Ubiquitination of Tumor Necrosis Factor Receptor Associated Factor 4 (TRAF4) by Smad Ubiquitination Regulatory Factor 1 (Smurf1) Regulates Motility of Breast Epithelial and Cancer Cells*

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Background: Ubiquitin E3 ligase Smurf1 plays an important role in cell migration and tumor metastasis.

Results: Smurf1 ubiquitinates TRAF4 at K190, which is required for TRAF4 localization at the cell junction. It also promotes cell migration and activates Rac1.

Conclusion: Smurf1 regulates cell migration through ubiquitination of TRAF4.

Significance: TRAF4 ubiquitination is a key regulatory step in controlling breast epithelial and cancer cell migration.

SUMMARY

Smad ubiquitin regulatory factors (Smurfs) are HECT-domain ubiquitin E3 ligases that regulate diverse cellular processes, including normal and tumor cell migration. However, the underlying mechanism of the Smurfs’ role in cell migration is not fully understood. Here we show that Smurf1 induces ubiquitination of tumor necrosis factor receptor-associated factor 4 (TRAF4) at K190. Using the K190R mutant of TRAF4, we demonstrate that Smurf1-induced ubiquitination is required for proper localization of TRAF4 to tight junctions in confluent epithelial cells. We further show that TRAF4 is essential for the migration of both normal mammary epithelial and breast cancer cells. The ability of TRAF4 to promote cell migration is also dependent on Smurf1-mediated ubiquitination, which is associated with Rac1 activation by TRAF4. These results reveal a new regulatory circuit for cell migration, consisting of Smurf1-mediated ubiquitination of TRAF4 and Rac1 activation.

INTRODUCTION

Ubiquitination is a post-translational modification of protein substrates by the covalent attachment of ubiquitin, a small polypeptide with 76 amino acid residues, through a series of enzymatic reactions (1). Depending on the number of ubiquitin moieties that are attached, proteins can be modified by mono-ubiquitination, in which a single ubiquitin moiety is attached to one or several lysine residues on the substrate, or polyubiquitination, in which the substrate protein is modified by a chain of more than four ubiquitin moieties linked through one of the seven lysine residues in ubiquitin (2). The most-studied polyubiquitin chain, with the K48 linkage, usually marks a protein for degradation in the proteasome. In contrast, monoubiquitination often regulates processes ranging from membrane transport to transcription (3). Because of diverse functions...
and physical contact with substrates, the third enzyme in the ubiquitination chain reaction, E3 ubiquitin ligase, is of the most importance. Mechanistically, E3 ubiquitin ligases are classified into two categories: the RING-domain-bearing E3 that acts as a scaffold to promote ubiquitin transfer from an E2 ubiquitin conjugase to a substrate and the HECT-domain-containing E3 that catalyzes ubiquitin transfer via a thioester intermediate formed on a conserved cysteine residue in the enzyme (4).

Smurf1 and Smurf2 are HECT-domain-containing E3 ubiquitin ligases, which were initially identified as regulators of Smads and TGF-β/BMP receptors (5–8). To date, the functions and the repertoires of known Smurf substrates have been expanded beyond TGF-β/BMP signaling. In in vitro cell culture systems and in mice, Smurf1 was reported to participate in the maintenance of bone homeostasis by targeting Runx2 and MEKK2, respectively (9–11), whereas Smurf2 was implicated in cell cycle progression, senescence, DNA damage response, and genomic stability through a variety of protein targets (12–14). In addition to their respective unique functions, Smurf1 and Smurf2 also share a common function by controlling non-canonical Wnt output in setting up planar cell polarity (15), a process not only important during embryonic development, but also in tumor cell invasion (16). Many Smurf protein targets involved in cell polarity, migration, and adhesion, including RhoA, Rap1B, hPEM-2, and Talin, have been identified (17–20).

In addition to their essential roles during development and normal physiological functions, several reports indicate that Smurf1 and Smurf2 expression are dysregulated in cancer cells (21–24). Elevated Smurf expression in breast cancer tissues was shown exclusively in the cytoplasm (22), whereas siRNA knockdown of Smurf1 or Smurf2 led to cell rounding and retardation of cell migration (22, 25). Smurf1 has been shown to promote lamellipodia formation and tumor cell migration by down-regulating RhoA activity and its downstream ROCK-MLC2 signaling at the cell periphery (25). Despite these myriad observations, the underlying mechanism of Smurfs’ regulation of cancer cell motility remains to be determined.

TRAF4, originally identified as a protein overexpressed in breast carcinomas (26), is one of six TRAF family adaptor proteins. TRAF proteins are known for their roles in immunity, inflammation, and apoptosis by functioning as scaffolds in the tumor necrosis factor receptor, Toll-like receptor, and interleukin-1 receptor signaling complexes (27). Although TRAF4 shares a common structural framework with other family members, its function does not fit the general signaling paradigm of other TRAFs (28). For instance, many TRAF gene knockout mice have compromised immune systems, but the immune responses in TRAF4-deficient mice appear to be normal (29). However, about one-third of TRAF4-deficient mice die in utero, with surviving pups displaying severe tracheal, skeletal, and neural tube closure defects (30). In endothelial cells, TRAF4 localizes at the leading edge of the focal adhesion complex, where it induces membrane ruffling through Rac1 and promotes cell migration (31). In normal mammary epithelial and breast cancer cells, TRAF4 was identified at sites of cell–cell contact and tight junctions (32). However, it is not clear what determines the subcellular localization of TRAF4 and whether TRAF4 regulates cancer cell migration.

Here we report that Smurf1 induces monoubiquitination of TRAF4 at K190, and demonstrate that this modification targets TRAF4 to cell–cell junctions in normal mammary epithelial cells. We further show that monoubiquitination of TRAF4 at K190 by Smurf1 is required for migration of normal mammary epithelial and breast cancer cells, and that this regulation is likely mediated by Rac1 activation.

EXPERIMENTAL PROCEDURES

Expression Plasmids—Full-length mouse TRAF4 cDNA (Image Clone 3598556, ATCC) and TRAF5 cDNA (MGC-13966, ATCC) were amplified by PCR and subcloned into the BamHI and SalI sites of pRK5-derived pRK2H or pRK2F vectors to generate N-terminal HA-tagged TRAF4, TRAF5, or FLAG-tagged
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TRAF4. Deletion mutants of the TRAF4 RING finger domain (AR), RING and Zinc finger domain (ΔRZ), RING, Zinc finger and N-TRAF domain (ΔRZN), and C-TRAF domain (ΔC) were similarly constructed and inserted into a pRK2H vector. The TRAF4-K190R mutant was generated using a PCR-based strategy. All PCR-amplified regions were verified by sequencing. Plasmids for HA-TRAF2, HA-TRAF3, HA-TRAF6, Myc-Smurf1, 6xMyc-Smurf1CA, Myc-Smurf2, and Myc-Smurf2CG have been described previously (8, 10, 33, 34). GFP-TRAF4 and GFP-TRAF4 K190R were constructed by inserting the BamHI/Sall fragments of full-length TRAF4 into the BgII/Sall sites of pEGFP-C1 (Clontech).

Cell Culture and Transfection Conditions—HEK293 (CRL-1573, ATCC) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Immortalized normal human breast epithelial MCF-10A (CRL-10317, ATCC) cells were cultured in DMEM/F12 supplemented with 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 500 ng/ml hydrocortisone, and 100 ng/ml cholera toxin. Breast cancer cell line MCF-7 was obtained from the DCTD Tumor Repository of the National Cancer Institute and maintained in Eagle’s minimum essential medium (MEM) with Earle’s balanced salt solution supplemented with 10 micrograms/ml insulin and 10% FBS. Transfections of HEK293 and MCF10A cells were done according to the manufacturer’s instructions using FuGENE® 6 (Roche Applied Science) and Lipofectamine® Plus (Invitrogen), respectively.

RNAi Experiments—Control non-silencing siRNA or TRAF4 siRNA (Hs_Traf4.5) were obtained from Qiagen. Oligofectamine Reagent (Invitrogen) was used for transfection of siRNAs into cells according to the manufacturer’s directions. Briefly, 12 μl of 20 μM siRNA duplex in 88 μl of Opti-MEM® was mixed with 12 μl of Oligofectamine Reagent in 88 μl of Opti-MEM. After incubation for 20–25 min at room temperature, the 200-μl siRNA-Oligofectamine mixture was added to cultured cells (40–50% confluence) in 800 μl of Opti-MEM in a 6-well plate. Regular medium (1 ml) containing 20% FBS was added to the transfected cells 3 h after transfection. Cells were harvested for Western blotting or other assays 2 days after transfection.

Retroviral Infection—Retroviral vector, pBABE-puro, was used to express FLAG-tagged TRAF4 and TRAF4-K190R. Vector construction information and the sequences are available upon request. The pBABE-FLAG-Smurf1 vectors were described previously (35). Viral production and infection were done as described previously (35). Stable MCF7 and MCF10A cells were established after puromycin selection (4–8 μg/ml) following infection with retroviruses.

Western Blotting and Immunoprecipitation—The methods for Western blotting and immunoprecipitation have been described previously (34, 36). The antibodies used were : Smurf1 (H-60, Santa Cruz), TRAF4 (H-72 and C-20, Santa Cruz), Rac1(C-11, Santa Cruz), Myc (9E10, Santa Cruz), Ub (P4D1, Santa Cruz), HA11 (Covance), and FLAG (M2, Sigma).

Wound Healing and Migration Assays—Cell migration was assessed using the Ibidi™ Culture-Insert “wound healing” system according to the manufacturer’s directions. A 70-μl cell suspension (6x10^5/ml) was added to each well of a 6-well dish and incubated at 37 °C overnight or for 24 h. After cell attachment, the Culture-Insert was removed to create a cell-free gap. The cell culture dish was then filled with regular cell culture medium to initiate cell migration. Pictures were taken at 24 h for the MCF7 cells and at 12 h for the MCF10A cells (unless otherwise specified). The gap widths were measured and quantified at these time points.

The transwell cell migration assay was performed using 6-mm Biocoat Cell Culture Control Inserts (BD Biosciences). MCF7 cells were starved overnight in 0.2% FBS medium, then 2X10^6 cells in 0.2 ml of 0.2% FBS medium were added to an insert that was then placed in
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0.6 ml 10% FBS medium. After incubation for 24 h at 37 °C, Culture-Inserts that had migrated cells to the underside of the membrane were stained with the Diff-Quik stain kit (Dade Boehringer). All cells on the membrane were counted under a microscope.

Ubiquitination Site Mapping by Mass Spectrometry—To map the Smurf-dependent ubiquitination site on TRAF4, HEK293 cells transfected with FLAG-TRAF4 and Myc-Smurf1 or vector control were lysed in 1X RIPA buffer, followed by immunoprecipitation with FLAG M2 agarose (Sigma). The beads were boiled in 1% SDS solution for 5 min, then diluted to 0.1% SDS in a solution containing 20 mM DTT and analyzed by SDS-PAGE and Coomassie blue staining. The band corresponding to ubiquitinated TRAF4, detected only in cell lysates transfected with both FLAG-TRAF4 and Myc-Smurf1, was excised. The gel slice was subjected to digestion with trypsin and the peptides were extracted and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the Proteomics Center at Boston Children’s Hospital.

Immunofluorescence—MCF10A cell lines transfected with GFP-vector, GFP-TRAF4, or GFP-TRAF4K190R were seeded in a four-chamber vessel (BD Falcon). Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. After washing three times with phosphate-buffered saline, cells were blocked in 20% goat:donkey (1:1) serum for 1 h at room temperature, then incubated with Alexa Fluor 594 phalloidin (Invitrogen) at a 1:1000 dilution for 1 h at room temperature or with occludin (BD Biosciences) at 1:100 in goat serum overnight at 4 °C. Cells were then washed with phosphate-buffered saline for 15 min, incubated with the second antibody at a 1:1000 dilution for 1 h at room temperature, and mounted with medium containing DAPI (Vector Lab) to visualize the nuclei. Cells were examined using an LMS 510 confocal microscope (Zeiss). Imaging with GFP, Alexa Fluor red, and DAPI blue was performed with a Plan-Apochromat 63x 1.4 NA oil immersion objective using 488-, 568-, and 405-nm excitation, and emissions were collected using BP 505-550, LP 585, and BP 420-480 filters. Images were acquired using the microscope’s software (Zeiss).

Rac1 Activity Assay—Rac1 activation was assessed using the Rac1/Cdc42 assay (Millipore), per the manufacturer’s instructions. Cells were lysed in MLB buffer (provided in kit) and 10 μg of PAK1 PBD agarose beads were used to perform affinity precipitation at 4 °C for 60 min with gentle rotation. After washing the agarose beads three times with MLB buffer, they were resuspended in sample buffer. The proteins obtained from beads were resolved by SDS-PAGE and detected by Western blotting for Rac1 (Santa Cruz).

RESULTS

Smurf1 Induces TRAF4 Ubiquitination—TRAF4 was identified previously by several groups as an interacting partner for Smurf1 and Smurf2 through yeast two-hybrid interaction screens (37, 38, 39). Subsequently, the in vivo interaction between TRAF4 and Smurf1 was confirmed through co-immunoprecipitation assays (38, 39), with one group showing that Smurf1 was capable of inducing polyubiquitination of all six TRAF proteins (39). Consistent with this report, we co-precipitated Myc-tagged Smurf1 with all five HA-tagged TRAFs from TRAF2 to TRAF6 that were transiently expressed in HEK293 cells (Fig. 1A). However, we were surprised to notice an additional, slow-migrating band of TRAF4 when it was co-expressed with Smurf1 (Fig. 1A). This slow-migrating TRAF4 band also appeared in the total lysate sample, but the ratio of this slow-migrating band to that of the regular TRAF4 increased markedly in the Smurf1-immunoprecipitated sample (Fig. 1A), implying a role for Smurf1 in the formation of this modified form of TRAF4. Smurf2 showed a similar, albeit relatively weaker, activity in inducing this TRAF4 modification (Fig. 1B). The ability of Smurf1 and Smurf2 to induce this form of TRAF4 modification is dependent on the intrinsic E3 ligase activity of Smurfs as
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neither the E3 ligase-deficient mutant of Smurf1 (C710A) nor that of Smurf2 (C716G) was able to induce such a slow-migrating band of TRAF4 (Fig. 1B). These results suggested that the slow-migrating band was likely the ubiquitin-modified TRAF4. To determine whether this was the case, we used the P4D1 antibody that recognizes endogenous ubiquitin to immunoprecipitate the ubiquitinated proteins from HEK293 cells that were co-transfected with FLAG-tagged TRAF4 and either Myc-tagged Smurf1 or Smurf2. Western blot analyses with anti-Flag detected both forms of TRAF4 in the total cell lysate, but only the slow-migrating form in the ubiquitinated samples (Fig. 1C), thus confirming that TRAF4 is ubiquitinated by Smurfs. In contrast to previously reported results with Xenopus TRAF4, which was degraded when co-expressed with exogenous Smurf1 in HEK293T cells (38), we did not see any meaningful degradation of the mammalian TRAF4 when it was co-expressed with either Smurf1 or Smurf2 (Fig. 1, A and B). Moreover, although we detected the smear of the polyubiquitinated TRAF4 when it was co-expressed with Smurfs and HA-ubiquitin (data not shown), the slow-migrating form of TRAF4 that we detected without the forcibly expressed HA-ubiquitin was a discrete band (Fig. 1). These observations indicate that the Smurf-mediated modification of TRAF4 is monoubiquitination.

K190 Is a Major Site of the Smurf1-induced Ubiquitination of TRAF4—To identify specific lysine residues in TRAF4 that were ubiquitinated by Smurf1, we isolated the FLAG-TRAF4 and Myc-Smurfl immunocomplexes from transfected HEK293 cells. After resolving them by SDS-PAGE, we identified the monoubiquitinated TRAF4 with Coomassie blue stain (Fig. 2A), and submitted it for tandem mass spectrometry (MS/MS) analysis. The MS/MS results yielded the discovery of a branched TRAF4 peptide containing K190, which corresponds to the covalent bond of the C-terminal ubiquitin glycine to the ε-amino group of the lysine residue (Fig. 2B).

To confirm that K190 is the site for Smurf1-induced monoubiquitination, we mutated this residue to arginine, and expressed the TRAF4 K290R mutant with Smurf1 in HEK293 cells. Western blot analyses indicated that the ability of TRAF4 K190R to be monoubiquitinated by Smurf1 was significantly curtailed comparing to that of the wild-type TRAF4 (Fig. 2C). Only a faint band of ubiquitinated TRAF4 was detected when K190 was mutated, suggesting that either additional lysine residues were targeted or in the absence of K190, a new lysine residue not previously engaged in the context of wild type TRAF4 was targeted, which often occurs for many other ubiquitinated proteins (40). Nevertheless, this result indicated that K190 on TRAF4 is a major site for Smurf1-induced ubiquitination.

The E3 Ligase Activity of TRAF4 Is Not Required for Smurf1-induced Ubiquitination—Like other members of the TRAF family, TRAF4 contains a RING domain at the N-terminus, seven zinc-finger domains in the middle, and a TRAF domain at the C-terminus that is further divided into N-terminal domain (NTD) and C-terminal domain (CTD) (Fig. 3A). Although the TRAF4 RING domain has an intrinsic E3 ligase activity, the RING domain deletion mutant, TRAF4ΔR, was still monoubiquitinated by Smurf1 (Fig. 3B), indicating that the E3 ligase activity of TRAF4 is not essential for this modification. However, the TRAF4 mutant, TRAF4-ARZ, which lacks the RING domain and the zinc finger domain that contains K190 (Fig. 3A), was completely refractory to Smurf1-mediated monoubiquitination (Fig. 3B). Another TRAF4 mutant with a CTD deletion, TRAF4-ΔC, was also incapable of undergoing Smurf1-mediated monoubiquitination (Fig. 3B). Thus, Smurf1-mediated monoubiquitination of TRAF4 requires the zinc-finger domains and the CTD.

K190 Mono-ubiquitination Directs TRAF4 to the Plasma Membrane and the Cell–Cell Junctions—Monoubiquitination is known to regulate endocytosis, membrane trafficking, and subcellular localization of the affected protein substrates (2, 3). In sparsely growing cells, TRAF4 primarily resides in the cytoplasm, often appearing in speckled intracellular vesicles (32). However, in confluent epithelial cells, TRAF4 was found at the plasma membrane and cell–cell
junctions (32). To investigate whether K190 monoubiquitination affects the subcellular localization of TRAF4, we generated wild-type GFP-TRAF4 and GFP-TRAF4 K190R fusion constructs and transiently expressed them in human mammary gland epithelial MCF10A cells. GFP itself showed a ubiquitous fluorescence pattern, evenly distributed between the cytoplasm and the nucleus (Fig. 4A). GFP-TRAF4, on the other hand, decorated the plasma membrane (Fig. 4A) and partially overlapped with the tight junction marker occludin (Fig. 4B), as reported previously (32). In contrast, GFP-TRAF4 K190R was found only in the cytoplasm (Fig. 4A). Thus, K190 monoubiquitination is required for the localization of TRAF4 to the plasma membrane and the cell–cell junctions.

**TRAF4 Knockdown Inhibits Breast Cancer Cell Motility**—Epithelial cell junctions play important roles in coordinating fundamental cellular activities, such as cytoskeletal organization, cell migration, and adhesion. Since TRAF4 is localized at the cell–cell junctions, we asked if it promotes cell migration in a wound healing assay in breast cancer MCF7 cells. A gap in the confluent monolayer of MCF7 cells was generated by lifting a Culture-Insert (Fig. 5A), and the extent of cell migration toward the gap area was assessed. MCF7 cells transfected with non-silencing control siRNA (siNS) showed about 75% gap closure after 48 h and full closure at 72 h, whereas the gap closure in the MCF7 cell culture transfected with siTRAF4 was markedly retarded at 24 and 48 h, and still incomplete at 72 h (Fig. 5B). Knockdown of TRAF4 also inhibited cell migration in a transwell migration assay (Fig. 5C), but showed no effect on MCF7 cell proliferation (Fig. 5D). Taken together, these results indicate that reducing TRAF4 expression specifically inhibits cell motility.

**Monoubiquitination of TRAF4 by Smurf1 Is Required for Promoting Cell Migration**—Smurf1 is known to promote breast cancer cell migration (22, 25, 41), so we wondered if this Smurf1 function is mediated by monoubiquitination of TRAF4 at K190. To address this query, we established three stable lines of MCF7 cells carrying empty pBABE-puro vector, pBABE-FLAG-TRAF4, or pBABE-FLAG-TRAF4-K190R, respectively (Fig. 6A). As part of the wound healing assays performed on these stable cell lines, we also transfected them with siNS or siSmurf1 to assess the requirement for Smurf1. Under normal Smurf1 expression conditions in siNS-transfected cells, forced expression of TRAF4 greatly accelerated MCF7 cell migration to the extent that the gap was completely closed within 24 h (Fig. 6B). In contrast, forced expression of TRAF4-K190R actually retarded MCF7 cell motility, comparing the extent of gap closure in cultures of pBABE vector and FLAG-TRAF4-K190R stable cells (Fig. 6B). After knockdown Smurf1 expression using siSmurf1, the wound gap established in the FLAG-TRAF4 stable cell culture failed to close, and the healing process in the stable cell cultures containing pBABE vector and FLAG-TRAF4-K190R was similarly impaired (Fig. 6B). The requirement for Smurf1-mediated ubiquitination of TRAF4 K190 for cell motility was also observed using mammary epithelial MCF10A cells (Fig. 7). Taken together, these results indicate that the Smurf1-mediated ubiquitination of TRAF4 at K190 promotes cell migration.

**TRAF4 K190 Ubiquitination Is Required to Activate Rac1**—In endothelial cells, a membrane-bound, myristoylated form of TRAF4 activates Rac1 and causes intense membrane ruffles (31). To determine if Smurf1 and TRAF4 also act through Rac1 in promoting cell migration in epithelial cells, we first examined whether Rac1 is activated by TRAF4 and Smurf1 in MCF10A cells. Western blot analyses indicated that endogenous Rac1 was activated to its GTP-bound form by forced expression of wild-type Smurf1 or TRAF4, but not their respective mutants (Fig. 8A). We then examined whether Rac1 is required for the Smurf1 and TRAF4 cell migration functions by using MCF10A cell wound healing assays. Treating the stable lines of MCF10A cells that express Smurf1 and TRAF4, respectively, with a Rac1 chemical inhibitor, NSC23766, we found that blocking Rac1 activity retarded the Smurf1 or TRAF4-induced cell migration (Fig. 8, B and C).
These results indicate that activation of Rac1 is the underlying causes of TRAF4-mediated cell migration.

DISCUSSION

In this study, we showed that Smurf1 induces monoubiquitination of TRAF4 at K190. Like other members of TRAF family, TRAF4 contains a RING domain that has E3 ligase activity; however, its intrinsic E3 ligase activity is not required for monoubiquitination since the RING domain deletion mutant of TRAF4 was ubiquitinated readily by Smurf1. Moreover, members of the TRAF family can catalyze polyubiquitination that either targets the substrates for proteasome-mediated degradation or assembling these substrates into signaling complexes for kinase activation (42). In contrast, monoubiquitination by Smurf1 is required for targeting TRAF4 to the tight junctions at cell–cell contacts in a confluent monolayer of epithelial cells. The localization of TRAF4 to cell junctions correlated well with its function in promoting cell migration and activating Rac1 because the TRAF4 K190R mutant was ineffective in promoting cell migration and Rac1 activation. This property of TRAF4 in epithelial cells is very similar to its role in endothelial cells. Previous studies on endothelial cells indicated that TRAF4 associates with the focal complex and membrane raft (31). This localization of TRAF4 in the leading edge of the focal complex is important for activating Rac1 and increases the oxidant production that facilitates endothelial cell migration. It will be interesting to determine if monoubiquitination at K190 is required for targeting TRAF4 to the focal complex.

Mice lacking TRAF4 displayed incomplete neural tube and branchial arch closure, which was attributed to defects in ontogenic migration (30). Human TRAF4 was originally discovered via its expression in breast cancer tissues (26). It was also found frequently amplified in male breast cancer (43). Detailed studies on TRAF4 expression patterns in breast cancer tissues revealed that cytoplasmic expression of TRAF4 is higher in invasive ductal carcinoma, but nuclear expression of TRAF4 is suppressed in breast carcinoma (44), suggesting a link between TRAF4 and breast cancer progression. However, TRAF4 has not been assigned to the family of genes that regulate breast cancer cell migration. We found here that TRAF4 is required for breast epithelial and cancer cell migration. The ability of TRAF4 to promote cell migration requires Smurf1-mediated ubiquitination.

Like TRAF4, the expression of both Smurfs is also elevated in breast carcinomas (22). Smurf1 was previously known to promote lamellipodia formation and migration in metastatic breast cancer cells (22, 25). It was shown that Smurf1 is recruited to the leading edge by the polarity complex PAR6-αPKC and locally targets RhoA for proteasome-mediated degradation (17, 25). Here we showed that Smurf1 has the ability to induce mono-ubiquitination of TRAF4 and recruit it to the tight junctions, thereby activating Rac1, another Rho GTPase. Thus, Smurf1 can regulate breast cancer cell migration through its regulation of Rho GTPases, leading to down-regulation of RhoA at the leading edge while activating Rac1.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Smurfs induce TRAF4 ubiquitination. A. Physical interaction between Smurf1 and TRAFs. Lysates of HEK293 cells transfected with HA-TRAFs and Myc-Smurfl or a vector control were subjected to immunoprecipitation using anti-Myc monoclonal antibody. Smurfl-bound TRAF proteins were detected by anti-HA-HRP. The expression levels of transfected HA-TRAFs in whole cell lysates (WCL) are shown in the bottom panel. B, E3 ligase activity is required for a Smurf-induced TRAF4 mobility shift. HEK293 cells were transfected with HA-TRAF4 and wild-type (WT) or E3 ligase-defective (CA or CG) Myc-Smurfl or Myc-Smurfl2. The expression levels of transfected proteins in whole cell lysates (WCL) were detected by Western blotting (shown in bottom panel). C, Smurfl- and Smurfl2-induced ubiquitination of TRAF4. Ubiquitinated proteins in HEK293 cells transfected with FLAG-TRAF4, Myc-Smurfl1, or Myc-Smurfl2 were isolated by anti-ubiquitin antibody P4D1, and subjected to anti-FLAG Western blotting to verify that the FLAG-TRAF4 was ubiquitinated.

FIGURE 2. K190 of TRAF4 is a major site of Smurf1-mediated ubiquitination. A, Preparative ubiquitinated TRAF4 protein was isolated from transfected HEK293 cells after immunoprecipitation, SDS-PAGE, and Coomassie blue staining. An immunoblot of portions of the same samples that were used for mass spectrometry is shown in the right panel. The protein band analyzed by mass spectrometry is marked by an arrowhead. B, The sequences of the TRAF4-ubiquitin peptide conjugate detected by mass spectrometry. Arrows indicate sites of trypsin cleavage. C, Mutation of K190 to R (KR) leads to decreased ubiquitination of TRAF4.

FIGURE 3. RING domain is not required for Smurf1-mediated TRAF4 ubiquitination. A, Schematic diagram of TRAF4 deletion mutants. B, Western blot of lysates of HEK293 cells transfected with HA-TRAF4 or its deletion mutants in the absence or presence of Myc-Smurfl1.

FIGURE 4. Ubiquitination at TRAF4 K190 is required for localization of TRAF4 at cell-cell contact. A. Immunofluorescence of GFP-TRAF4 or GFP-TRAF4 K190R (KR) in confluent MCF10A cells. Cells were probed for F-actin with Alexa Fluor® 594-phalloidin and nuclear DNA stained with DAPI. B, Co-localization of GFP-TRAF4 with the tight junction marker occludin. Confluent MCF10A cells transfected with GFP-TRAF4 were fixed and immunostained for endogenous occludin. XZ and YZ images were derived from Z-stack sections along the white lines. Scale bar: 10 μm.

FIGURE 5. TRAF4 is required for MCF7 breast cancer cell migration. A, Western blot shows knockdown of endogenous TRAF4 in MCF7 cells. siNS: non-silencing control siRNA. B, Ibidi wound healing and migration assay of MCF7 cells transfected with either control or TRAF4 siRNAs. C, Transwell migration assay of MCF7 cells transfected with either control or TRAF4 siRNAs. Cells at the bottom of the transwell chambers were counted. Data represent mean ± SD (n = 3). D, MTT assay of MCF7 cells transfected with either control or TRAF4 siRNAs. Data represent mean ± SD (n = 3).

FIGURE 6. Smurf1-mediated ubiquitination is required for TRAF4 to promote MCF7 breast cancer cell migration. A, Expression of FLAG-TRAF4 and FLAG-TRAF4 K190R and endogenous Smurfl in MCF7 cells. B, Ibidi wound healing and migration assay of MCF7 cells stably expressing FLAG-TRAF4 or FLAG-TRAF4 (K190R) in the presence of either control or Smurfl siRNAs. Quantified gap distances are shown in the bottom panel. Data represent mean ± SD (n = 3).

FIGURE 7. TRAF4 but not the TRAF4 K190R mutant promotes mammary gland MCF10A epithelial cell migration. A, Expression of FLAG-TRAF4 and endogenous TRAF4 in MCF10A cells. B, Ibidi wound healing and migration assay of MCF10A cells stably expressing FLAG-TRAF4 or FLAG-TRAF4 (K190R) transfected with either control or TRAF4 siRNAs. Quantified gap distances are
shown in the bottom panel. Data represent mean ± SD (n = 3).

FIGURE 8. Smurf1 and TRAF4, but not their mutants, activate Rac1, which is required for cell migration. A, Rac1 activity assay in MCF10A cells stably expressing FLAG-Smurfl or FLAG-TRAF4. B, Inhibition of Rac1 activity inhibited Smurf1-mediated MCF10A cell migration. C, Inhibition of Rac1 activity inhibited TRAF4-mediated MCF10A cell migration.
Ubiquitination of TRAF4 regulates cell migration

Figure 1

Figure 2

Figure 3
Figure 4

A

|          | GFP-C2 | Phalloidin | DAPI | Overlay |
|----------|--------|------------|------|---------|
| GFP-C2   | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| GFP-TRAF4| ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| GFP-TRAF4KR| ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |

B

| Occludin | GFP-TRAF4 | DAPI | Overlay |
|----------|-----------|------|---------|
|          | ![Image](image13) | ![Image](image14) | ![Image](image15) |

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Ubiquitination of TRAF4 regulates cell migration

Figure 5

A  

B

Time (hr): 0 24 48 72

siNS  siTRAF4  siNS  siTRAF4

Tubulin  

C

Cell Number

D

Time (hr): 24 48 72

siNS  siTRAF4

MTT assay (Abs)
Ubiquitination of TRAF4 regulates cell migration

Figure 6

A

| siNS:  | + | - | + | - | + | - |
|-------|---|---|---|---|---|---|
| siSmurf1: | - | + | - | + | - | + |
| FLAG | | | | | |
| Smurf1 | | | | | |
| Tubulin | | | | | |

B

| siNS | siSmurf1 |
|------|----------|
| pBabe | | |
| F-TRAF4 | | |
| F-TRAF4KR | | |

Gap width (µm)

siNS | siSmurf1

pBabe | TRAF4 | TRAF4KR
Figure 7

A

| siNS: | + | - | + | - | + | - |
| siTRAF4: | - | + | - | + | - | + |

TRAF4

FLAG

Tubulin

B

| siNS | siTRAF4 |
|------|---------|
| pBabe | pBabe |
| F-TRAF4 | F-TRAF4 |
| F-TRAF4KR | F-TRAF4KR |

Gap width (μm)

siNS

siTRAF4

F-TRAF4

F-TRAF4KR

Ubiquitination of TRAF4 regulates cell migration
Ubiquitination of TRAF4 regulates cell migration

Figure 8

A

|          | F-Smurf1 | F-TRAF4 |
|----------|----------|---------|
|          | WT CA    | WT KR   |
| GTP-Rac1 |          |         |
| Rac1     |          |         |
| FLAG     |          |         |

B

- Rac1 inhibitor (NSC23766)

pBabe

F-Smurf1

C

- Rac1 inhibitor (NSC23766)

pBabe

F-TRAF4
Ubiquitination of Tumor Necrosis Factor Receptor Associated Factor 4 (TRAF4) by Smad Ubiquitination Regulatory Factor 1 (Smurf1) Regulates Motility of Breast Epithelial and Cancer Cells

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