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The RING Finger Protein11 Binds to Smad4 and Enhances Smad4-dependant TGF-β Signalling

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Abstract. *Background: In breast carcinomas, prolonged signalling through the TGF-β receptor promotes latent tumour progression, metastasis and the epithelial-to-mesenchymal transition of tumour cells. Previously, it has been found that the 154 amino acid RING finger protein, RNF11, was overexpressed in high-grade breast tumours and was capable of modulating TGF-β signalling. Materials and Methods: Utilizing cellular and biochemical assays, key interactions and molecular roles for the RNF11 protein in the TGF-β pathway were explored. Results: It is shown that RNF11 is required for TGF-β signalling and is capable of enhancing the Smad-TGF-β signalling pathway directly. Further, that endogenous RNF11 and Smad4 proteins associate and co-localize in a TGF-β-enhanced manner. This study indicates that RNF11 induces an increase in Smad4 protein levels. In functional assays, it is observed that RNF11 enhances Smad4-dependant TGF-β signalling and that RNF11 alone can recapitulate Smad4-dependant apoptosis in cellular assays. Conclusion: RNF11 acts directly on Smad4 to enhance Smad4 function, and plays a role in prolonged TGF-β signalling and possibly in latent tumour progression.*

The TGF-βs belong to a super family comprised of the BMPs, activins and TGF-β signalling factors, which are multifunctional regulators of cell growth and function. TGF-β has been implicated in cell differentiation, and is an effective *in vitro* inhibitor of cell growth, as well as a potent *in vivo* tumour suppressor (1-6). TGF-β signalling proceeds through the binding of the TGF-β ligand to the TGF-β receptor. The TGF-β receptor complex is comprised of a homodimer of a type 1 TGF-β receptor (TβR-I) and a homodimer of a type 2 TGF-β receptor (TβR-II). Transphosphorylation of the type 1 receptor by the type 2 receptor initiates the first steps in TGF-β signalling (7). Receptor associated signal-transducing factors, called R-Smads (such as Smad2 and Smad3) associate with the phosphorylated TβRI *via* the adapter molecule SARA. The Smads complexed to SARA are internalized through early endosomal vesicles that form intracellular speckles. This allows R-Smads to become activated through phosphorylation by the activated TβRI (1). Phosphorylated receptor Smads associate with Smad4 (8). During this process, Smad4 becomes mono-ubiquitinated on lysine 507 (K507), which leads to high-affinity interactions with the R-Smads, and has been shown to be indispensable for competent TGF-β signalling to occur (9). 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 (8, 10, 11).

Previously, it was found that 154 amino acid RING finger protein, RNF11, was overexpressed in breast tumours and was capable of antagonizing Smurf2 mediated inhibition of TGF-β signalling (5, 17). RNF11 has been shown to be indispensable for TGF-β signalling, wherein *RNF11* knock-down abrogates the TGF-β signal (12). RNF11 has modular domains and motifs including a RING-H2 finger domain, a PY motif and a predicted ubiquitin interacting motif (UIM) (13, 14) (Figure 1). The RING-H2 domain and the UIM motif of RNF11 are predicted to be involved in the ubiquitin pathway (13), while it has been previously demonstrated that the PY motif is essential for RNF11 binding to HECT-type E3 ligases, such as Smurf2 (5). In this current study, it is demonstrated that not only is RNF11 required for TGF-β signalling, but it also directly enhances TGF-β signalling. It is shown that RNF11 binds directly to Smad4, the common Smad for TGF-β, activin and BMP signalling. The interaction between RNF11 and Smad4 does not require the RNF11 PY motif, indicating that a ternary complex that includes other common TGF-β signalling factors may not be

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Key Words: RNF11, Smad4, TGF-β, ubiquitination, signal transduction, breast cancer.

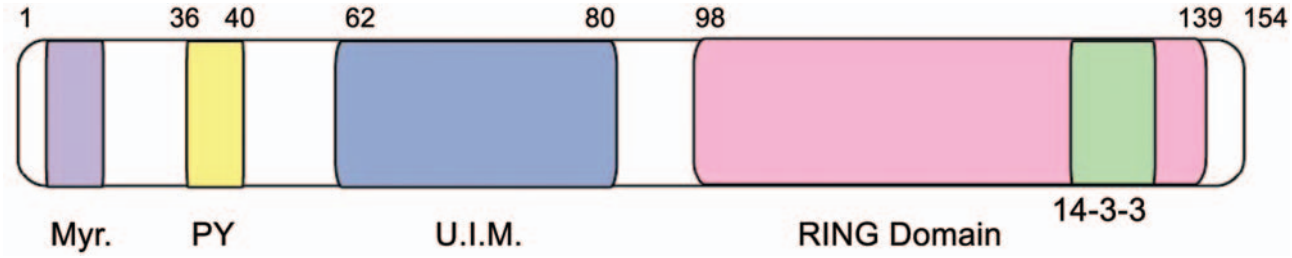

Figure 1. *The RNF11 protein contains a PY motif, UIM and RING(H2) domain. An outline of the RNF11 protein is shown with amino acid sequence numbers labelled across the top. Key motifs are shaded and include: 1) the PY motif (PPPY) which is required for interactions with proteins containing WW domains (shaded yellow); 2) the ubiquitin-interacting motif (UIM) which is required for the binding of ubiquitin and ubiquitinated proteins (shaded blue); 3) the RING(H2) domain, which is required for associations with E2 ubiquitin ligases, and catalytic ubiquitin ligase activity (shaded pink). Other key motifs that may be important for RNF11 protein function, localization and stability are highlighted for illustrative purposes, and include an N-terminal myristoylation site (labelled "Myr.", shaded purple) and a 14-3-3 binding site located in the RING domain (shaded green).*

required for Smad4-RNF11 complex formation. Endogenous Smad4 and RNF11 are shown to interact directly, and co-localize to sub-cellular speckles in response to TGF-β stimulation. Evidence that these sub-cellular speckles might be lysosomes or early endosomes, thereby indicating that RNF11 relocalizes upon TGF-β signalling in a manner similar to other TGF-β signalling factors, is hereby presented. It is shown that the RNF11-Smad4 interaction is enhanced in the presence of excess ubiquitination factors. Molecular studies indicated that RNF11 acts to increase and/or stabilize Smad4 steady state levels. Consistent with molecular studies, functional studies show that RNF11 is capable of enhancing Smad4-dependant TGF-β signalling and R-Smad-Smad4 signals. Cellular studies indicate that RNF11 can initiate apoptosis in a similar manner to that described for Smad4-dependant apoptosis. These observations imply a role for RNF11 in Smad4-dependant signal transduction.

Materials and Methods

Plasmid constructs transfections, luciferase assays and statistical analysis. Plasmid constructs were previously described (5, 15, 16). The sequence for RNF11 silencer was previously described (12). Transfections and luciferase assays are also as previously described (5, 15, 16). Briefly, transfections were carried out by seeding cells in a 6-well dish format 24 hours' pre-transfection with an average of approximately 5×10^5 cells per well. The cells were transfected with the indicated vectors using Lipfectamine2000 (Invitrogen; Carlsbad, CA, USA) with a ratio of 2:1 Lipofectamine2000:DNA with a total amount of DNA equal to 4 μg/ transfection. Total DNA quantities were kept constant between experimental groups using pCMV-GFP or pSG5 empty vector. For luciferase assays, 12-24 hours post transfection, cells were stimulated with 10 ng/mL of recombinant TGF-β-1 (PeproTech Inc., Rocky Hill NJ, USA). Twelve hours post TGF-β-1 stimulation, cells were assayed using the dual luciferase detection kit (Promega, Madison Wisconsin, USA) according to the manufacturer's instructions and bio-luminescence was detected using a Berthold Luminometer (Calmbacher Str. 22 D-75323 Bad Wildbad, Germany). Luminescence values were normalized to internal renella control and expressed as averages of 3 independent repeats with standard deviations. Statistical analysis was done using Excel spreadsheet applying Student's *t*-test for comparing experimental groups. *P* significance values are quoted in comparison to a reference group labelled with an asterix.

Cell culture. HEK293 and HepG2 cells were grown as previously described (15). Briefly, HEK293 cells were acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in DMEM, high glucose supplemented with 10% fetal calf serum (FCS). HepG2 cells were grown in MEM supplemented with Earl Salts, L-pyruvate, L-glutamine, non-essential amino acids and 10% fetal calf serum (FCS). Smad4-negative HCT116 cells were a generous gift of Dr. B. Vogelstein, and were maintained in McCoys 5A media supplemented with 10% FCS.

Protein binding, immunoprecipitaion, immunoblotting, and immunofluorescence. Anti-Flag mouse monoclonal antibody (M2, Sigma) and anti-Flag rabbit polyclonal (M1, Sigma) were purchased from Sigma and used according to the manufacturers' specifications. Anti-GST mouse monoclonal was also purchased from Sigma. Anti-Smad4 (mouse monoclonal) was purchased from BD Biosciences. Glutathione beads were acquired from Sigma and prepared according to the manufacturers specifications. ProteinA/G agarose beads (Sigma) were used for immuno-precipitations. RNF11 antibody is previously described (5) and was used at a dilution of 1:500. Lysotracker Red was obtained from Invitrogen-Molecular Probes and used according to the manufacturers specifications. For immuno-fluorescence and Lysotracker Red experiments, HepG2 cells were grown on glass cover slips for 24 hours. Cells were stimulated with 10 ng/mL of TGF-β-1 for the indicated times with or without Lysotracker Red. Cells were then fixed with 4% PFA and blocked with 10% goat serum overnight at 4˚C. Fixed and blocked cells were then probed with the indicated antibodies for 8 hours at 4˚C. Washes were done with PBS and repeated 3 times. Anti-mouse and anti-rabbit secondary fluorescent antibodies were obtained from Sigma and used according to manufactures specifications. Final washes used PBS and were repeated 5 times. Cover slips were mounted on slides which were then observed in a Zeiss Axiovert 200M.

Survival and Annexin V assays. MCF cells and MDA-MB-468 cells were grown as previously described (15, 17). Briefly, MCF7 and MDA-MB-468 cells were grown in DMEM supplemented with 10% FCS. Cells were transfected with the indicated vectors. For the survival assay, MCF7 cells were plated and transfected in triplicate with the indicated amount of RNF11 expression vector or irrelevant DNA control. Live cells were harvested 36 hours post transfection in 1 mL of PBS and were counted in a hemocytometer. For the apoptosis study, 24 hours post transfection, cells were stained with annexin-V and propidium iodide to detect apoptosis using the annexin-V-FLUOS staining kit (Roche Diagnostics) as per the manufacturer's instructions. The average percentage of annexin-V-positive cells is reported for each group. Three independent counts were taken for the purposes of establishing standard deviations and averages.

Results

RNF11 enhances TGF-β signalling and specifically binds Smad4. It has been previously shown that the overexpression of RNF11 could counter the inhibition of Smurf2 E3 ligase, which normally attenuates the TGF-β signal (5). In order to better understand the role of RNF11 in TGF-β signalling, RNF11 was overexpressed or knocked down in HepG2 cells and TGF-β responsiveness was assayed using a standard luciferase reporter assay. Smurf2 was used as a control for the loss of TGF-β signalling (Figure 2, lane 3). Knocking-down RNF11 with RNF11-siRNA results in a greater inhibition of TGF-β signalling (Figure 2, lane 4) than what was observed with overexpression of Smurf2 alone. Furthermore, overexpression of RNF11 enhances TGF-β signalling (Figure 2, lane 5). Enhancement of TGF-β signalling by RNF11 expression was observed to range from 20% to 50% above TGF-β alone (seen in repeat experiments, data not shown).

These initial observations lead to the conclusion that RNF11 is important for TGF-β signalling, and that the over-expression of RNF11 can have a direct enhancement on the TGF-β pathway. The level of RNF11-dependant enhancement on TGF-β signalling may represent the inhibition of endogenous Smurf2 function, or a direct augmentation of the TGF-β-Smad pathway by RNF11. To further evaluate the role of RNF11's in the TGF-β pathway, several binding assays with RNF11 and some common mediators of TGF-β signalling were performed. In these screens, it was discovered that RNF11 was able to associate with Smad4 (Figure 3), but not Smad2 or Smad7 (data not shown). These screens were conducted using standard GST pulldown assays. Here, it is shown that GST-RNF11, but not the GST tag alone, was capable of binding to Flg-Smad4 (Figure 3).

RNF11 binds to endogenous Smad4 through ubiquitin-enhanced mechanism. RNF11 contains motifs that predict a role for RNF11 in ubiquitination and ubiquitin binding. The

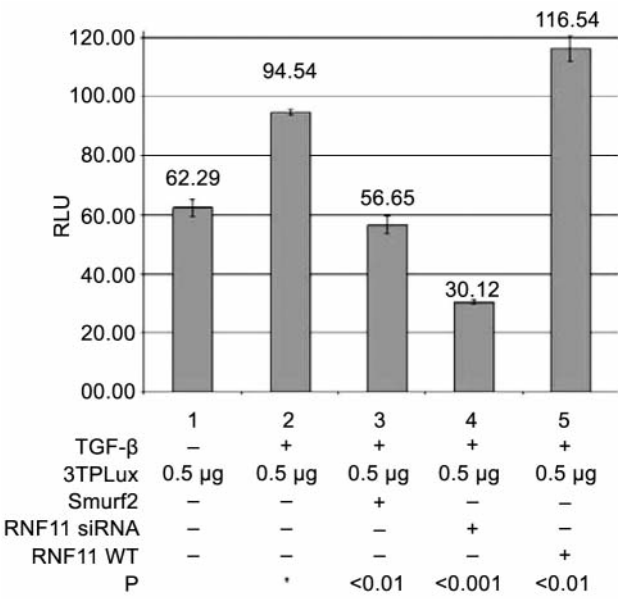

Figure 2. *RNF11 is required for TGF-β signalling and is sufficient for TGF-β signalling enhancement. HepG2 cells were transfected with the indicated expression vectors, along with the beetle-luciferase gene driven by the Smad-responsive promoter, 3TP-Lux. Lysates were analyzed for beetle-luciferase reporter gene levels, and normalized to renella-luciferase internal control. Each luciferase reporter experiment was conducted in triplicate and repeated at least twice. Representative data are shown. Smurf2 opposes TGF-β signalling and is used as a control for the loss of TGF-β signalling (lane 3). Knock-down of endogenous RNF11 with RNF11 siRNA expression vector results in a loss of TGF-β responsiveness (lane 4). Over-expression of RNF11 in HEPG2 cells enhances TGF-β signalling by at least 30%-50% (lane 5).*

amino acid sequence of RNF11 including the RING domain and a predicted UIM are shown in Figure 1. RING domains are known to be important for the catalytic addition of ubiquitin to substrates, and UIMs are known to allow for interactions with ubiquitin and ubiquitinated proteins. Studies have shown that ubiquitin modifications of Smad4 are important for Smad4 binding and function (9). In order to address the mechanism by which RNF11 binds to Smad4, co-immunoprecipitation experiments were conducted in HEK293 cells with RNF11 or the RNF11 PY mutant, in the presence of ubiquitination factors and endogenous Smad4. Since RNF11 can interact with Smurf2 *via* the PY motif and Smurf2 can form ternary complexes with Smad4 and other TGF-β signalling factors (35-37), the RNF11 PY mutant was used in order to determine if ternary complexes may be responsible for RNF11-Smad4 binding. It was observed that wild-type RNF11 or its PY mutant was able to bind to endogenous Smad4 (Figure 4a). Additionally, the expression of Ubc-H5b and ubiquitin enhanced the binding of endogenous Smad4 to both wild-type and PY mutant RNF11

(Figure 4a, lanes 6 and 7 *vs.* 3 and 4). Taken together, this may indicate ubiquitin-enhanced mechanism through which RNF11 and Smad4 interact directly.

RNF11 binds to Smad4 in a TGF-β-enhanced manner. To determine if endogenous RNF11 and endogenous Smad4 proteins interact, RNF11 was immunoprecipitated (IP) from HepG2 cells that were either stimulated or not stimulated with TGF-β, followed by immunoblotting for Smad4 (Figure 4b). It was observed that Smad4 was detectable in the IP when cells were stimulated with TGF-β (lane 1 *vs.* lane 2), but not in the absence of TGF-β. This may indicate that RNF11 and Smad4 normally associate after TGF-β signalling has been initiated. As a control HCT116 Smad4-negative cells were also used in these experiments (lane 3).

Endogenous RNF11 co-localizes with endogenous Smad4 in a TGF-β-enhanced manner. In order to better determine whether RNF11 and Smad4 associate with each other in cells, immunoflourenscence microscopy was conducted for endogenous RNF11 and Smad4 in TGF-β-responsive HepG2 cells in the search for co-localization of these two proteins. With no TGF-β stimulation, RNF11 remained predominantly in the cytoplasm (Figure 5a). RNF11 appeared to localize to diffuse speckled regions within the cytoplasm and to areas around the perinuclear space. Smad4 appeared to exhibit a diffuse localization pattern with both nuclear and cytoplasmic distributions. In the absence of TGF-β, only minor amounts of Smad4 and RNF11 co-localized, predominately around the perinuclear spaces (Figure 5a, merged image, white areas). However, in response to TGF-β stimulation, RNF11 and Smad4 appear to have an enhanced co-localization pattern (Figure 5b, merge image, white areas marked by red arrows). This pattern seems to preferentially appear as speckles in the cytoplasm and as strong perinuclear streaks. Upon TGF-β stimulation, Smad4 was seen to accumulate in the nucleus (Figure 5b). This is in agreement with the known behaviour of Smad4 in response to TGF-β signalling and serves as a positive control for the initiation of TGF-β signalling in the cell. These observations are in agreement with molecular interaction studies that indicate that RNF11 and Smad4 interact with one another at some point after TGF-β signalling is initiated.

RNF11 localizes to lysomal and/or endosomal compartments in response to TGF-β signalling. In intracellular co-localization studies, RNF11 and Smad4 formed cellular speckles in response to TGF-β signalling. Previous studies have reported that the Smad proteins and SARA associate during TGF-β signalling in endosomal compartments that appear as cellular speckles (1). It was postulated that RNF11 may also localize to these endosomal compartments when TGF-β signalling is initiated. In order to address this question, wild-type RNF11

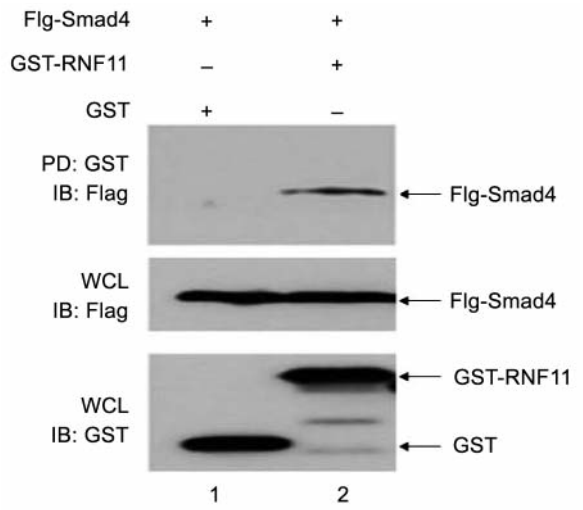

Figure 3. *RNF11 Binds to Smad4. Exogenously expressed RNF11 and Smad4 interact. Pull-down interaction experiments were conducted with GST-RNF11 and various Flg-Smads. A specific interaction was observed with Flg-Smad4 and GST-RNF11 but not with the GST tag alone. GST-RNF11 did not interact with Flg-Smad2 or Flg-Smad7 (data not shown).*

was expressed in HepG2 cells. HepG2 cells were stimulated with TGF-β and treated with Lysotracker Red, which marks lysosomal and endosomal compartments as fluorescent red. Here it was seen that in the absence of the TGF-β signal, RNF11 (green) was diffusely localized in the cell and remained excluded from endosomal bodies (red) (Figure 6a). In contrast, upon stimulation of these cells with TGF-β, RNF11 co-localized with the lysosomal/endosomal bodies (Figure 6b, merged). These observations may indicate that in response to TGF-β, the RNF11 protein behaves in a similar manner to what has been observed for other TGF-β signalling factors, which also relocate to endosomal bodies (38-40).

RNF11 enhances Smad4-dependant TGF-β signalling and R-Smad-Smad4 function. In order to functionally test the biological significance of the RNF11-Smad4 interaction, a Smad4-dependant luciferase reporter system was used. In this system, reporter gene expression is dependant on the exogenous addition of Smad4 to HCT116 cells that have been genetically engineered to lack Smad4. RNF11, but not the control expression vector (expressing Green Fluorescent Protein), induced a significant increase in Smad4-dependant luciferase reporter gene activity (Figure 7a). This may indicate that RNF11 acts to enhance TGF-β signalling through an association with Smad4 that leads to enhanced Smad4 function.

Smad4 normally associates with R-Smads, such as Smad2 and Smad3. To further evaluate the hypothesis that RNF11 could enhance Smad4 function, Smad4 and Smad2 were expressed together and in the presence of increasing amounts

of RNF11 in HEPG2 cells (Figure 7b). Synergistic activation of the SBE4-lux reporter gene can be observed with the coincident expression of Smad2 and Smad4. There was a significant enhancement in reporter activity with the addition of RNF11 to Smad2/4 co-expressing cells in a dose-dependant manner (Figure 7b, lanes 3 *vs*. 4 and 5).

Reports have shown that Smurf2 can degrade Smad2. Furthermore, ternary complexes containing Smad2 and Smurf2 have been shown to degrade Smad2-associated transcription factors, such as Smad4 (9, 35, 36). To further evaluate the means by which RNF11 enhances Smad2/4 activity, the RNF11 PY mutant that cannot bind to Smurf2 and is most likely incapable of binding to other HECT-type E3 ligases (5, 17) was employed. Furthermore, it was shown above that the RNF11 PY mutant is capable of binding the endogenous Smad4 protein (Figure 4a). The RNF11-PY mutant showed a greater ability to enhance Smad2/4 transcriptional activity than the WT RNF11 protein (Figure 7c). This indicates that RNF11 directly enhances Smad2-Smad4 function, without the need to block Smurf2 activity. This observation implies a mechanism for the RNF11-dependant enhancement of Smad4 and Smad4-R-Smad activities that is independent of HECT-Type E3 ligases.

RNF11 increases Smad4 steady state levels. RING finger proteins are known to have integral roles in the ubiquitin-proteasome pathway (31-34), and it has been previously shown that RNF11 interacts with the Ubc-H5 class of E2 ubiquitin-conjugating enzymes (5, 15). Other reports have shown that mono- and oligo-ubiquitination of Smad4 can lead to an enhancement of Smad4 transcriptional activity and stabilize Smad4 protein levels (9). As such, the possible role of RNF11 in the ubiquitin-dependant enhancement of Smad4 protein levels was investigated. Smad4 was co-expressed with ubiquitination factors (HA-ubiquitin and UBC-H5b) in the presence or absence of RNF11 or the RNF11-AKT phospho-mutant. Smad4 levels in the whole cell lysates of transfected HEK293 cells increased in the presence of RNF11 (Figure 8a). Densitometry normalized to actin was used to quantify the relative levels of Smad4 in these lysates. Densitometry revealed a 17% increase in Smad4 protein levels when Ubc-H5b and ubiquitin were co-expressed with Smad4 (Figure 8b). The addition of RNF11 wild-type protein lead to a 50% accumulation of Smad4 protein (Figure 8b). When the RNF11 AKT phosphorylation mutant was used, which has previously been shown to be more active in the enhancement of TGF-β activity (15), an approximate 40% increase in exogenous Smad4 protein levels was observed (Figure 8b). These observations highlight a possible mechanism by which RNF11 enhances Smad4-dependant TGF-β signalling; specifically through the stabilization and accumulation of Smad4 protein levels.

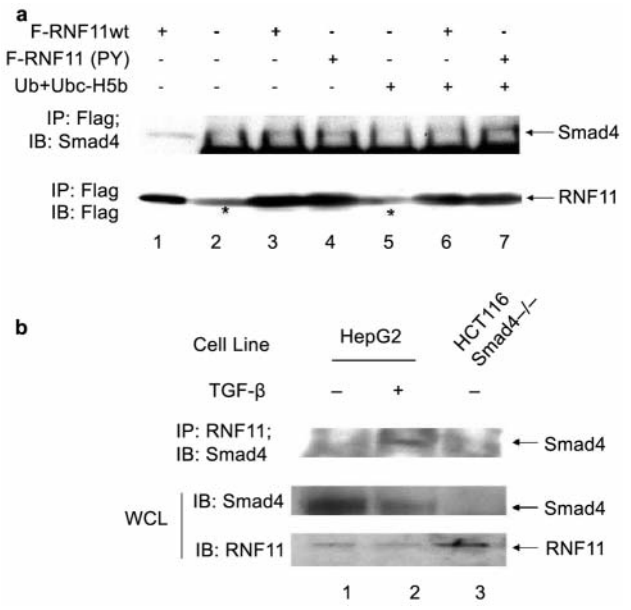

Figure 4. *RNF11 Interacts with endogenous Smad4 in a TGF-β- and Ub-enhanced manner. a) Wild-type Flg-RNF11 and PY mutant Flg-RNF11 bind to endogenous Smad4. WT RNF11, but not Flg-empty vector was seen to bind endogenous Smad4 (lane 2 vs. lane 3). In order to control for possible ternary complex formations between RNF11, Smad4 and other molecules, RNF11-PY mutant that cannot bind Smurf2 was used. RNF11-PY mutant was also observed to bind Smad4 (lane 4). The presence of additional ubiquitination factors (Ubc-H5b and ubiquitin) enhances the binding of Smad4 to RNF11 WT and PY mutant (lanes 6 and 7). Hek293 cells were transfected with the indicated expression vectors: 50μg of the whole cell lysate from sample lane #3 were loaded in the first lane of the same gel as a reference for the position of endogenous Smad4 and exogenous RNF11 on the immunoblot. The blot was stripped and re-probed with anti-flag antibody to show Flg-RNF11 was present in the precipitate (bottom panel). The apparent molecular weight of RNF11 is approximately 25kDa which causes the protein to co-migrate with the immunoglobulin light chain. *Indicates IgG light chain background band that co-migrates with the RNF11 protein. b) Endogenous RNF11 and Smad4 interact in a TGF-β enhanced manner. RNF11 was immunoprecipitated from HepG2 cells that were either stimulated or not stimulated with TGF-β, followed by immuno-detection for Smad4. Upon TGF-β signalling, Smad4 co-immunoprecipitates with RNF11 (lane 2 vs. lane 1). HCT116 Smad4 negative cells were used as a control for Smad4-(lane 3).*

Discussion

TGF-β is known to be a potent regulator of cellular growth and differentiation, and plays important roles in tumour suppression and tumour progression (19-24). Previously, it was found that RNF11 is overexpressed in breast tumours and its high expression correlated with invasive breast carcinomas (5). There are many reports documenting the link between prolonged TGF-β signalling and/or activated Smad4 activity with highly aggressive, metastatic breast cancer (21, 22, 24-26). It is believed that dysregulated RNF11 may

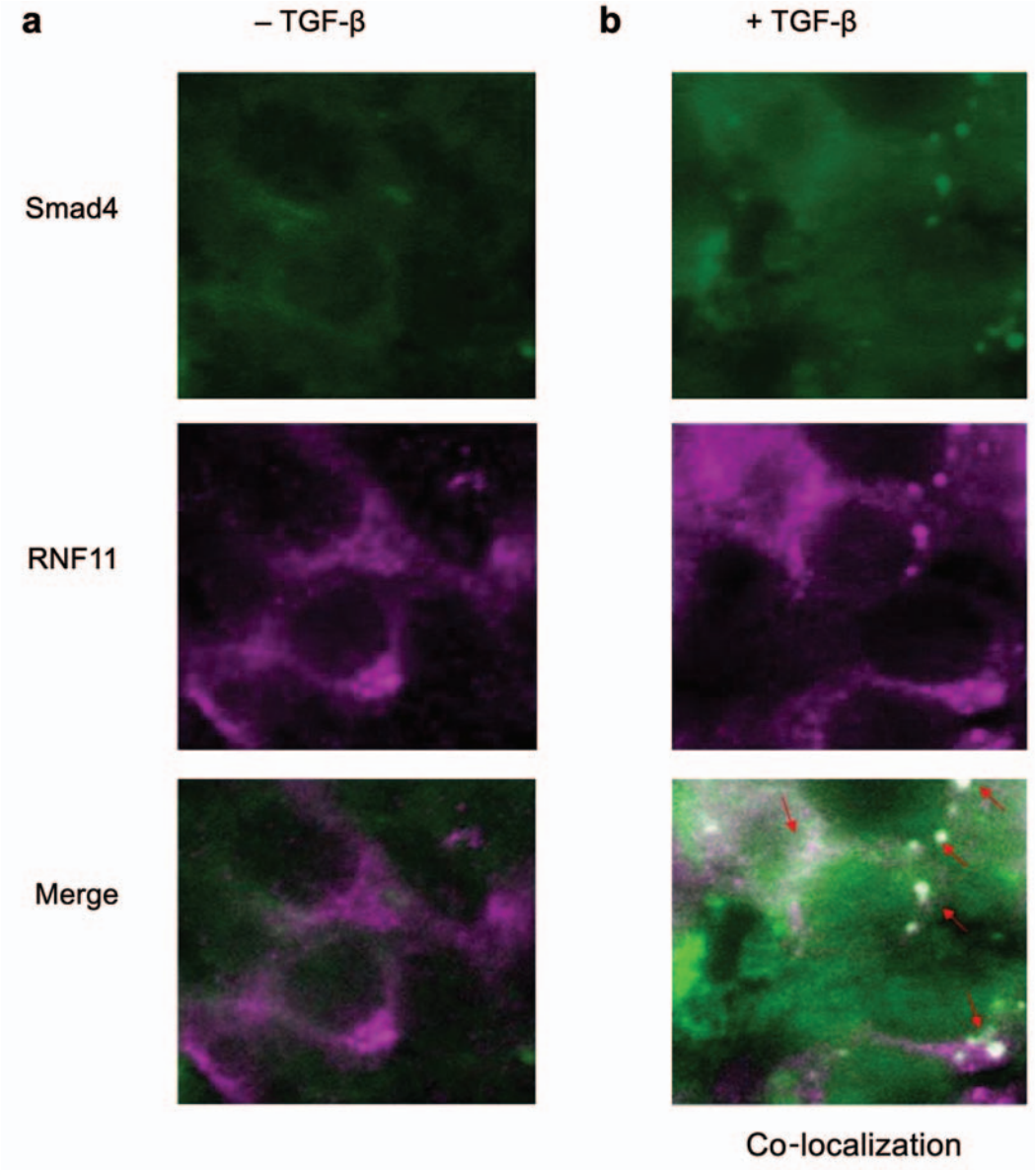

Figure 5. *Endogenous RNF11 and Smad4 co-localize in vivo in a TGF-β-enhanced manner. HepG2 cells were grown on cover slips and treated with TGF-β1 (10 ng/mL) for 24 hours and restimulated for 15 min before fixation with 4% PFA. Endogenous RNF11 and Smad4 were detected by immuno-fluorescence as outlined in the materials and methods. a) Without the addition of TGF-β, RNF11 (purple) is localized diffusely throughout the cell cytoplasm, while Smad4 (green) is observed both in the nucleus and cytoplasm. In the absence of TGF-β, both proteins sparsely co-localize (white areas). b) When cells are treated with TGF-β, RNF11 and Smad4 co-localize to the peri-nuclear spaces and to punctuate spots around the nucleus and in the cytoplasm (white areas). Red arrows are used to highlight areas of co-localization.*

provide a functional link in the transition between benign and invasive breast cancer.

RNF11 represents a unique RING finger protein, with no other family members yet described. The combination of a PY motif, UIM and RING domain implicate RNF11 in TGF-β signalling and ubiquitin pathways, respectively (13, 15, 27). The RNF11 PY motif is identical to the Smad6 and Smad7 PY motif, and is very similar to the PY motifs found in Smads 1, 2, 3 and 5. Here it is shown that RNF11 can enhance TGF-β signalling through association and stabilization of the Smad4 protein. Furthermore, RNF11 binding to Smad4 and activation of R-Smad-Smad4 function is not dependant on the presence of the RNF11 PY motif, which may indicate that these effects are independent of ternary complexes that may involve HECT-type E3 proteins. Smad4 enhancement through an RNF11-dependant

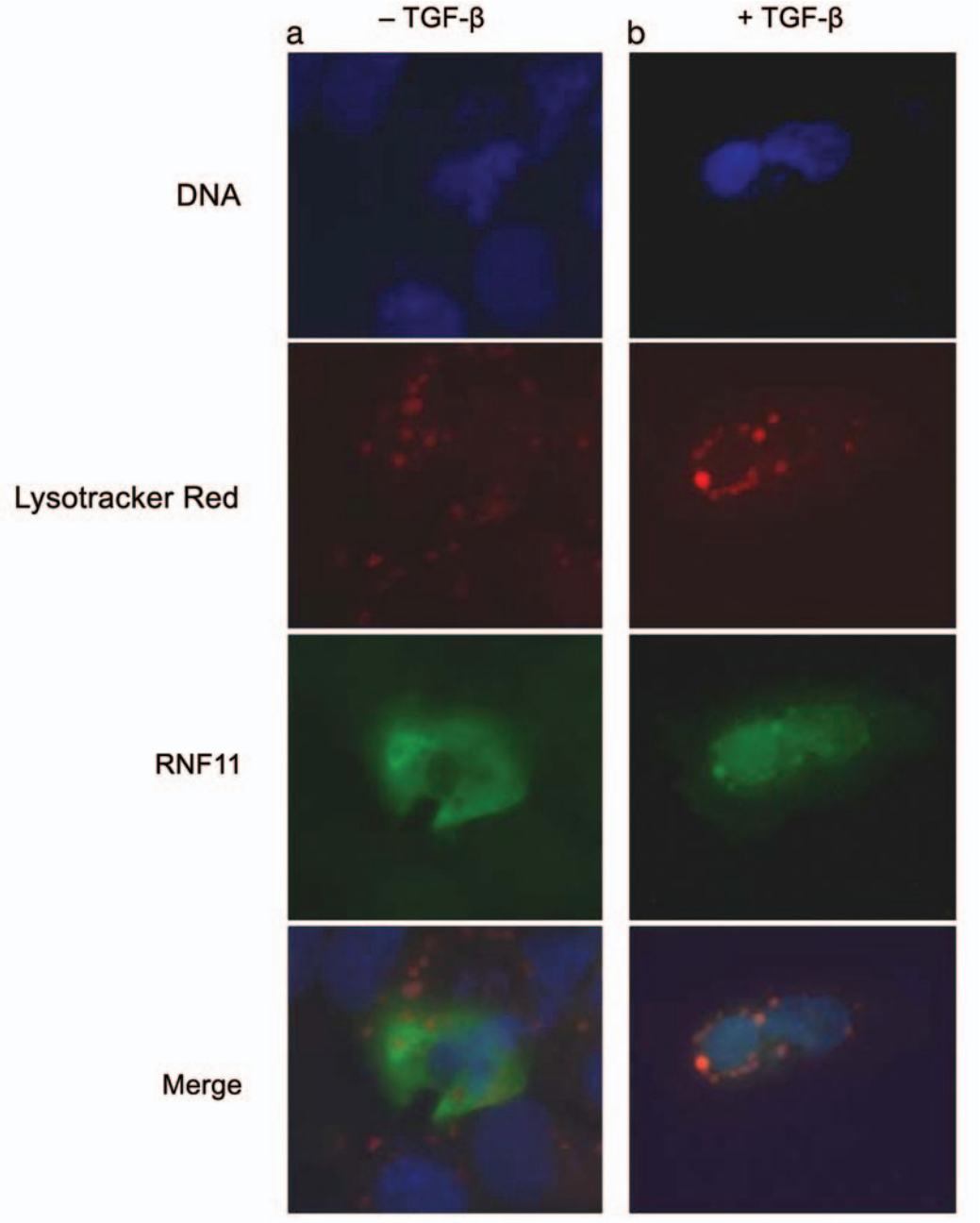

Figure 6. *RNF11 localizes to lysosomal and/or endosomal compartments in response to TGF-β signalling. a) HepG2 cells were transfected with Flg-RNF11 and treated with Lysotracker Red, which marks lysosomal and endosomal compartments in fluorescent red. RNF11 is labelled green. Merged images show that RNF11 was excluded from lysosomal/ endosomal compartments in the absence of TGF-β signalling. b) HepG2 cells transfected with Flg-RNF11 were stimulated with TGF-β-1 and treated with Lysotracker Red. Merged images show that RNF11 relocated to lysosomal/ endosomal bodies upon TGF-β signalling (merged signal, orange).*

mechanism has implications for the TGF-β, BMP and activin signalling pathways.

The RNF11-Smad4 interaction appears to be specific and is regulated by TGF-β signalling and the ubiquitin pathway. In order to help rule out the presence of ternary complexes that may mediate the interaction between RNF11 and Smad4, RNF11 PY mutant was employed in some interaction and functional assays. In the presence of ubiquitination factors, RNF11 and Smad4 seem to have a greater affinity for one another. The RNF11 PY mutant showed the highest levels of interaction with Smad4 in the presence of ubiquitination factors. This observation may

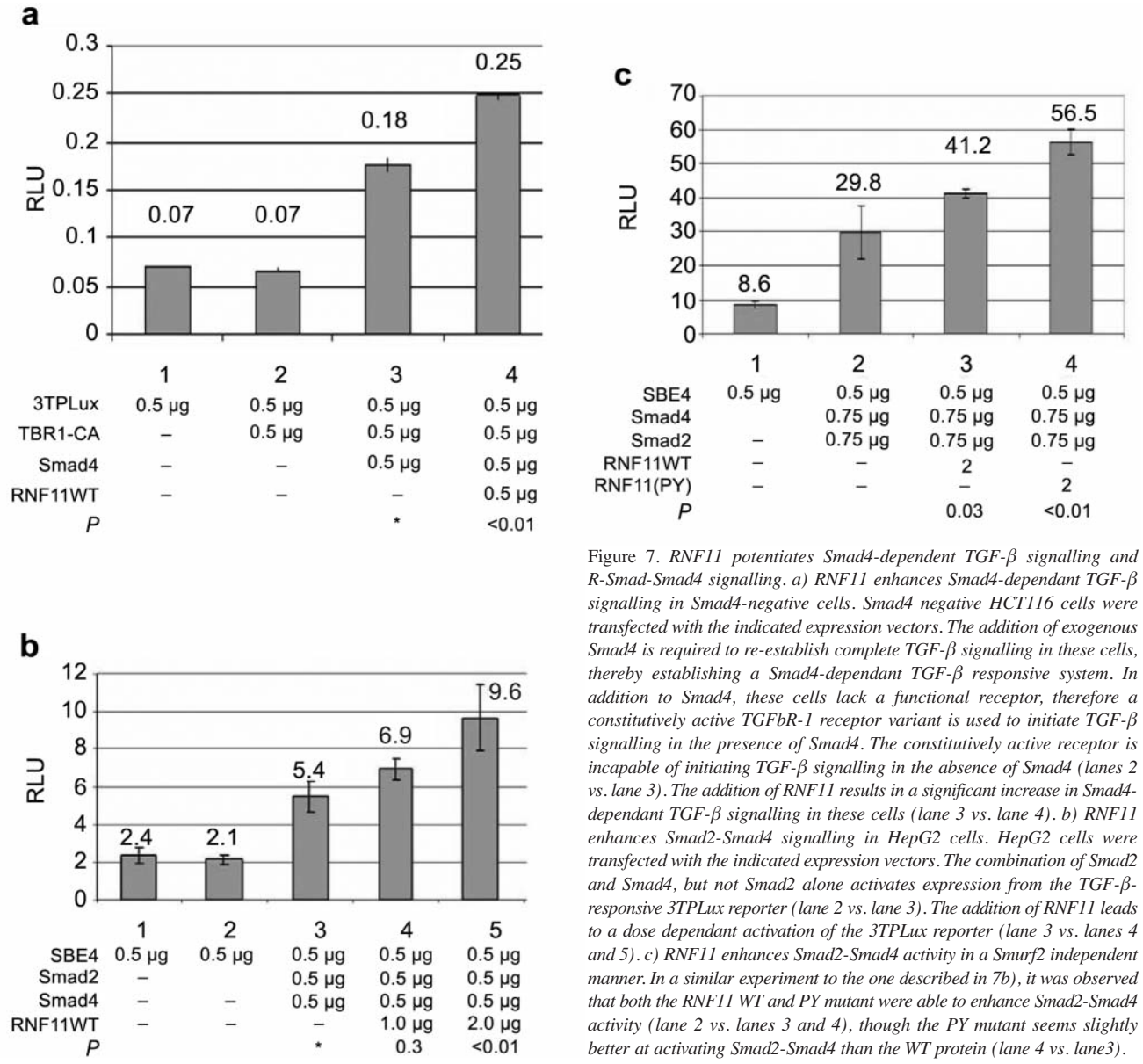

Figure 7. *RNF11 potentiates Smad4-dependent TGF-β signalling and R-Smad-Smad4 signalling. a) RNF11 enhances Smad4-dependant TGF-β signalling in Smad4-negative cells. Smad4 negative HCT116 cells were transfected with the indicated expression vectors. The addition of exogenous Smad4 is required to re-establish complete TGF-β signalling in these cells, thereby establishing a Smad4-dependant TGF-β responsive system. In addition to Smad4, these cells lack a functional receptor, therefore a constitutively active TGFbR-1 receptor variant is used to initiate TGF-β signalling in the presence of Smad4. The constitutively active receptor is incapable of initiating TGF-β signalling in the absence of Smad4 (lanes 2 vs. lane 3). The addition of RNF11 results in a significant increase in Smad4-dependant TGF-β signalling in these cells (lane 3 vs. lane 4). b) RNF11 enhances Smad2-Smad4 signalling in HepG2 cells. HepG2 cells were transfected with the indicated expression vectors. The combination of Smad2 and Smad4, but not Smad2 alone activates expression from the TGF-β-responsive 3TPLux reporter (lane 2 vs. lane 3). The addition of RNF11 leads to a dose dependant activation of the 3TPLux reporter (lane 3 vs. lanes 4 and 5). c) RNF11 enhances Smad2-Smad4 activity in a Smurf2 independent manner. In a similar experiment to the one described in 7b), it was observed that both the RNF11 WT and PY mutant were able to enhance Smad2-Smad4 activity (lane 2 vs. lanes 3 and 4), though the PY mutant seems slightly better at activating Smad2-Smad4 than the WT protein (lane 4 vs. lane3).*

indicate that RNF11 can mediate several distinct interactions simultaneously; however, in the absence of the PY domain, certain interactions are favoured (such as the one with Smad4). These observations are consistent with findings from other studies, which have observed that ubiquitination factors alone could enhance Smad4 activity (9). It is possible that this may occur through the enhanced binding of RNF11 to Smad4 following Smad4 ubiquitination. Interestingly, enhancement of Smad4 function occured with the addition of RNF11 alone without the need to express exogenous ubiquitination factors, which may indicate that RNF11 is targeted to Smad4 before Smad4 is ubiquitinated, but after TGF-β signalling is initiated. In line with these observations, previous studies have shown that an increase in Smad4 expression and activity is capable of inducing apoptosis in sensitive breast cancer cells (30). When RNF11 is expressed in Smad4-sensitive MCF7 cells, survival can be affected and apoptosis induced to the same levels as observed by previous works (Figure 9a and b) (30). Likewise, when the RNF11 Ring domain is mutated, which is known to disrupt Ring domain function and E2 binding, a drastic reduction in RNF11-induced apoptosis in MCF7 cells is observed. This indicates that RNF11 functions to activate Smad4 *via* the RNF11 Ring domain. Moreover, when RNF11 is expressed

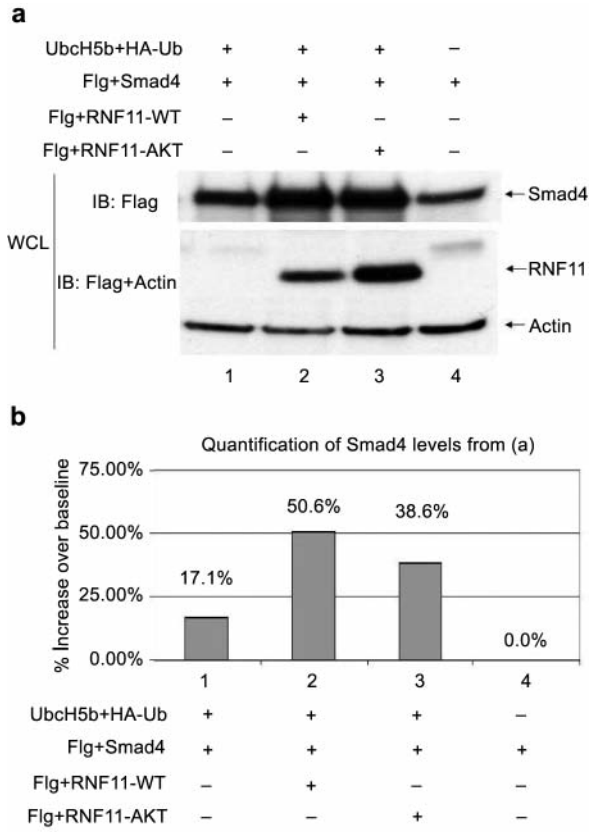

Figure 8. *RNF11 Increases Smad4 steady state levels. a) Hek293 cells were transfected with the indicated mammalian expression vectors. Equal amounts of whole cell lysate were resolved on SDS-PAGE and probed with the indicated antibodies. Exogenous Smad4 steady state levels increased with the addition of ubiquination factors (lane 4 vs. lane 1). RNF11 or its AKT mutant, but not control vector showed a further increase in Smad4 steady state levels (lane 1 vs. lane 2 and lane 3). b) Densitometry reveals that RNF11 WT or AKT mutant can increase Smad4 steady state levels approximately 40% – 50% above baseline Smad4 protein levels. Densitometry was performed on scanned films from (a). Values for Smad4 band intensity were normalized to actin and then plotted graphically. All groups were normalized to the Smad4 only group (lane 4), which was set as a reference baseline and is therefore reported as 0% increase above baseline.*

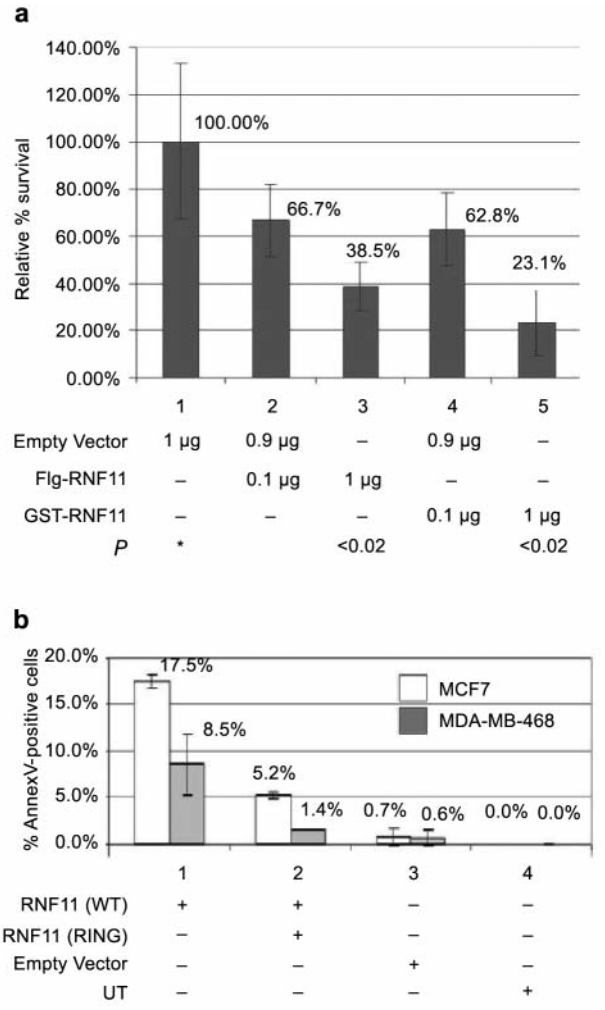

Figure 9. *RNF11 enhances Smad4-dependant apoptosis in breast cancer cell lines. a) MCF7 cells were plated and transfected in triplicate with the indicated amount of RNF11 expression vector or irrelevant DNA control. Survival in all groups was normalized to the empty vector control group, which was set at 100% survival. Flg-RNF11 and GST-RNF11 induced a loss of survival in MCF7 cells in a dose-dependant manner. b) RNF11 wild-type expression vector induces significantly more apoptosis in MCF7 cells versus the Smad4-negative MDA-MB-468 human breast carcinoma cells (lane 1). Furthermore, RNF11 WT induced more apoptosis than did the RING mutant RNF11 or control vector in both cell lines (lane 1 vs. lanes 2, 3 and 4). Cells were transfected with Flg-RNF11 WT (lane 1), Flg-RNF11-RING mutant (lane 2), irrelevant DNA control (lane3) or not at all (UT: untransfected control, lane 4). Three independent counts were taken for the purposes of establishing standard deviations and averages.*

in the Smad4-negative cell line, MDA-MB-468, RNF11-induced apoptosis was drastically reduced (Figure 9b). Collectively, these observations indicate that RNF11 may illicit some of its biological effects through the direct activation of Smad4.

In this study, it was shown that RNF11 was capable of direct enhancement of TGF-β signalling in the range of 20% to 50% above what is commonly observed with TGF-β alone. Likewise, RNF11 increases Smad4 steady state levels by roughly the same amount. This direct enhancement of Smad4 protein levels is believed to represent a previously undescribed mechanism for TGF-β regulation. It is also reported that RNF11 can bind to Smad4 directly, and that this binding is regulated through TGF-β signalling and perhaps though a possible ubiquitin-mediated mechanism. Previous studies have reported that the ubiquitination, stabilization and activation of Smad4 can be accomplished in the presence of excess ubiquitination factors (9), though a specific E3 ubiquitin ligase

responsible for this effect has yet to be elucidated. Here it is shown that RNF11 is capable of binding Smad4 and enhancing Smad4 transcriptional activities in the absence of excess ubiquitination factors. It is therefore postulated that RNF11 may use endogenous E2s and ubiquitin to elicit similar effects on Smad4 to the ones previously observed in the presence of exogenous E2 and ubiquitin.

Upon TGF-β stimulation the SARA protein, an adapter involved in TGF-β signal transduction, localizes to speckled compartments in the cytoplasm (28,29). Likewise upon TGF-β stimulation, Smad4 and RNF11 dramatically co-localize to speckled compartments in the cytoplasm and to streaks around the nucleus. The co-localization pattern for RNF11 and Smad4 appear similar to the pattern observed for SARA. In the absence of TGF-β stimulation, RNF11 and Smad4 weakly co-localize. In large scale interaction screens, RNF11 and Smad4 have been found to interact with SARA (12, 28, 29), though it should be noted that the interaction between RNF11 and SARA has yet to be confirmed by a more detailed study. It is conceivable that SARA may act as a scaffold for RNF11 and Smad4 associations. Though it cannot be ruled out that other proteins, such as SARA, might be involved in mediating Smad4-RNF11 interactions, the attempt to use RNF11 mutants that remove protein interactions known to form ternary complexes is a first step to understanding the RNF11-Smad4 interaction. At the least, this study demonstrates that RNF11 and Smad4 can form associations that are aided in part through the ubiquitin pathway and are biologically responsive to TGF-β signalling. The net result of these interactions seems to yield a more stable and active Smad4 protein.

Previous studies have shown that breast cancer bone metastasis is highly reliant on the Smad-TGF-β pathway and in particular on Smad4 (22). It is then conceivable that RNF11 dysregulation in breast carcinoma is a precipitating factor leading to an invasive state *via* a direct activation of Smad4. Indeed, it has been shown that RNF11 is indispensable for TGF-β signalling, where knock-down studies have revealed that TGF-β signalling is severely impaired in the absence of RNF11 (12) (Figure 2). The interaction of RNF11 with Smad4 is novel and may represent the most potent means by which RNF11 influences TGF-β and TGF-β family member signalling. Future studies are designed to uncover the molecular events that underlie Smad4 activation through RNF11. In light of these findings combined with previous observations, it is believed that RNF11 may represent a novel target for cancer therapeutics in patients with advanced metastatic disease.

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