1173

Hershko A, Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* 67: 425 – 479

Hoodless PA, Haerry T, Abdollah S, Stapleton M, O’Connor MB, Attisano L, Wrana JL (1996) MADR1, a MAD-related protein that functions in BMP2 signaling pathways. *Cell* 85: 489 – 500

Jackson PK, Eldridge AG, Freed E, Furstenthal L, Hsu JY, Kaiser BK, Reimann JD (2000) The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. *Trends Cell Biol* 10: 429 – 439

Joazeiro CAP, AM Weissman (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102: 549 – 552

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 TGFβ receptor for degradation. *Mol Cell* 6: 1365 – 1375

Kitching R, Gish G, Burger A, Landberg G, Seth A (2003) The RING-H2 protein RNF11 is differentially expressed in breast tumors and interacts with HECT-type E3 ligases. *Biochim Biophys Acta*, in press

Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP (1998) Tissue microarrays for high-throughput molecular profiling of tumor specimens [see comments]. *Nat Med* 4: 844 – 847

Landberg G, Ostlund H, Nielsen NH, Emdin S, Burger AM, Seth A (2001) Downregulation of the potential suppressor gene IGFBP-rP1 in human breast cancer is associated with inactivation of the retinoblastoma protein, cyclin E overexpression, and increased proliferation in estrogen receptor negative tumors. *Oncogene* 20: 3497 – 3505

Li H-X, Seth A (2003) An RNF11:Smurf2 complex mediates ubiquitination of the AMSH protein, in review process.

Longnecker R, Merchant M, Brown ME, Fruehling S, Bickford JO, Ikeda M, Harty RN (2000) WW- and SH3-domain interactions with Epstein – Barr virus LMP2A. *Exp Cell Res* 257: 332 – 340

Maeda I, Ohira T, Koizumi H, Fukuda M (2001) *In vitro* ubiquitination of cyclin D1 by ROC1-CUL1 and ROC1-CUL3. *FEBS Lett* 494: 181 – 185

Mehra A, Wrana JL (2002) TGF-beta and the Smad signal transduction pathway. *Biochem Cell Biol* 80: 605 – 622

Mizuide M, Hara T, Furuya T, Takeda M, Kusunagi K, Inada Y, Mori M, Imamura T, Miyazawa K, Miyazono K (2003) Two short segments of Smad3 are important for specific interaction of Smad3 with c-Ski and SnoN. *J Biol Chem* 278: 531 – 536

Seki N, Hattori A, Hayashi A, Koruma S, Sasaki Y, Sugano S, Muramatsu MA, Saito T (1999) Cloning and expression profile of mouse and human genes, Rnf11/RNF11, encoding a novel RING-H2 finger protein. *Biochim Biophys Acta* 1489: 421 – 427

Suzuki C, Murakami G, Fukuchi M, Shimamura T, Shiroma Y, Imamura T, Miyazono K (2002) Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. *J Biol Chem* 277: 39919 – 39925

Thrower JS, Hoffman L, Rechsteiner M, Pickart CM (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J* 19: 94 – 102

Winberg G, Matskova L, Chen F, Plant P, Rotin D, Gish G, Ingham R, Entenberg L, Pawson T (2000) Latent membrane protein 2A of Epstein–Barr virus binds WW domain–ubiquitin ligases that ubiquitinate B-cell tyrosine kinases. *Mol Cell Biol* 20: 8526 – 8535

Yagi R, Chen LF, Shigesada K, Murakami Y, Ito Y (1999) A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator. *EMBO J* 18: 2531 – 2562

Yendamuri S, Kuroki T, Trappasso F, Henry AC, Dumon KR, Hubeckner K, Williams NN, Kaiser LR, Croce CM (2003) WW domain containing oxidoreductase gene expression is altered in non-small cell lung cancer. *Cancer Res* 63: 878 – 881

Zheng N, Schuman BA, Song L, Miller JJ, Jeffrey PD, Wang P, Chu C, Koepp DM, Elledge SJ, Pagano M, Conaway RC, Conaway JW, Harper JW, Pavletich NP (2002) Structure of the Cul1-Rbx1-Skp1-F-boxSkp2 SCF ubiquitin ligase complex. *Nature* 416: 703 – 709