ELSEVIER

European Journal of Cancer 41 (2005) 2549–2560

European Journal of Cancer

www.ejconline.com

RNF11 is a multifunctional modulator of growth factor receptor signalling and transcriptional regulation

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Available online 13 October 2005

Abstract

Our laboratory has found that the 154aa RING finger protein 11 (RNF11), has modular domains and motifs including a RING-H2 finger domain, a PY motif, an ubiquitin interacting motif (UIM), a 14-3-3 binding sequence and an AKT phosphorylation site. RNF11 represents a unique protein with no other known immediate family members yet described. Comparative genetic analysis has shown that RNF11 is highly conserved throughout evolution. This may indicate a conserved and non-redundant role for the RNF11 protein. Molecular binding assays using RNF11 have shown that RNF11 has important roles in growth factor signalling, ubiquitination and transcriptional regulation. RNF11 has been shown to interact with HECT-type E3 ubiquitin ligases Nedd4, AIP4, Smurf1 and Smurf2, as well as with Cullin1, the core protein in the multi-subunit SCF E3 ubiquitin ligase complex. Work done in our laboratory has shown that RNF11 is capable of antagonizing Smurf2-mediated inhibition of TGFβ signalling. Furthermore, RNF11 is capable of degrading AMSH, a positive regulator of both TGFβ and EGFR signalling pathways. Recently, we have found that RNF11 can directly enhance TGFβ signalling through a direct association with Smad4, the common signal transducer and transcription factor in the TGFβ, BMP, and Activin pathways. Through its association with Smad4 and other transcription factors, RNF11 may have a role in direct transcriptional regulation. Our laboratory and others have found nearly 80 protein interactions for RNF11, placing RNF11 at the cross-roads of cell signalling and transcriptional regulation. RNF11 is highly expressed in breast tumours. Deregulation of RNF11 function may prove to be harmful to patient therapeutic outcomes. RNF11 may therefore provide a novel target for cancer therapeutics. The purpose of this review is to discuss the role of RNF11 in cell signalling and transcription factor modulation with special attention given to the ubiquitin-proteasomal pathway, TGFβ pathway and EGFR pathway.

Keywords: RNF11; Cancer; Growth factor; Ubiquitination; Signalling; Transcription

1. Introduction

Cellular growth and development are regulated by a balance between the internal genetic programming inherent to a cell, and the interpretation of environmental cues in the form of growth factor signalling. The ultimate manifestation of these inherent and instructive programs is that from the thousands of possible proteins that a cell could express, only certain patterns of gene expression will be observed, thereby allowing cells to take on unique and different roles within an organism. The convergence of genetic programs with instructive signals occurs at the level of transcription factors. The modulation of gene expression allows cells to express new protein factors or alter the levels of factors already expressed by the cell, which ultimately defines the function and capabilities of the cell. Miscues from the environment or genetic alterations that lead to improper

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doi:10.1016/j.ejca.2005.08.020

gene expression can change the intended program outcome in a cell, leading potentially to disease. In an attempt to understand the genetic alterations common in breast cancer, our laboratory has cloned several unique genes that were found to be overexpressed in breast tumour cells [1]. One of these genes was found to be the highly conserved RING finger protein 11 (RNF11) [2]. RNF11 seems unique in that it can modulate cell signalling and transcription factor activity via apparently separate mechanisms of action. RNF11 is capable of modulating protein function through ubiquitination [3] and perhaps through sumoylation (Seth lab, unpublished observations). Our observations suggest that RNF11 sits at the "cross-roads" of growth factor signalling and transcriptional gene regulation.

2. Discovery and regulation of the RNF11 gene

The RNF11 gene was originally cloned from a library enriched for tumour cDNAs [1]. The mRNA for human RNF11 was found to be expressed at high levels in breast and prostate cancer cells [2,4]. Also, the RNF11 protein is found to be highly expressed in high-grade breast tumours [4]. *In vivo*, RNF11 is expressed in varying levels in virtually all tissues tested. For instance, RNF11 is expressed at low levels in lung, liver, thymus, spleen, colon and peripheral blood lymphocytes. However, it is highly expressed in heart, brain, testis, ovaries, and skeletal muscles. Phylogenetic analysis shows that RNF11 does not have any immediate family members with a related structure [2], suggesting that RNF11 has an integral and non-redundant role in cell physiology. In order to gain insight into the regulation and tissue-specific function of RNF11, we investigated the RNF11 promoter region. By bioinformatic prediction, we found three conserved Ets1 transcription factor binding sites in the human and mouse RNF11 promoters [5]. Ets1 is produced by a variety of cell types and is required for proper embryonic development, angiogensis, hormone production, lymphocyte development and immune cell activation [6–8]. We showed that RNF11 and Ets1 were co-regulated at the transcript level in bone cells, and that Ets1 can bind to and activate transcription from an RNF11 Ets1 binding site [5]. Furthermore, the closely related Ets2 was unable to bind and activate transcription from the RNF11 Ets binding site [5].

3. RNF11 protein structure and binding partners

RNF11 is a modular protein that contains at least three domains (Fig. 1), comprised of an N-terminal PY motif which binds WW-domain containing proteins, an internal ubiquitin-binding domain [9], and a C-terminal RING(H2) domain, that interacts with UbcH5 E2 [4].

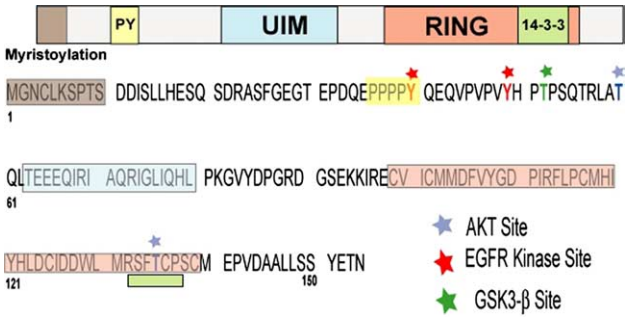

Fig. 1. Protein sequence and domain structure of RNF11. RNF11 is a 154 amino acid protein with modular domains and multiple phosphorylation sites. RNF11 contains an N-terminal myristoylation sequence, which allows the anchorage of RNF11 to membranes. The PY motif (PPPPY) contains an EGFR consensus phosphorylation site at the tyrosine. When phosphorylated, this sequence becomes a consensus-binding site for Fyn-SH2-mediated binding. Furthermore, the PY motif is important for interactions with WW domain containing proteins, such as, AIP4, Nedd4, and Smurf1/2. The central ubiquitin interaction motif (UIM) is involved in binding to ubiquitin or ubiquitinated proteins. The C-terminal RING domain interacts with E2 ubiquitin conjugating enzymes, other RING-finger proteins and has sumo- or ubiquitin-ligase activity. Situated within the RING domain is a 14-3-3 binding site. In order to confer 14-3-3 binding to RNF11, the threonine within this site is phosphorylated by AKT.

The gene is comprised of 3 exons, which interestingly contain each of the aforementioned domains, and the structure of which is highly conserved throughout evolution [2]. At the protein level, RNF11 is virtually unchanged from frog to human, with the greatest degree of divergence seen at the level of insect and worm. Interestingly, only the N-terminal domain that contains the PY motif seems to diverge at the level of invertebrates, perhaps indicating a unique function specific for vertebrate biology is encoded in the N-terminus of RNF11 [2]. The N-terminal PY motif binds WW-domain containing proteins, such as the HECT-type ubiquitin E3 ligases, Smurf1 and Smurf2, AIP4 and Nedd4 [2,4,10]. The internal region contains an ubiquitin interaction motif (UIM), while the C-terminal region contains a RING (H2) finger. The UIM and PY domains serve to mediate interactions between proteins [2,4,10]. In the case of the UIM, ubiquitinated proteins (either mono-, di-, or poly-ubiquinated) can interact with proteins that contain a UIM [9]. By analogy, the UIM serves the same function that SH2 domains serve in phosphotyrosine-mediated binding between proteins, whereas in the case of the UIM domains, ubiquitin serves as the tag. The RING domain is comprised of eight residues that coordinate two zinc ions, and are known to serve various functions, such as mediating protein–protein interactions, protein–DNA interactions, and can have ubiquitin and/or sumo ligase activity [11–20]. Ring finger proteins for the most part represent regulatory factors that impart control on receptors, transcription factors and signalling proteins via the

Table 1
RNF11 interaction partners

#	Protein name	Description and function	Refs.	Predicted role	Binding confirmed
1	EPN2	Clathrin-mediated endocytosis	[22]	Traffic	
2	EPN3	?	[22]	?	
3	FLJ35794	?	[22]	?	
4	GGA3	Ubiquitous coat proteins: regulate trafficking proteins b/w trans-Golgi network & lysosome	[22]	Traffic	
5	HERC1	Membrane transport processes	[22]	Traffic	
6	NEDD4	Ubiquitination – HECT-type E3 ligase	[3,22]	Protein metabolism	Yes
7	NY-REN-25	Protein–protein interactions	[22]	Protein–protein interaction	
8	RPS27A	Ub-ribosomal fusion protein	[3,22]	Protein metabolism	Yes[a]
9	SARA	Smad anchor for receptor activation	[22]	Signalling	?
10	SMURF2	Ubiquitination – HECT-type E3 ligase	[3,22]	Signalling/protein metabolism	Yes
11	UBA52	Ub-ribosomal fusion protein: N-term-Ub; C-term L40 ribosomal protein	[22]	Protein metabolism	Yes[a]
12	UBC	Ubiquitin C	[3,22]	Protein metabolism	Yes[a]
13	UREB1	Contains HECT domain – funx?	[22]	Protein metabolism	
14	WWP1	Similarity to Nedd-4-like ubiquitin-protein ligase	[22]	Protein metabolism	
15	WWP2	In the NEDD4-like protein family – funx?	[22]	Protein metabolism	
16	cbl-b	Similar to c-cbl proto-oncogene product	[22]	Protein metabolism	
17	EPSIN	EH domain-binding mitotic phosphoprotein: endocytosis and cytoskeletal machinery	[22]	Traffic	
18	FLJ21588	ASC-1 complex subunit: may be involved in binding ubiquitin-conjugating enzymes	[22]	Protein metabolism	
19	GGA1	Ubiquitous coat proteins: regulate trafficking proteins b/w trans-Golgi network & lysosome	[22]	Traffic	
20	MYO6	Myosin VI	[22]	Traffic	
21	NAF1	Hvirion-associated nuclear-shuttling protein	[22]	Traffic	
22	POLI	Similar to *Saccharomyces cerevisiae* DNA polymerase eta (Rad30)	[22]	DNA binding	
23	RABEX5	Putativeputative Rab5 GDP/GTP exchange factor homologue	[22]	Traffic	
24	RP42protein	Similar to Mus musculus RP42 protein1...259 RP42 protein; 1...259 RP42 homolog	[22]	?	
25	SMURF1	Ubiquitination – HECT-type E3 ligase	[3,22]	Signalling/protein metabolism	Yes
26	TRIAD3	Apoptosis/survival	[22]	Survival	
27	UBB	Ubiquitin B	[22]	Protein metabolism	Yes
28	UBE2N	Ubiquitination: E2	[22]	Protein metabolism	
29	TAX1BP1	?	[22]	?	
30	A1U	Ub-like	[22]	Protein metabolism	
31	AUP1	Ancient ubiquitous protein – cellular metabolism	[22]	Protein metabolism	
32	DKFZP547N043	dJ876B10.3 hypothetical protein DKFZp547N043	[22]	?	
33	ENDOFIN	Endosome-associated FYVE-domain protein	[22]	Traffic	
34	ERCC6	DNA-binding protein – transcription-coupled excision repair	[22]	DNA binding	
35	FLJ12392	Hypothetical protein FLJ32746	[22]	?	
36	FLJ32746	Hypothetical protein FLJ32746	[22]	?	
37	HERC2	Potential guanine nucleotide exchange factor and E3 ubiquitin protein ligase	[22]	Protein metabolism	
38	KIAA0323	1...719 similar to KIAA0323	[22]	?	
39	LOC133957	?	[22]	?	
40	LOC51619	Ubiquitin-conjugating enzyme HBUCE1 – E2	[22]	Protein metabolism	
41	NDP52	Nuclear domain 10 protein – ?	[22]	Traffic	
42	NEDD4L	NEDD4-like ubiquitin ligase 3	[22]	Protein metabolism	
43	NEMO	NF-κB essential modulator NEMO	[22]	Signalling	
44	OPTN	?	[22]	?	
45	PDCD6IP	Apoptosis/cell survival	[22]	Signalling	
46	PTPRC	Member of the protein tyrosine phosphatase (PTP) family	[22]	Signalling	
47	QARS	Aminoacyl-tRNA synthetases	[22]	Protein metabolism	
48	SDCBP	Syndecan binding protein (syntenin) – ?	[22]	?	

(*continued on next page*)

Table 1 (*continued*)

#	Protein name	Description and function	Refs.	Predicted role	Binding confirmed
49	STAM2	Closely related to STAM	[22]	Signalling	
50	TNFAIP3	Inhibit NF-κB activation as well as TNF-mediated apoptosis	[22]	Signalling + survival	
51	UBE2D2	E2 ubiquitin conjugating enzyme	[22]	Protein metabolism	
52	UBQLN2	Ubiquitin-like protein (ubiquilin)	[22]	Protein metabolism	
53	USP5	De-ubiquitinating enzyme ubiquitin isopeptidase	[22]	Protein metabolism	
54	GDBR1	Putative glialblastoma cell differentiation-related	[22]	Protein metabolism	
55	SNRP70	(U1)ribonucleoprotein – ?	[22]	?	
56	AMSH	Associated molecule with the SH3 domain of stam	[3]	Signalling	Yes
57	ZBRK1	ZINC FINGER AND BRCA1-INTERACTING PROTEIN WITH A KRAB DOMAIN 1	[3]	DNA binding + survival	
58	EPS15	Epidermal growth factor receptor substrate 15	[3]	Signalling	
59	UbcH5a	E2	[3]	Protein metabolism	Yes
60	UbcH5b	E2	[3]	Protein metabolism	Yes
61	UbcH5c	E2	[3]	Protein metabolism	Yes
62	AIP4	Atrophin-1-interacting protein 4, Itch, itchy homolog E3 ubiquitin protein ligase (mouse), NAPP1, NFE2-associated polypeptide	[3]	Protein metabolism	Yes
63	Smad4	Mothers against decapentaplegic homolog 4	[3]	Signalling + DNA binding	Yes
64	Culin1	Cullin homolog 1	[3]	Protein metabolism	Yes
65	14-3-3	14-3-3 Protein	[3]	Traffic	Yes
66	VCY2IP-1 gene	VCY2 interacting protein-1, a MAP-like protein	[3]	Traffic/chromosomal activity + RNA binding	
67	Hypothetical protein FLJ11626	Accession No. XM_050609	[3]	?	
68	Plasminogen activator, tissue (PLAT)	Tissue plasminogen activator	[3]	Protein metabolism/ activation of protein function	
69	cDNA DKFZp586E1521	Hypothetical protein DKFZp586E1521.1 – human (fragment). Accession T43499	[3]	?	
70	Inversin (INVS)	Inversion of embryonic turning	[3]	Cell growth/signalling/ protein metabolism	
71	Clone IMAGE:4346864	?	[3]	?	
72	cDNA FLJ11302 fis	Homo sapiens cDNA FLJ11302 fis Accession AK002164	[3]	?	
73	Polymerase (RNA) II (DNA directed) polypeptide J (POLR2J), transcript variant c, mRNA	Homo sapiens DNA directed RNA polymerase II polypeptide J-related gene (POLR2J2), transcript variant 2	[3]	DNA binding/transcription	
74	dUTP pyrophosphatase (DUT)	Deoxyuridine triphosphate nucleotidohydrolase	[3]	DNA stability/dTTP synthesis	
75	Vimentin; VIM	Intermediate filament, cytoskeletal protein	[3]	?/Traffic	
76	Homo sapiens similar to 1 beta dynein heavy chain	Cytoskeletal protein	[3]	?/Traffic	
77	Ribosomal protein S26	Ribosomal protein S26; RPS26	[3]	Protein metabolism	

Yeast-2-hybrid screens reveal multiple interacting partners for RNF11. Functional categories describe the various activities that these potential RNF11 binding partners have. Based on these general function categories, the graph in Fig. 2 was generated. Confirmed binding partners are marked.

[a] Ubiquitin and ubiquitin precursors.

ubiquitin and sumo pathways. RNF11 also contains many phosphorylation sites which may modulate its activity, as well as a 14-3-3 binding site which we have shown to be important to the normal function and intracellular localisation of the RNF11 protein (Fig. 1) [21]. The implications of these domains and motifs are discussed below.

Using yeast-2-hybrid methodology, several binding partners for RNF11 have been elucidated (Table 1) [3,22]. We have found at least 6% of the total number of characterised proteins that bound to RNF11 are transcription factors (Fig. 2). Of these we have confirmed the interaction with Smad4 (Seth lab, unpublished observations), the common signalling and transcription factor in the TGFβ, BMP and Activin pathways (discussed in more detail below). Interestingly, RNF11 itself may have transcriptional activity. In the yeast two-hybrid screen, RNF11 bait protein was found to be transcrip-

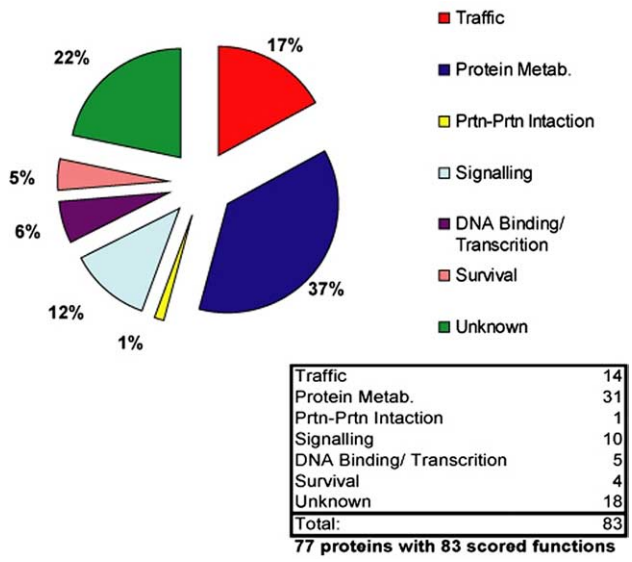

Fig. 2. RNF11 has multiple binding partners. Through two-hybrid screens, 77 interacting proteins have been elucidated for RNF11. These interactions place RNF11 predominately in the ubiquitin-proteasomal pathway, with roles in protein trafficking, cell signalling and transcriptional regulation.

tionally active on its own, thus the RING domain of RNF11 had to be mutated to conduct the screen [3].

In order to get a better view of RNF11 function, we have catalogued all the known binding partners for RNF11, totalling 77 proteins [3,22] (Fig. 2 and Table 1). Upon analysing the types of protein interactions that RNF11 is involved in, one can quickly see that RNF11 has a large role in protein metabolism, trafficking and transcription. Judging from the modular domain structure and its partner proteins, RNF11 is likely involved in numerous pathways and regulatory modalities. The major pathways that we have found RNF11 to modulate are discussed below.

4. The role of RNF11 in ubiquitin-modifications of target proteins

The ubiquitin and ubiquitin-like pathways are integral to the normal function of eukaryotic cells [13,16,23–28]. Protein turn-over, trafficking, and the modulation of transcription factor function have been ascribed to ubiquitination [29–31]. Ubiquitination proceeds in a stepwise format, starting with the activation of free ubiquitin. Ubiquitin is activated in an ATP-dependent manner by an ubiquitin-activating enzyme known as E1. The activated ubiquitin is then transferred to an ubiquitin-conjugating enzyme E2, which then interacts with a specific E3 ubiquitin-protein ligase resulting in the specific ubiquitination of target proteins (reviewed extensively [32–34]). Specificity in targeting proteins for ubiquitination lies mostly in the E3 enzyme. There are two major classes of E3 enzymes. The first are the HECT-type E3 ubiquitin ligases [35]. These include Smurf1 and Smurf2, AIP4, and Nedd4 among others. RNF11 is known to interact with all of the aforementioned E3 proteins [2,4,10]. The other class of E3 ubiquitin ligases are the RING-finger E3s, which are further divided into two groups, those that have intrinsic ubiquitination activity, and those that function in a multi-subunit complex [13,15]. Well known examples of RING-finger E3s that have intrinsic ubiquitination activity include Cbl proteins which target receptor tyrosine kinases (RTK) such as the B-cell receptor [36,37] and EGFR for degradation [38], MDM2 which targets p53 for degradation [13,15], and BCA2 (breast cancer associated protein 2) that we show is a RING-finger ubiquitin ligase [39]. The second group of RING-finger proteins are the small RING-finger proteins, such as Roc1 (also known as Rbx1) and APC11, which form the catalytic core of a multi-subunit E3 complex with Cullin proteins [40–42]. These complexes are important in cell cycle control, signal transduction, protein turn over, and many other processes [13]. Like Roc1 and APC11, we observe that RNF11 interacts with Cullin1 and participates in ubiquitination with Cullin1 (Seth lab, unpublished observations).

RNF11 is known to interact with both E2 conjugating enzymes and E3 ubiquitination ligases [4]. We have shown that RNF11 is capable of targeting certain E3 ubiquitination enzymes to substrates for degradation [3,4]. The best-described example is that of RNF11 interaction with Smurf2 E3 ligase. Smurf2 has been shown to interact with Smad7 and target the TGFβ receptor for degradation [43]. Furthermore, Smurf2 can interact with Smad2, thereby targeting Smad2 for degradation, as well as the Smad2 associated transcription factor SnoN [44]. The dual mechanism by which Smurf2 disrupts TGFβ signalling makes Smurf2 a highly effective negative regulator of the TGFβ pathway. We have shown that not only can RNF11 disrupt Smurf2-mediated ubiquitination of the TGFβ receptor (Seth lab, unpublished observations), but also RNF11 can target Smurf2 to the AMSH protein for degradation [3]. Therefore, RNF11 rescues TGFβ signalling and causes the degradation of AMSH through a Smurf2-mediated pathway, the implications of which are discussed further below.

5. RNF11 enhances TGFβ signalling at the receptor level and at the transcription factor level

The TGFβs belong to a super family comprised of the BMPs, Activins, and TGFβ growth factors. TGFβ has been implicated in cell differentiation, and as an

effective *in vitro* inhibitor of cell growth, as well as a potent *in vivo* tumour suppressor (reviewed extensively [45–50]).

TGFβ signalling proceeds through the binding of the TGFβ ligand to the TGFβ receptor. The TGFβ receptor is comprised of a homodimer of a type 1 TGFβ receptor (TβR-I) and a homodimer of a type 2 TGFβ receptor (TβR-II). The TβRII receptor has constitutive kinase activity, and is capable of binding TGFβ in the absence of the TβRI [51,52]. Ligand binding to TβRII induces its association with the TβRI receptor. Upon aggregation of the TβRI receptor to the TβRII receptor, transphosphorylation of the type 1 receptor by the type 2 receptor initiates the first steps in TGFβ signalling [52]. Receptor associated signal-transducing factors, called R-Smads (such as Smad2 and Smad3), associate with the phosphorylated TβRI and in turn become activated through phosphorylation (reviewed [45]). Phosphorylated receptor Smads associate with the common Smad, Smad4 [53]. The complex of Smad4 and R-Smads then translocates to the nucleus, where these complexes associate with auxiliary transcription factors, or independently initiate transcription from cognate promoters [53–55].

Regulation of TGFβ signalling is accomplished through the action of the inhibitory Smads (I-Smad), Smad7 in TGFβ signalling and Smad6 in BMP signalling, which bind to the receptor in place of the receptor Smads, but do not transduce the TGFβ signal [56–58]. Expression of the inhibitory Smad7 is activated by TGFβ signalling, thereby initiating a negative feedback loop that in turn shuts down TGFβ signalling [56,57,59]. Smad7 also recruits the HECT-type E3 ubiquitin-ligase, Smurf2 to the receptor complex via interactions mediated through the Smad7 PY domain, which in turn initiates TGFβ receptor destruction via proteosomal degradation [43]. Additionally, Smurf2 has been shown to interact with Smad2 (via the Smad2 PY domain) and promote Smad2 degradation as well as the degradation of the Smad2-associated transcription factor, SnoN [44,60]. Additionally, other HECT-type E3 ligases can negatively regulate TGFβ signalling. In the same manner that Smurf2 binds to Smad7 to degrade the TGFβ receptor, others have found through a yeast two-hybrid screen that the HECT-type E3 ligase, WWP1 also binds Smad7. The association of Smad7 with the WWP1 protein also leads to TGFβ receptor degradation and the downregulation of the TGFβ signal [61]. WWP1 has also been shown to play a possible role in the ubiquitin-mediated degradation of Smad4 [62]. Colland et al. [22] have found that RNF11 also interacts with WWP1. By analogy to what we have already observed with Smurf2, it is likely that RNF11 could also antagonise the effects of WWP1 on TGFβ signalling.

We have shown that RNF11 can interact with Smurf1 and Smurf2. Interestingly, the interaction between RNF11 and Smurf2 seems to play a dual role. Firstly, RNF11 abrogates Smurf2-mediated ubiquitination of the TGFβ receptor (Seth lab, unpublished observations), thereby enhancing TGFβ signalling [4]. Concomitantly, RNF11 cooperates with Smurf2 to degrade AMSH, an ubiquitin isopeptidase (a de-ubiquitination enzyme, DUB) that enhances TGFβ signalling and EGFR-endosomal recycling [3,63]. This may indicate a role for RNF11 in maintaining an appropriate balance between activation and quiescence of the TGFβ and EGFR pathways.

In breast carcinomas, signalling through the TGFβ receptor has been implicated in early tumour suppression and in late tumour progression [64–68]. *In vivo*, the increased expression of TGFβ2 was shown to enhance cell survival and metastasis of TA3 mouse mammary carcinoma cells in an autocrine manner. Furthermore, many breast cancer cell lines show an increased expression of TGFβ but are refractory to TGFβ-induced cell cycle arrest. Conversely, retrograde studies looking at human breast carcinomas, have found a significant correlation between the loss of the TGFβ receptors and/or signalling factors with high-grade breast carcinomas reviewed by Reiss and Barcellos-Hoff [67].

We observe that overexpression of RNF11 is capable of enhancing TGFβ signalling initiated at the receptor level. In a complimentary experiment, RNF11 siRNA knock-down was shown to severely repress TGFβ responsiveness of the endogenous PAI promoter, indicating that endogenous RNF11 plays an indispensable role in allowing TGFβ signalling to proceed [22] (Fig. 3).

Interestingly, RNF11 also binds directly to Smad4, the common Smad for TGFβ, BMP and Activin signalling. We also show that RNF11 can enhance the transactivation activity of Smad4 and of Smad2–Smad4 transcription complexes (Seth lab, unpublished observations). We believe that this enhancement is not simply due to a blockage in Smurf2 function, since the RNF11 PY mutant (which does not bind Smurf2, or other WW-domain containing proteins) is better at enhancing Smad2–Smad4 transcriptional activity when compared to wild-type RNF11. It is conceivable that RNF11 may directly augment the transcriptional abilities of these transcription factors, and possibly participate in transcription itself. As we have noted earlier, RNF11 has been shown to have intrinsic transcriptional activity [3]. RNF11-dependant modulation of TGFβ signalling represents a novel and as yet undescribed mechanism of regulating TGFβ signalling, and may contribute to early TGFβ-dependant breast tumour suppression and late-stage TGFβ-dependant breast tumour progression.

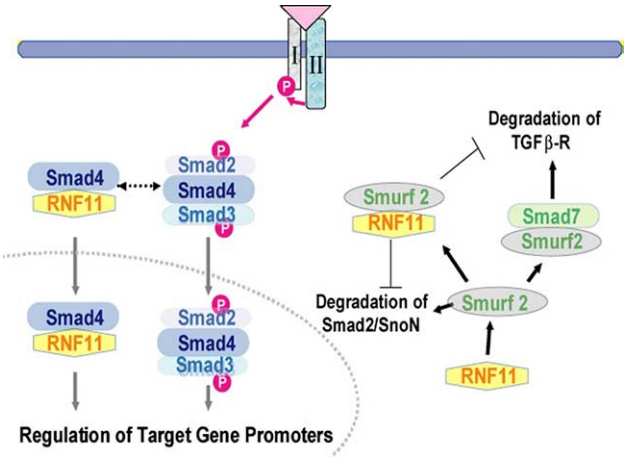

Fig. 3. RNF11 enhances TGFβ signalling through blockage of Smurf2 activity and direct enhancement of Smad4 activity. Upon TGFβ receptor activation, the intracellular signalling factors Smad2, Smad3 (termed R-Smads), become activated and associate with Smad4. The complex of Smad4 with activated Smad2 or 3, then translocates to the nucleus where transcriptional activation of TGFβ responsive genes is initiated. Regulation of TGFβ signalling is accomplished through the actions of inhibitory Smads (Smad6 or Smad7, termed I-Smads) that bind to the receptor in place of the R-Smads, but do not transduce the TGFβ signal. The inhibitory Smads also recruit the HECT-type E3 ligases, Smurf1 and Smurf2, to the receptor complex, which in turn initiates TGFβ receptor destruction via proteasomal degradation. Additionally, Smurf2 has been shown to interact with Smad2 and promote Smad2 degradation. RNF11 can block the activity of Smurf2, thereby rescuing the TGFβ receptor from degradation, as well as Smad2 and associated SnoN from degradation. RNF11 can also bind directly to Smad4 and directly regulates Smad4 transcriptional activity.

6. Negative feedback regulation of EGFR signalling by RNF11

The epidermal growth factor signalling cascade is a key mediator of cellular growth, differentiation, migration and survival [69,70]. The EGF receptor (EGFR) family is comprised of four members designated EGFR (ErbB1), ErbB2, ErbB3, and ErbB4 [69,71,72]. The intrinsic protein tyrosine kinase activities of these receptors (termed receptor tyrosine kinases, or RTK) and their abilities to engage and activate intracellular signalling pathways are controlled by members of the EGF family of growth factors reviewed by [70].

The regulatory arm of RTK signalling is initiated by ligand-activated RTKs which undergo rapid endocytosis. The endocytosed receptors go through a sorting process, which determines receptor fate and signal intensity [38,73–77]. The receptors can be targeted to the lysosome for degradation, which terminates receptor signals. Alternatively, the internalised receptors can be recycled back to the cell surface for continued signalling. Endosomal recruitment of RTKs is dependant on ubiquitination of the RTK by its cognate E3. c-CBL is known to mono- or multi-ubiquitinate the EGFR, and therefore downregulate EGFR signalling via lysosomal targeting of the ubiquitinated EGFR [73–75,38]. The process of targeting the EGFR to the lysosome for destruction can be broken down further. Ubiquitinated EGFR associates with Eps15 through the Eps15 UIM. Eps15 then mediates the endocytosis of the EGFR where the complex is sequestered by the endosomal Hrs/Clathrin coat, following which further ubiquitin-mediated interactions with components of the ESCRT machinery lead to translocation into internal vesicles [70,73,74]. The process of ubiquitination and degradation of the EGFR is balanced by the activity of AMSH, a de-ubiquitinating (DUB) enzyme that counters the activity of c-Cbl on the EGFR [63].

We have shown that RNF11 is capable of recruiting Smurf2 to degrade AMSH [3]. Furthermore, RNF11 interacts with Eps15 [3]. In order for EPS-15 to form a complex with Hrs, it must be mono-ubiquitinated following phosphorylation by the EGFR. Thus, it may be that via its interaction with E2 and E3 ligases RNF11 may mediate the mono-ubiquitination of EPS-15. This means that RNF11 could promote the internalisation and degradation of the EGFR via two distinct mechanisms, either by degradation of AMSH and/or mediating the mono-ubiquitination of EPS-15. Thus in the presence of RNF11, endocytosis and degradation of the activated EGFR would occur. This is in agreement with our observations regarding RNF11 in the EGFR pathway, which seem to indicate that RNF11 downregulates EGFR signalling (Seth lab, unpublished observations). We hypothesise that RNF11 maybe mediating a balance between the pro-growth signals received by EGFR signalling, and the pro-arrest signals from TGFβ. The observation that EGFR signalling may in fact oppose RNF11 expression at the protein level, may in part, explain why tumour cells become unresponsive to growth arrest signals induced by TGFβ.

7. Phosphorylation and intracellular localisation of RNF11

RNF11 contains multiple potential regulatory sites (Fig. 1). There are two EGFR kinase sites and two AKT phosphorylation sites [21]. One of the EGFR phosphorylation sites is located in the PY domain, and recognises the terminal tyrosine for phosphorylation. When the PY domain is phosphorylated it becomes a consensus Fyn SH2-binding site (http://scansite.mit.edu). Likewise, one of the AKT phosphorylation sites resides in an optimal 14-3-3 binding site located in the RING domain of RNF11. Indeed, we have shown that AKT-dependent 14-3-3 binding is important in mediating RNF11 function, and may provide further insight into the relevance of elevated RNF11 expression observed in breast cancer [21].

14-3-3 Proteins have roles in signal transduction, cell cycle control, mitogen-activated protein kinase (MAPK) activation, apoptosis, and gene regulation reviewed by [78]. The 14-3-3 protein family is comprised of seven highly related genes, which bind a variety of proteins [78,79]. RNF11 contains one of the optimal binding sequences for the 14-3-3 proteins, RxxS/T(p)xP, where the internal threonine (T135) must be phosphorylated for 14-3-3-binding. Interestingly, this threonine is also part of an AKT phosphorylation consensus sequence and we have shown that AKT can phosphorylate this site [21]. Indeed, we have shown that 14-3-3 binding to RNF11 is mediated by AKT phosphorylation of the 14-3-3 binding motif [21]. AKT is a serine/threonine kinase that controls signalling pathways which play an integral role in many cellular functions (reviewed extensively [80–84]). AKT is activated by a variety of growth factors, including EGF [70,85,86], which provides another link between the EGFR pathway and RNF11 function. Once activated, AKT phosphorylates several apoptotic factors to inactivate them, and promote cell survival [70,80,82,85–87]. AKT activation has been linked with TGFβ resistance in cells through an AKT-dependent phosphorylation and sequestering of Smad3 [88–91]. AKT has also been shown to be important in many cancers including breast cancer (reviewed extensively [80–83]).

In our study of AKT mediated 14-3-3 binding to RNF11, we observed that 14-3-3 alters both the intracellular localisation and activity of RNF11 [21]. RNF11 bound 14-3-3 better in WM239 cells that have a constitutively active AKT (due to the loss of PTEN), but not in the parental WM35 cells, which have low AKT activity. Furthermore, we showed that wild-type, but not the mutant RNF11 that lacks the AKT phosphorylation site (T135E mutant), is able to bind 14-3-3. Interestingly, in the presence of active AKT (whether ectopically expressed or endogenously enhanced) wild-type RNF11 was observed to be less stable than the T135E mutant, suggesting that RNF11 is regulated at the protein level by AKT phosphorylation and 14-3-3 binding. RNF11 is a predominantly cytoplasmic protein, with moderate nuclear localisation. When RNF11 is co-expressed with a constitutively active AKT, we found that RNF11 shifts from being cytoplasmic to almost exclusively nuclear. This could indicate active transport into the nucleus or that cytoplasmic RNF11 is being degraded rapidly. Lastly, we looked at the physiological function of AKT on RNF11 in TGFβ signalling. As we had observed before, RNF11 is capable of enhancing TGFβ signalling directly and by inhibition of Smurf2-mediated downregulation of the TGFβ signal. Here, we used the T135E mutant that could not be phosphorylated by AKT and could not associate with 14-3-3, and we observed that the T135E mutant is better at enhancing TGFβ signalling than is the wild-type protein [21]. This observation would indicate that RNF11 function is negatively regulated by AKT phosphorylation and 14-3-3 binding (Fig. 4).

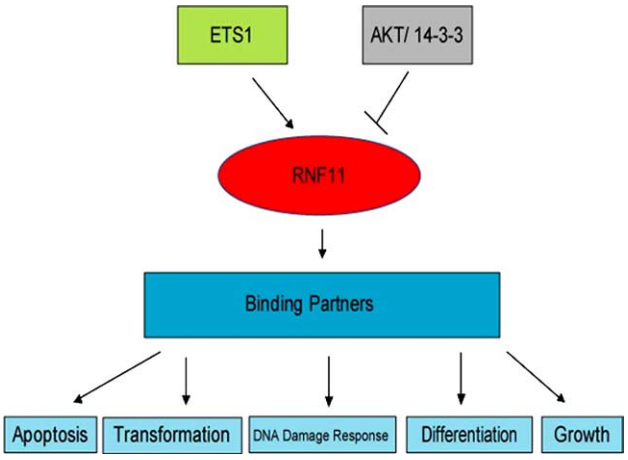

Fig. 4. RNF11 is regulated at the transcription level and the protein level, and interacts with many factors that determine cell fate. RNF11 is upregulated by the Ets1 transcription factor. Furthermore, AKT phosphorylation of RNF11 leads to its association with 14-3-3 and a downregulation of RNF11-action. Through the association with various binding partners, RNF11 mediates cell growth, apoptosis, differentiation transformation and responses to DNA damage.

8. RNF11 enhances DNA damage and signalling responses

Apoptosis is an integral feature of cell physiology. Apoptosis can be initiated through external signalling, such as through death receptors [92,93], or via internal cues, such as DNA damage [94–96]. In the latter case, there are several genes that have been found to "sense" DNA damage and either induce DNA repair or apoptosis. Among these is the prototypical breast cancer associated protein, BRCA1 [97]. Mutations in BRCA1 have been linked to breast, ovarian, and prostate cancers [97–100].

DNA damage triggers a complex signalling pathway that activates various cellular responses that signal the cell to arrest growth and express proteins that help in the repair of the damaged DNA. Coordinated regulation of genes that play essential roles in these responses is a key step in maintaining genomic integrity [101,102]. To attain precise control over the expression of these genes, the availability and/or activity of transcription activators and repressors is closely regulated through different mechanisms, which include acetylation, phosphorylation, and ubiquitination. It has been shown that the function of many important transcription regulators, such as NF-κB [103], p53 [104,105], c-Jun [106], and β-catenin [107], among others, are regulated by the ubiquitin pathway. Deregulation of these transcription factors

through inappropriate ubiquitination has been demonstrated for many cancers.

The BRCA1 protein contains a RING-finger domain and regulates transcription of DNA damage repair and growth arrest genes, such as p21 and Gadd45a [100,108–110]. However, because BRCA1 lacks the intrinsic ability to recognise DNA regulatory sequences, it must associate with sequence-specific binding transcription factors. We are particularly interested in the association of BRCA1 and ZBRK1, a CRAB-domain containing protein that regulates the DNA damage response gene Gadd45a (growth arrest and DNA damage-inducible gene) [110–113]. ZBRK1 functions as a BRCA1-dependant transcriptional repressor [110,113]. In response to DNA damage (such as by UV radiation), ZBRK1 is ubiquitinated and degraded by the ubiquitin-proteosome pathway, thereby releasing the expression of Gadd45a [110]. Interestingly, the E3 ligase responsible for ZBRK1 ubiquitination is still unknown. We found ZBRK1 in a yeast two-hybrid screen [3] (Table 1), and it is intriguing to think that RNF11 may have a role to play in ZBRK1 ubiquitination or in transcriptional repression through ZBRK1 binding. In addition to the phosphorylation sites already mentioned above, RNF11 harbours many DNA-damage kinase group motifs (http://scansite.mit.edu), and this may indicate a role for RNF11 in the DNA damage-response cascade.

To investigate this possibility further, we conducted apoptosis assays with RNF11 in human breast cancer cell lines and in hepatocarcinoma cells. We found that RNF11 has a pro-apoptotic effect on cells that remain sensitive to DNA damage-induced apoptosis or TGFβ-induced apoptosis (Seth lab, unpublished observations). It is conceivable that through the DNA damage pathway, RNF11 may participate with ZBRK1 in inducing the expression of DNA repair and pro-apoptotic factors.

9. Conclusions

RNF11 is an especially interesting protein, with many binding partners and diverse functions. We have come to find RNF11 to be involved in the ubiquitin-proteosome pathway, in protein trafficking, cell signalling and transcription. Because we find that RNF11 is so highly conserved throughout evolution, and lacks any immediate family members, we postulate an essential role for RNF11. RNF11 may have ancient links with early growth factor signalling.

TGF-β1 is a molecule that is fundamental for the homeostatic maintenance between cell growth and apoptosis [114]. The balance between survival, proliferation and apoptosis is central to many physiological processes and its deregulation can lead to disease. TGFβ1 is unique in that it can engage both the growth factor receptor, EGFR and the TGFβ receptor [114]. These two pathways, depending on the cell type, are generally antagonistic to one another and mediate a crucial balance between growth, differentiation and apoptosis (as discussed previously). As stated, RNF11 seems to serve both of these pathways. It is interesting to note that while RNF11 has a net positive effect on TGFβ signalling, it seems to have an opposing effect of EGFR signalling. Even clearer, are the effects that these two pathways have on the RNF11 protein. TGFβ signalling clearly enhances the steady-state levels of RNF11, while EGFR signalling decreases RNF11 steady-state levels (Seth lab, unpublished observations). Furthermore, it is clear that TGFβ signalling is adversely affected by the removal of RNF11 [22]. When taken together, one can postulate a scenario where EGFR signalling impacts negatively on TGFβ signalling through RNF11 and vice versa. Therefore RNF11 may represent a lynchpin for these two signalling modalities.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported in part by funding from CBCRA and the Canadian Foundation for Innovation.

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