Receptor tyrosine kinases (RTKs) are important drivers of cancers. In addition to genomic alterations, aberrant activation of wild type RTKs plays an important role in driving cancer progression. However, the underlying mechanisms of how RTKs drive prostate cancer remain incompletely characterized. Here we show that non-proteolytic ubiquitination of RTK regulates its kinase activity and contributes to RTK-mediated prostate cancer metastasis. TRAF4, an E3 ubiquitin ligase, is highly expressed in metastatic prostate cancer. We demonstrated here that it is a key player in regulating RTK mediated prostate cancer metastasis. We further identified TrkA, a neurotrophin RTK, as TRAF4-targeted ubiquitination substrate that promotes cancer cell invasion and inhibition of TrkA activity abolished TRAF4-dependent cell invasion. TRAF4 promoted K27 and K29-linked ubiquitination at the TrkA kinase domain and increased its kinase activity. Mutation of TRAF4-targeted ubiquitination sites abolished TrkA tyrosine auto-phosphorylation and its interaction with downstream proteins. TRAF4 knockdown also suppressed NGF-stimulated TrkA downstream p38 MAPK activation and invasion-associated gene expression. Furthermore, elevated TRAF4 levels significantly correlated with increased NGF-stimulated invasion-associated gene expression in prostate cancer patients, indicating that this signaling axis is significantly activated during oncogenesis. Our results revealed a post-translational modification mechanism contributing to aberrant non-mutated RTK activation in cancer cells.
TRAF4-mediated ubiquitination of NGF receptor TrkA regulates prostate cancer metastasis

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Abstract

Receptor tyrosine kinases (RTKs) are important drivers of cancers. In addition to genomic alterations, aberrant activation of wild type RTKs plays an important role in driving cancer progression. However, the underlying mechanisms of how RTKs drive prostate cancer remain incompletely characterized. Here we show that non-proteolytic ubiquitination of RTK regulates its kinase activity and contributes to RTK-mediated prostate cancer metastasis. TRAF4, an E3 ubiquitin ligase, is highly expressed in metastatic prostate cancer. We demonstrated here that it is a key player in regulating RTK mediated prostate cancer metastasis. We further identified TrkA, a neurotrophin RTK, as TRAF4-targeted ubiquitination substrate that promotes cancer cell invasion and inhibition of TrkA activity abolished TRAF4-dependent cell invasion. TRAF4 promoted K27 and K29-linked ubiquitination at the TrkA kinase domain and increased its kinase activity. Mutation of TRAF4-targeted ubiquitination sites abolished TrkA tyrosine auto-phosphorylation and its interaction with downstream proteins. TRAF4 knockdown also suppressed NGF-stimulated TrkA downstream p38 MAPK activation and invasion-associated gene expression. Furthermore, elevated TRAF4 levels significantly correlated with increased NGF-stimulated invasion-associated gene expression in prostate cancer patients, indicating that this signaling axis is significantly activated during oncogenesis. Our results revealed a post-translational modification mechanism contributing to aberrant non-mutated RTK activation in cancer cells.
INTRODUCTION

Ubiquitination is an important post-translational modification regulating protein degradation, trafficking, activity, and protein-protein interaction. Dysregulation of the ubiquitin pathways has been implicated in a number of diseases including cancer (1-5). Targeting the ubiquitination machinery has been considered to be an effective therapeutic strategy (3, 6, 7).

The RING domain E3 ubiquitin ligase, TRAF4, is emerging as a key regulator in cancer development, metastasis, and chemoresistance (8-15). It was originally identified as a gene upregulated in metastatic breast cancer (16). TRAF4 belongs to the TRAF family that consists of seven members. They are adaptor/scaffold proteins that couple tumor necrosis factor (TNF) receptors and interleukin receptors to downstream signaling pathways. Unlike other TRAFs, TRAF4 weakly interacts with very few TNF receptor family members (17). It also does not have a significant contribution to the development and normal function of the immune system except for facilitating immune cell migration (18). The biological function of TRAF4 has remained elusive. It is expressed at basal levels in most adult tissues (17) but is overexpressed and amplified in a variety of human cancer (11). We found that TRAF4 has a significantly higher expression level in metastatic prostate cancer compared to primary tumor, and plays an important role in prostate cancer cell invasion.

After screening TRAF4 ubiquitination substrates in prostate tumors, we identified Tyrosine Receptor Kinase A (TrkA, also named NTRK1) as a prominent substrate for TRAF4-mediated ubiquitination. TrkA is a receptor tyrosine kinase (RTK) that binds to nerve growth factor (NGF) at the cell membrane. It activates Ras/MAPK, PI3K and PLCγ signaling pathways to promote cell survival, proliferation and invasion (19). In addition to the nervous system, NGF is also abundant in prostate cancer and its receptor has been linked to prostate cancer proliferation and metastasis (19-25). Targeting genetically altered constitutively active protein kinases has led to dramatic clinical responses in several cancers. Although TrkA activating mutations through genomic rearrangement and deletion have been documented in in a number of cancers (26-31), its mutations were not identified
in prostate cancer (32). The pathways leading to aberrant activation of non-mutated TrkA remained poorly understood. Herein we present evidence that TRAF4 promotes TrkA ubiquitination at its kinase domain through atypical K27 and K29-ubiquitin linkages. This post-translational modification hyper-activates TrkA kinase activity, and alters its phosphorylation status. Our study deciphered that the TRAF4-regulated signaling cascade is an important driver for prostate cancer metastasis.

RESULTS

TRAF4 plays a role in prostate cancer metastasis

To investigate the expression of TRAF4 in prostate tumor specimens, we used a prostate cancer tissue cDNA array consisting of 39 prostate cancer and 9 normal prostate tissue samples. TRAF4 mRNA was highly expressed in prostate tumors compared to normal tissues (Figure 1A). We also found that TRAF4 protein levels were significantly higher in 7 out of 10 human prostate tumors compared to matched normal prostate tissues (Figure 1B). Since TRAF4 has been reported to be associated with cell migration and cancer metastasis (9, 15, 18, 33, 34), we also analyzed its expression in several publically available prostate cancer datasets which contain significant cases of metastatic cancers (35-39). Consistent with our analysis of tumors (Figure 1A and B), TRAF4 expression was significantly elevated in prostate tumors compared to adjoining prostate tissues (Figure 1C-F). Interestingly, the patients with metastatic disease all had enhanced levels of TRAF4 compared to localized tumors (Figure 1C-F). These findings suggest the possibility of a critical role of TRAF4 in aggressive metastatic prostate cancers.

To determine whether TRAF4 plays a role in prostate cancer metastasis, we first analyzed the effect of TRAF4 knockdown in prostate cancer cell migration and invasion. TRAF4 knockdown was achieved using pool siRNA or two different shRNAs. PC3 cells, a highly invasive prostate cancer line, were subjected to TRAF4 or control knockdown followed
by seeding with equal numbers in a transwell invasion chamber either with (for invasion) or without matrigel (for migration assays) in a serum-free medium for 12-16 hours. No significant difference in cell proliferation was observed under these conditions (data not shown). We found that the numbers of migratory and invasive PC3 cells were significantly decreased upon TRAF4 knockdown compared to non-targeting controls (Figure 2A and B, respectively). A similar observation also was seen in another metastatic prostate cancer line DU145 (Supplemental Figure 1A).

We next examined the role of TRAF4 in prostate cancer cell metastatic potential in an experimental lung metastatic mouse model by injecting prostate tumor cells via tail vein. PC3 luciferase expressing cells selected for stable knockdown of TRAF4 or non-targeting shRNA control were injected via tail vein into 4-5 weeks old male NOD/SCID mice. The number of injected tumor cells in the circulation were compared between the control and shTRAF4 groups at 5 minutes after the tail-vein injection by bioluminescence imaging (BLI) of the mouse lung (0 week) (Figure 2C). Although injected with the same number of cells, the shTRAF4 cells display higher luminescence intensities compared to shcontrol cells due to higher expression levels of the luciferase gene in shTRAF4 cells (Supplemental Figure 1B). Both groups showed gradual decrease in luminescence signals 1-3 weeks after injection, indicating that most of the injected cells did not survive in the lung. At 9 weeks, the luminescence signals at the lung areas of control shRNA injected mice steadily increased (Figure 2C), indicating the growth of successfully colonized tumor cells. In contrast, the signals from the shTRAF4 cells-injected mice continued to fade away. This difference is unlikely due to a difference in proliferation rates of tumor cells since comparable Ki67 staining intensities were found in the luminescence signal positive tumor areas of both mice (Supplemental Figure 1C). These results suggest that the injected shTRAF4 cells have reduced ability to colonize into the lung compared to control cells. H&E staining of the lungs also showed that both the size and number of metastatic nodules were significantly reduced in TRAF4 knockdown cells (Figure 2D). We found that some of the shRNA control cells but
not shTRAF4 cells injected mice developed luminescence signals at other areas 8-9 weeks post injection in addition to the lung, an indication of further metastasis. Tumor bone metastasis was confirmed via immunohistochemistry using an anti-luciferase antibody in shRNA control mice (Figure 2E). However, we did not find any observable bone metastasis in shTRAF4 injected mice. These results suggest that TRAF4 plays an important in vivo role in prostate cancer cell metastasis.

**The RING domain is critical for TRAF4-mediated cell invasion in prostate cancer cell lines**

Next we investigated the molecular mechanisms by which TRAF4 drives prostate cancer cell invasion and eventually systemic metastasis. TRAF4 is a RING domain E3 ubiquitin ligase which plays an important role in TRAF4-mediated ubiquitination. To determine whether the E3 ubiquitin ligase activity of TRAF4 is important for its invasive function, we deleted the RING domain and compared the effect of this truncated protein on the invasion ability of a poorly invasive prostate cancer cell line, LNCaP, with the full length TRAF4. LNCaP cell lines stably expressing vector control, TRAF4 WT or TRAF4ΔRING mutant were used in an invasion assay. The expression levels of flag-TRAF4 WT and flag-TRAF4ΔRING mutant were comparable (Figure 3A right panel). As shown in Figure 3A, WT TRAF4 overexpression significantly increased the cell invasion ability, confirming the role of TRAF4 in mediating cell invasion. In contrast, the RING domain deletion mutant of TRAF4 lost the ability to promote cell invasion (Figure 3A). These results suggest that the TRAF4-RING domain vital for its E3 ubiquitin ligase activity also is critical for driving prostate tumor cell invasion.

**TRAF4 interacts with and ubiquitinates the neurotrophin receptor TrkA**

To identify TRAF4 targeted ubiquitination substrate that mediates TRAF4’s ability to promote cell invasiveness, we performed an unbiased screen on a ubiquitin array, which measures the ubiquitination levels of 49 different proteins. Among them we found TrkA as one of the top hit candidate proteins showing significantly enhanced ubiquitination upon
TRAF4 overexpression in PC3 cells (Figure 3B). TrkA is a tyrosine kinase receptor that is activated upon binding to its ligand nerve growth factor (NGF), a member of the neurotrophin family that regulates brain development and function. TrkA was previously reported to also regulate prostate cancer cell metastasis (19-25). To test the hypothesis that TrkA is an important downstream mediator of TRAF4-dependent prostate tumor cell invasion, we examined the invasion potential of DU145 or PC3 cells followed by TrkA depletion. Our data revealed that TrkA silencing significantly reduced cell invasion, similar to the effect of TRAF4 knockdown (Figure 3C, Supplemental Figure 2A and 2B), indicating that TrkA likely is a potential TRAF4 ubiquitination substrate involved in cell invasion.

We further tested the role of TrKA in prostate cancer cell metastasis in vivo using a TrKA specific inhibitor GW441756 (40). PC3 luciferase expressing cells were injected via tail vein into male NOD/SCID mice. The mice were then randomized into control and drug treatment groups. The drug treatment mice group received GW441756, 10mg/kg i.p. twice a week while the control group received solvent only. We did not observe any significant change in mouse weight between the two groups (Supplemental Figure 2C). 9 weeks after injection, the TrkA inhibitor treatment group has significantly reduced tumor compared to the control group (Figure 3D). These results suggest that TrKA inhibition in PC3 cells has an inhibitory effect on prostate cancer metastasis.

To test whether TRAF4 can interact with TrkA to promote its ubiquitination in cells, we transiently transfected flag-tagged TrkA or empty vector along with HA-tagged TRAF4. Immunoprecipitation of flag-tagged TrkA revealed a direct association with HA-tagged TRAF4 compared to cells expressing only flag-vector as a control (Figure 4A). Next we carried out an ubiquitination assay in 293T cells by transiently transfecting HA-ubiquitin, flag-TrkA, and V5-TRAF4 or its RING domain deletion mutant. The levels of TrkA ubiquitination were detected through immunoprecipitation using a flag-specific antibody followed by a Western blot analysis using a HA-specific antibody. The results confirmed that the wild type TRAF4 but not the RING domain deletion mutant promoted the ubiquitination of TrkA in cells (Figure 4B). To confirm that the TRAF4-TrkA interaction exists in prostate tumor cells, we
immunoprecipitated endogenous TrkA from DU145 cells and identified a strong interaction with TRAF4 (Figure 4C). Since NGF stimulation triggers TrkA activation, we investigated whether TrkA ubiquitination is regulated by NGF induction and whether TRAF4 plays a role in the ubiquitination process of endogenous TrkA. As shown in Figure 4D, NGF treatment significantly increased TrkA ubiquitination, whereas TRAF4 knockdown dramatically reduced the levels of ubiquitinated-TrkA. We also performed an in vitro ubiquitination assay using purified flag-TrkA from 293T cells, purified TRAF4, HA-Ubiquitin, ubiquitin activating enzyme UBE1, and ubiquitin conjugating enzyme UbcH5a to demonstrate that TRAF4 can directly promote TrkA ubiquitination (Supplemental Figure 3A). These results suggest that TRAF4 plays an important role in NGF-induced TrkA ubiquitination in prostate cancer cells.

TrkA undergoes internalization following activation at the cell membrane. To determine where TRAF4-mediated ubiquitination event happens, we isolated the cytosolic and membrane protein fractions from cells transfected with flag-TrkA with or without TRAF4 overexpression and then examined the TrkA ubiquitination. Although TrkA is located at both cytoplasm and cell membrane, TRAF4-mediated TrkA ubiquitination mainly occurs at the cell membrane (Figure 4E).

Ubiquitination chain linkage involves one or more of seven ubiquitin lysine residues (Lys-6, Lys-11, Lys-27, Lys-29, Lys-33, Lys-48, and Lys-63). The type of ubiquitin linkage determines the fate of proteins in the cell. The most common ubiquitin linkage is K48 and it is usually associated with protein degradation. Interestingly, we did not observe any significant change in the TrkA protein level upon TRAF4 overexpression in the absence of proteasome inhibitors, suggesting that TRAF4-mediated TrkA ubiquitination unlikely promotes its protein degradation (Supplemental Figure 4A right panel). It has been reported previously that TRAF4 is capable of mediating K63-linked ubiquitination (8, 9), suggesting that TRAF4 may target TrkA ubiquitination through non-classical ubiquitin linkage. To determine which lysine linkage is involved in TRAF4 mediated TrkA ubiquitination, we utilized a series of ubiquitin mutants that only contain one of the seven lysine residues while all other lysine residues are mutated into arginine residues. As shown in Figure 4F, the
ubiquitin mutants containing only K27 or K29 residue were able to promote TrkA ubiquitination similar to the wild type ubiquitin, suggesting that TRAF4 mediated TrkA ubiquitination occurs through the K27 and K29 ubiquitin linkages.

**TrkA plays a role in TRAF4-promoted cell invasion**

To understand how TRAF4 promoted cell invasion and how TrkA plays a role in this function, we carried out a reverse phase protein array (RPPA) study using cell lysates from PC3 cells expressing two different shTRAF4 or control shRNA plasmids. A total of 207 antibodies recognizing different proteins or protein phosphorylation forms were analyzed in the RPPA study. Among the proteins that are down-regulated or have reduced phosphorylation levels in TRAF4 knockdown cells compared to control cells, we found a number of EMT and invasion-associated proteins (Table 1). We further validated the RPPA results using qRT-PCR to examine the effect of siTRAF4 on the expression of these target genes (Figure 5A). Interestingly, many of them including COX-2, Slug, IL-6 and Integrinβ1, were also up-regulated upon NGF stimulation (Figure 5B). To determine whether TrkA plays a role in TRAF4-regulated expression of these target genes, we knocked-down TrkA using siRNAs followed by measurement of target gene expression. TrkA knockdown cells showed a pattern of reduced gene expression of TRAF4 target genes as was observed with siTRAF4 treatment (Figure 5C). We also found that knockdown of TRAF4 abolished NGF-stimulated gene up-regulation (Figure 5D), confirming that TRAF4 is important for NGF-induced gene expression.

Since TrkA is a tyrosine kinase, we then determined whether inhibition of TrkA kinase activity affects TRAF4-mediated cell invasion. We first examined whether the TrkA selective inhibitor, GW441756, affects cell growth in our experiemental condition. A MTT assay was performed on PC3 cells treated with a series of concentration of GW441756 for two days. No significant effect of the inhibitor treatment on cell growth was found (Supplemental Figure 3B). We next chose a 0.5 μM of GW441756 concentration to examine its effect on TRAF4-promoted cell invasion. As shown in Figure 5E, overexpression of TRAF4 significantly
increased PC3 cell invasion. Treatment of GW441756 for two days abolished the TRAF4-mediated effects. These results substantiate that the interplay of TRAF4 and TrkA regulates cell invasion-associated gene expressions and cell invasion.

**Lysine residues at the TrkA kinase domain are responsible for TRAF4-mediated TrkA ubiquitination**

Since TRAF4 regulates TrkA ubiquitination, we next investigated the ubiquitination target sites in TrkA to understand the importance of this post-translational modification on its function. TrkA is a 140 KDa transmembrane receptor containing extracellular domains involved in NGF binding, a transmembrane domain (TM), a juxtamembrane domain (JM), a tyrosine kinase domain (TK) and a short C-terminal domain (CT). A schematic representation of these domains is shown in Figure 6A. To determine which domain is targeted by TRAF4 for ubiquitination, we generated different deletion mutants of TrkA and then tested their relative ubiquitination levels in TRAF4 overexpressed cells (Figure 6A). The TrkA ubiquitination was abolished upon deletion of the TK domain (ΔTK and ΔJM vs. WT and ΔCT). These results suggested that the TK domain is the likely region targeted by TrkA ubiquitination.

Next we determined the ubiquitination sites in the TK domain. Ubiquitin ligases often target multiple neighboring lysine residues for ubiquitination (41-43). There are 10 lysine residues present in the TK domain. Three of them (K523, K544 and K547) are located close to each other to form a lysine cluster in the crystal structure (Supplemental Figure 3C) (44). To test whether this lysine cluster is the TRAF4-targeted ubiquitination site, we used site directed mutagenesis to generate lysine to argine mutants of TrkA. Mutation of two of these three lysine residues to argine (K523_44R or K544_47R) significantly reduced TRAF4-mediated ubiquitination while mutation of all three lysine residues completely abolished the ubiquitination (Figure 6B). These results suggested that TRAF4 targets TrkA ubiquitination at a lysine cluster containing K523, K544 and K547 residues.
Interestingly, the three lysine residues we identified are located in proximity to the kinase activation loop (Supplemental Figure 3C). It is likely that post-translational modification at the lysine cluster affects the kinase activity. We next determined whether TRAF4 directly regulates TrkA kinase activity through an in vitro kinase assay. Flag tagged wild type TrkA or the K523_44_47R mutant was purified from TRAF4 overexpressing cells or vector control cells using a flag antibody. A flag tagged serine kinase PKCδ was purified following the same procedure to serve as a negative control. Comparable levels of TrkA, its ubiquitin mutant, or PKCδ, were then used in an in vitro luminescence kinase assay utilizing a poly (Glu4, Tyr1) synthetic peptide as a substrate (Figure 6C). The luminescence value from the PKCδ kinase reaction was used as a background reading and subsequent kinase assay readings were adjusted according to it. We found that TRAF4 overexpression significantly enhanced in vitro kinase activity of wild type TrkA, whereas in TrkA ubiquitination-deficient mutant TRAF4 had no effect on the kinase activity (Figure 6C). The K547 residue was previously reported to be an important site for ATP binding and the K547A mutant of TrkA was considered as a kinase dead mutant (45). It is not clear whether K547R mutation could behave similarly to K547A mutation to inactivate the kinase activity or not. To avoid the potential complication caused by K547R mutation, we also tested the in vitro kinase activity of the K523_44R double mutant, which has significantly reduced ubiquitination compared to the WT TrkA (Figure 6B). Similar to the triple mutation, the double mutation abolished TRAF4-mediated enhancement on TrkA in vitro kinase activity (Supplemental Figure 3D). These results suggest that TRAF4-mediated TrkA ubiquitination at the lysine cluster (K523_44_47) adjacent to the kinase domain hyperactivates TrkA-kinase activity.

**TRAF4-mediated ubiquitination affects TrkA tyrosine phosphorylation**

Next we assessed how TRAF4-mediated ubiquitination affects the NGF-induced signal transduction cascade. The ubiquitination mainly occurs at the cell membrane (Figure 4E). It was reported previously that TrkA ubiquitination at a different site regulates the
receptor internalization (46). We did not observe a major change of TrkA subcellular localization when TRAF4 is overexpressed (Supplemental Figure 4A), suggesting that TRAF4-mediated ubiquitination does not have a significant effect on receptor trafficking. When TrkA is activated, several of its tyrosine residues are phosphorylated. The tyrosine phosphorylation could either release autoinhibition that is important for kinase activity (Y674/675) or serve as a docking site for binding to adaptor proteins to activate downstream signaling cascades (Y490 or Y785). Y674/675 phosphorylation correlates positively with the TrkA kinase activity and it precedes the phosphorylation of other Tyr residues (47). To test whether TRAF4-mediated ubiquitination affects TrkA tyrosine phosphorylation, we analyzed the levels of TrkA tyrosine phosphorylation in the absence or presence of TRAF4 overexpression. As shown in Figure 7A, TRAF4 overexpression increased the levels of TrkA tyrosine phosphorylation at Y674/675, Y490 and Y785 sites in the presence of NGF, consistent with its ability to enhance TrkA kinase activity (Figure 6C). More importantly, mutation of the three TRAF4 targeted ubiquitination sites (K523_544_547R) abolished the tyrosine-phosphorylation of TrkA. We observed higher basal level of TrkA WT phosphorylation in the absence of TRAF4 overexpression compared to the mutant phosphorylation (Figure 7A, lane 1 vs. lane 3). This is probably due to the presence of endogenous TRAF4. A similar result was observed for the K523_544R mutant (Supplemental Figure 4B). Interestingly, K547R single mutation appears to have minor effects on TrkA phosphorylation levels, suggesting that K547R mutation by itself did not abolish its kinase activity (Supplemental Figure 4C) in contrast to K547A mutation as previously reported (48). These results suggest that TRAF4-mediated TrkA ubiquitination regulates TrkA tyrosine phosphorylation.

Upon NGF stimulation, the binding of phosphatases, such as SHP-1, to TrkA is transiently induced which counter balances TrkA activation (49). This could affect the phosphorylation levels of TrkA. To determine whether TRAF4-mediated ubiquitination affects phosphatase recruitment, we performed a co-IP experiment using a TrkA-specific antibody.
As shown in Supplemental Figure 4D, no significant difference was found between the association of SHP-1 with TrkA in the absence and presence of TRAF4 overexpression. Next, we asked whether mutation of the ubiquitination sites affects the ability of TrkA to recruit adaptor proteins. Y490 phosphorylation serves as a docking site for recruiting the shc adaptor protein and is necessary for subsequent activation of downstream Ras/MAPK and PI3K pathways (49-51). A co-IP experiment was performed to examine the interaction between shc and TrkA or its mutant in the presence of NGF. There are three shc isoforms, p66, p52 and p46. P52 and p46 but not p66 play a role in mediating growth factor signaling. As shown in Figure 7B, p52 and p46 but not p66 were indeed associated with flag-TrkA wild type. The K523_544_547R mutant, however, had a significantly reduced ability to interact with p52/p46 shc. TrkA dimerizes upon NGF stimulation. The reason we did not observe complete loss of the interaction between shc and the TrkA mutant is likely due to heterodimerization between the mutant TrkA and endogenous wild type receptor.

In the RPPA analysis (Table 1), we found that the p38 MAPK phosphorylation levels were down-regulated when TRAF4 was knocked-down. P38 MAPK plays an important role in EMT, invasion, extravasation, and organ colonization during cancer metastasis [11, 44]. Since TrkA ubiquitination mutant has reduced interaction with the shc adaptor protein, we examined whether TRAF4 regulates shc downstream p38 kinase activation upon NGF induction. As shown in Figure 7C, the level of p38 phosphorylation (Thr180/Tyr182) increased when cells were stimulated with NGF. TRAF4 knockdown significantly reduced NGF-induced p38 phosphorylation. This result confirms that TRAF4 plays a role in regulating NGF-induced TrkA signaling pathways.

The levels of TrkA phosphorylation and its downstream p38 phosphorylation were also significantly reduced in metastatic mouse tumors derived from tail-vein injected shTRAF4-PC3 cells compared to controls (Fig S4E), suggesting that TRAF4 expression levels regulate TrkA phosphorylation and its downstream signaling in vivo.
Taken together, our results suggested that TRAF4-mediated TrkA ubiquitination is important for TrkA function.

Correlation of TRAF4 expression and TrkA regulated gene transcription in human prostate cancer samples

To determine whether the regulation of TRAF4 on TrkA signaling also exists in human prostate cancer patients, we analyzed the expression of TRAF4 and two of the TrkA regulated genes involved in cancer metastasis, Slug and IL-6, in a prostate cancer cDNA array. As shown in Figure 7D and E, a significant correlation between expression levels of TRAF4 and Slug or IL-6 was observed (r=0.852, p<0.0001 and r=0.89, p= 2.53e-14, respectively). These results suggest that TRAF4 also can regulate TrkA signaling in human prostate cancers.

Altogether, our results demonstrate that TRAF4 promoted TrkA ubiquitination through K27 and K29 linkages at the tyrosine kinase domain. This post-translational modification enhanced TrkA kinase activity, its tyrosine phosphorylation levels, and subsequent downstream signaling activation to promote cancer metastasis.

DISCUSSION

Malfunction of the ubiquitination system can contribute significantly to cancer development and metastasis. In addition to the well-studied proteasome-dependent protein degradation, non-proteolytic ubiquitination is emerging as a pivotal player in cancers. Herein we demonstrated that the RING domain E3 ubiquitin ligase TRAF4 is highly expressed in metastatic prostate cancers and plays an important role in regulating prostate cancer invasion and metastasis. Its E3 ubiquitin ligase activity is essential for promoting cell invasion. We further revealed that TrkA, a member of the receptor tyrosine kinase superfamily, is a ubiquitination substrate of TRAF4 that mediates the effect of TRAF4 on prostate cancer cell invasion.
Receptor tyrosine kinases are important signaling molecules that regulate cell proliferation, survival, differentiation, apoptosis and migration. Aberrant activation of tyrosine kinases has been linked to a variety of cancers. In addition to genomic alterations, the activation of non-mutated kinases can contribute to cancer development and metastasis, especially in cancers with a low mutation rates such as prostate cancer (52-55). Global tyrosine phosphorylation levels are significantly increased in advanced prostate cancers even in the absence of kinase somatic mutations (56, 57). It was reported that several wild type tyrosine kinases, including Trk family members TrkB (NTRK2) and TrkC (NTRK3), can drive prostate cancer bone and visceral metastasis in vivo (58). Thus, mechanisms other than genomic alterations are important for aberrantly activating tyrosine kinases in prostate cancers. Our study here underscores a role of non-proteolytic ubiquitination in aberrant activation of wild type TrkA. Emerging evidence has documented the function of neurotrophins and their receptors in prostate cancer development and metastasis (21, 22, 24, 58, 59). Our results presented here provide strong evidence supporting the functional role of the TRAF4-TrkA axis in prostate cancer cell invasion.

Ubiquitination of several receptor tyrosine kinases has been reported previously, but the ubiquitination (mainly K48 or K63-linked) was associated with receptor protein turnover or receptor trafficking (46, 60-65). Similar to other RTKs, TrkA was found to be ubiquitinated by the RING type E3 ubiquitin ligase c-Cbl (64) and the HECT type ligase Nedd4-2, which promote its degradation through the proteasome (62). TRAF6 promotes K63-linked TrkA ubiquitination at the juxtamembrane domain and subsequent receptor internalization (46). Our results demonstrate that TRAF4–mediated ubiquitination occurs at the tyrosine kinase domain and it hyperactivates TrkA kinase activity, suggesting that TRAF4 targeted ubiquitination regulates TrkA function through a novel mechanism apart from other E3 ubiquitin ligases.

Interestingly, TRAF4 promoted TrkA ubiquitination through atypical K27 and K29-linked ubiquitin chain (Figure 4F). Unlike the well-studied K48 and K63 polyubiquitin chain, the functional roles of K27 and K29 ubiquitin linkages are less clear. It appears that they are
non-proteolytic and recently have been implicated in protein-protein association/dissociation, negative regulation of protein degradation, and protein aggregation (66-70). It is not clear exactly how the ubiquitination precisely affects TrkA kinase activity. The cellular localization of TrkA and its interaction with phosphatase SHP-1 were not significantly changed upon TRAF4 overexpression (Supplemental Figure 4A and 4D). We found that TrkA is ubiquitinated at the K523, K544 and K547 residues (Figure 6B). These lysine residues are located adjacent to each other in the N-lobe of the kinase domain, which is close to the center of the kinase active site: the DFG motif at the activation loop and the ATP binding site (Supplemental Figure 3C) (44). Conformational change of the activation loop, especially the position of the DFG motif, upon ligand binding is essential for tyrosine kinase activation (71).

Protein-protein interaction is one of the mechanisms to stabilize the active conformation of the activation loop of RTK or serine/threonine kinases during activation. Binding of cyclins to CDKs induces conformational change of the CDK kinase domain and stabilizes the active activation loop conformation (71). Similarly, one of the EGFR molecules in the ligand-bound EGFR dimer serves as an activator kinase to stabilize the active conformation of its partner through dimerization (71). It is possible that the K27 and K29-linked ubiquitin chain conjugation at the K523, K544 and K547 sites stabilizes the active conformation of the TrkA activation loop similar to the cases of cyclin/CDK and EGFR. The second possibility is that the positive charges introduced by the ubiquitin modification at these positions affect the position of the Asp residue at the DFG motif and stabilizes the active conformation of the activation loop, or stabilizes ATP binding, to sustain the kinase activation. Consistent with these speculations, we found that tyrosine phosphorylation (Y674/675) at the kinase domain, which is associated with kinase activation, was abolished when these lysine residues were mutated (Figure 7A).

It is known that NGF binds and activates TrkA which auto-phosphorylates tyrosine residues in TrkA (Figure 7F). This activates several important signaling pathways, such as PI3K/Akt, PLC-γ and MAPK, which regulate migration, invasion and metastasis. We found that overexpression of TRAF4 increased TrkA tyrosine phosphorylation in the presence of
NGF. The TrkA ubiquitination mutant not only was defective in tyrosine phosphorylation, but also has reduced ability to interact with downstream adaptor proteins. Consequently, we found that NGF-induced p38 MAPK phosphorylation and the expression of downstream invasion associated genes were suppressed in TRAF4 knockdown cells. All of these results strengthen the notion that TRAF4 mediated TrkA ubiquitination is required for TrkA phosphorylation and subsequent pathway activation (Figure 7F).

We also investigated whether other RTKs may be regulated by TRAF4 in a manner similar to TrKA, which might indicate that regulation of kinase activity by ubiquitination could be more generally applicable. We explored this concept using an array containing multiple receptor tyrosine kinases and found that TRAF4 overexpression increased ubiquitination of several receptor tyrosine kinases in addition to TrkA (Fig S4F). These new substrate kinases need to be further explored as additional therapeutic targets in prostate cancer.

Taken together, our study not only significantly expands our knowledge of TRAF4 in prostate cancer metastasis, but also provides a potential novel drug target for treating aggressive prostate cancers. Unlike genomic alteration or protein overexpression, wild type tyrosine kinase activation other than kinase overexpression is not easily diagnosed in patients. The study we present here revealed a new potential biomarker that could help for the prediction of TrkA activation in cancer patients.

METHODS

Animal and Human studies

All animal experiments were performed in accordance with the Animal Care Research Committee at Baylor College of Medicine. For in vivo studies, 5 to 6 week-old male SCID mice (The Jackson Laboratories) were used for experimental lung metastasis assays. Human tissue samples were obtained from the Human Tissue Acquisition and Pathology Core of the Dan L. Duncan Cancer Center and were collected from fresh radical prostatectomy specimens after obtaining informed consent under an Institutional Review
Board approved protocol. Cancer samples contained a minimum of 70% cancer and benign tissues were free of cancer on pathologic examination.

**Cell culture**

The human prostate cancer cell lines LNCaP, PC3, DU145 and HEK293T cells were obtained from ATCC (Manassas, VA, USA). PC-3, DU145 and LNCaP cells were maintained in RPMI1640 medium containing 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 unit/ml penicillin at 37°C and 5% CO2. Human embryonic kidney epithelial cell line HEK293T was maintained in DMEM supplemented with 10% FBS. For NGF-treatment experiments, cells were maintained in serum-free culture medium for stated period of time supplemented with 2 mM L-glutamine, 100U/ml penicillin and 100 μg/ml streptomycin and then treated with NGF (50µg/ml) for specified period of time.

**Reagents and antibodies**

Human Nerve Growth Factor Beta (NGF-β, Cat. 300-174P) was obtained from Gemini Bio-products, West Sacramento, CA. Primary antibodies were obtained as follows: anti-pTrkA(Y785) (Cat. 4168), anti-pTrkA(Y674/675) (Cat. 4621), anti-p38 (Cat. 9212), anti-p-p38 (Cat. 9211), anti-Shc (Cat. 2432), anti-SHP1 (Cat. 3759), anti-Na,K-ATPase (Cat. 3010) from Cell Signaling Technology (Beverly, MA); anti-TRAF4 (Cat. sc-10776), anti-HA-probe (Cat. sc-805), anti-Ub (Cat. sc-8017), anti-GAPDH (Cat. sc-32233) from Santa Cruz biotechnology (Dallas, TX); anti-TrkA (Cat. 06-574), anti-Shc (Cat. 06-203) from EMD Millipore (Massachusetts); anti-pTrkA(Y490) (Cat. ab85130), anti-Ki-67 (Cat. 66155) from purchased from Abcam (Cambridge, MA). HRP-conjugated secondary anti-mouse (Cat. 1706516) or anti-rabbit (Cat. 1706515) antibodies were obtained from Biorad (Hercules, CA). Monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody (Cat. 8592A), EZview™ Red ANTI-FLAG® M2 Affinity Gel (Cat. F2426) was obtained from Sigma-Aldrich (Allentown, PA). TRAF4 adenovirus (Cat. VH819961) was obtained from Vigen Bioscences (Rockville, MD).
GFP adenovirus was produced in the Gene Vector Core at Baylor College of Medicine. GW441756 (Cat. 141051) was purchased from abcam Cambridge, MA.

**Reverse transcription and quantitative real-time PCR**
Total RNA was extracted from the indicated cells by using RNeasy mini kit (Qiagen, Valencia, CA). The RNA concentration and purity were measured by NanoDrop 2000 UV-vis Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA). 2μg of total RNA was used to generate cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). Real time PCR was performed using SYBR green PCR master mix (Life-technologies, USA). Primers used are listed in supplementary table (Supplemental Table 1,2). For all RT-PCR analysis, β-Actin was used to normalize RNA input and expression levels were calculated according to the comparative C_T method (ΔΔCT).

**Construction of expression vectors and TrkA mutants**
The TRAF4, TrkA cDNA was cloned into Flag-tag pSG5 expression vector. All TrkA deletion as well as lysine mutants were also cloned into Flag-tag pSG5 expression vector. In addition, TRAF4 was also cloned into HA-tagged pCM5 expression vector. SHP-1 (8572), Wild type Ubiquitin and its mutant constructs were obtained from Addgene. TRAF4 shRNA were cloned into pLenti6/TR vector (Thermo-Fisher Scientific, Waltham, MA). Primers used for cloning are listed in supplementary table (Supplemental Table 1,2).

**Transfection and lentivirus infection**
Cells were transfected with plasmid DNA using Lipofectamine® 3000 and siRNA using Lipofectamine® RNAiMAX transfection reagent (both from Thermo-Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer’s protocol. Virus packaging was performed in HEK 293T cells after co-transfection of plasmid with the packaging plasmid psPAX2 and envelope plasmid pMD2.G using Lipofectamine 3000. Viruses were harvested
48h after transfection, and viral titers were determined. Target cells, were infected with recombinant lentivirus-transducing units in the presence of 8μg/mL polybrene (Sigma, MO).

**Immunoblotting**

Cells were harvested and protein was extracted from cells as previously described (72). The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) and samples were separated in SDS polyacrylamide gels, with various concentrations depending on the molecular weight of the protein under investigation. After probing with a primary antibody, the membrane was incubated with a secondary antibody conjugated with HRP. Finally, signal intensity was determined using the enhanced chemiluminescence reagents (Pierce, France). Endogenous GAPDH was used as the internal control.

**MTS assay**

The CellTiter 96® AQueous one solution cell proliferation assay (MTS) reagent (Cat. G358A) was obtained from Promega (Madison, WI, USA) and the assay was performed according to the manufacturer's instructions. Briefly, cells were seeded in 96 well plate and treated with or without TrkA inhibitor, GW441756. The plate was incubated at 37°C in a humidified, 5% CO₂ atmosphere. 20μL of CellTiter 96® AQueous one solution reagent was added to each well containing 100μL media and again incubated for 3h. Absorbance was measured at 490 nm using a microplate reader.

**Cell migration and invasion assay**

Cell invasion activity was determined in vitro using BD BioCoat tumor invasion system (Cat. 354483) from BD Biosciences (San Jose, CA), which contains an 8-μm polyethylene terephthalate membrane with a thin layer of reconstituted Matrigel basement membrane matrix per the manufacturer’s protocol. In brief, cells were harvested, resuspended in serum-free medium, and then transferred to the hydrated Matrigel chambers (≈25,000 cells per well). The chambers were then incubated for 16 h in culture medium with 10% FBS in the bottom chambers before examination. The cells on the upper surface were scraped and washed away, whereas the invaded cells on the lower surface were fixed and stained with
0.05% crystal violet for 2h. Finally, invaded cells were counted under a microscope and the relative number was calculated. Cell migration assay was performed by following the same procedures as cell invasion assay except that modified two-chamber transwell system was used in the migration assay (Cat. 354578) (BD Biosciences).

**Ubiquitination screen assay**

Targets of TRAF4 mediated ubiquitination were determined using R&D Systems Proteome Profiler™ Human Ubiquitin Array Kit (Cat. ARY027) which consists of 49 different proteins samples and Proteome Profiler Human Phospho-RTK Array Kit (Cat. ARY001B) (Minneapolis, MN, USA) consisting of 49 human receptor tyrosine kinases. Relative expression levels of ubiquitination of human proteins in samples were determined as per manufacturer’s protocol. Briefly, the human ubiquitin array nitrocellulose membranes spotted with 49 different antibodies to human ubiquitin target proteins were incubated with prepared cell lysates for one hour on a rocking platform shaker. After thorough wash to remove unbound proteins, the membrane was incubated with biotinylated pan anti-ubiquitin detection antibody cocktail at 4˚C for overnight. Next day the membrane was washed thoroughly followed by addition of streptavidin-HRP. The signal produced at each capture spot corresponding to the relative amount of ubiquitinated protein bound was exposed to autoradiography film and analyzed.

**In vitro ubiquitination assay**

Flag-TrkA was transiently transfected into 293T cells. The protein was then purified from 293T cell lysates using an anti-flag M2 beads and eluted from the beads using 3Xflag peptides. The purified protein was incubated with 100 ng of UBE1, 150 ng of UbcH5a, 5 μg of HA-Ubiquitin (Boston Biochem) in the absence or presence of 500 ng of TRAF4 (Novus Biologicals) with ubiquitination buffer (50 mM Tris-Cl, pH 7.4, 2 mM ATP, 5 mM MgCl2, 2 mM DTT) at 30°C for 90 minutes. The incubation mixture was then subjected to
immunoprecipitation using an anti-flag antibody followed by Western blot analysis using an anti-HA antibody.

**Tail vein injection and IVIS imaging**

The animal studies were conducted in accordance with National Institutes of Health animal use guidelines and the experimental protocol was approved by the Baylor College of Medicine (BCM) Animal Care Research Committee. To evaluate the role of TRAF4 in tumor metastasis, luciferase positive PC3 cells (control and TRAF4 knockdown by shRNA) were injected into SCID mice through tail vein ($1 \times 10^6$ cells per mouse). To study the effect of TrKA inhibition in tumor metastasis, we used a TrKA specific inhibitor GW441756. Luciferase positive PC3 cells were injected into SCID mice through tail vein ($1 \times 10^6$ cells per mouse). Thereafter, mice were treated with i.p. injection twice a week with solvent control or GW441756 (10mg/kg). Tumor metastasis in mice was assessed via in vivo bioluminescence measurement using the IVIS Imaging System (Perkin Elmer). For the luciferase detection imaging, 200 µl of 15 mg/mL D-luciferin (Caliper Life Sciences) in PBS was injected i.p. before imaging. The photometry of the tumor was calculated by software Living Image 3.1.0, (Caliper Life Sciences, Waltham, MA) and the results were used to generate the tumor metastasis progression. Eight weeks after injection, mice were sacrificed, lungs were collected, fixed in Bouin's solution and images were captured.

**Reverse phase protein array (RPPA) analysis**

Reverse phase protein array assays were carried out as described previously with minor modifications (Chang et al., 2015). Protein lysates were prepared from cultured cells with modified Tissue Protein Extraction Reagent (TPER) (Pierce) and a cocktail of protease and phosphatase inhibitors (Roche Life Science) (Chang et al., 2015). The lysates were diluted into 0.5 mg/ml of total protein in SDS sample buffer and denatured on the same day. The Aushon 2470 Arrayer (Aushon BioSystems) with a 40 pin (185 µm) configuration was used to spot samples and control lysates onto nitrocellulose-coated slides (Grace Bio-labs) using an array format of 960 lysates/slide (2880 spots/slide). The slides were processed as
described (Chang et. al.) and probed with a set of 204 antibodies against total and phosphoprotein proteins using an automated slide stainer Autolink 48 (Dako). Each slide was incubated with one specific primary antibody and negative control slide was incubated with antibody diluent instead of primary antibody. Primary antibody binding was detected using a biotinylated secondary antibody followed by streptavidin-conjugated IRDye680 fluorophore (LI-COR Biosciences). Total protein content of each spotted lysate was assessed by fluorescent staining with Sypro Ruby Protein Blot Stain according to the manufacturer’s instructions (Molecular Probes).

Fluorescence-labeled slides were scanned on a GenePix AL4200 scanner, each slide, along with its accompanying negative control slide, is scanned at an appropriate PMT to obtain optimal signal for this specific set of samples. The images were analyzed with GenePix Pro 7.0 (Molecular Devices). Total fluorescence signal intensities of each spot were obtained after subtraction of the local background signal for each slide and were then normalized for variation in total protein, background and non-specific labeling using a group-based normalization method as described (Chang et. al. 2015). For each spot on the array, the-background-subtracted foreground signal intensity was subtracted by the corresponding signal intensity of the negative control slide (omission of primary antibody) and then normalized to the corresponding signal intensity of total protein for that spot. Each image, along with its normalized data, was carefully evaluated for quality through manual inspection and control samples. Antibody slides that failed the quality inspection were either repeated at the end of the staining runs or removed before data reporting. Total 203 antibodies remained in the list. A complete list of validated antibodies can be found at: https://www.bcm.edu/centers/cancer-center/research/shared-resources/cprit-cancer-proteomics-and-metabolomics/reverse-phase-proteinarray).

The median of the triplicate experimental values (normalized signal intensity) is taken for each sample for subsequent statistical analysis. We determined significantly changed proteins between experimental groups by employing Student’s t-test (significant for p<0.05).
Kinase assay

The TrkA kinase assay was performed using the TrkA kinase enzyme system (Cat. V2931) and ADP-Glo™ Kinase Assay kit (Cat. V6930) (Promega; Madison, WI) as per manufacturer’s protocol. To compare the kinase activity of TrkA with and without TRAF4 overexpression and TrkA ubiquitin mutant with wild type TrkA, 293T cells were transfected with different plasmids using Lipofectamine 3000. Cells were grown in complete media followed by serum starvation for 4h. Thereafter cells were briefly induced with NGF before harvesting. Cell lysis was performed using M-PER™ mammalian protein extraction reagent (Thermo-Fisher Scientific, Waltham, MA). Over expressed flag-tagged proteins were immunoprecipitated using EZview™ Red ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich (Allentown, PA). After thoroughly washing the beads with wash buffer (1X PBS, 1mM DTT, 1X protease inhibitor), protein was eluted by incubating the beads at 4°C for 30min with 100uL of 3X FLAG® peptide (Sigma-Aldrich (Allentown, PA) at a 100ng/mL working concentration. Equal amount of protein was used to compare the kinase activity using TrkA kinase enzyme system and ADP-Glo™ Kinase Assay kit (Promega; Madison, WI). The TrkA reaction utilizes ATP and generates ADP. Then the ADP-Glo™ reagent terminates the kinase reaction and depletes the remaining ATP. Finally, the kinase detection reagent converts ADP to ATP and the newly synthesized ATP emits light using the luciferase/luciferin reaction. The light generated correlates to the amount of ADP generated in the kinase or ATPase assay, which is indicative of kinase activity.

Statistics

Unless otherwise indicated, all results represent the mean ± SEM, and statistical comparisons between different groups were performed using the 2-tailed Student’s t test or 1-way ANOVA with multiple comparisons corrections. For all statistical analyses, differences of P ≤ 0.05 were considered statistically significant, and experiments were repeated at least 3 times. GraphPad Prism software version 4.0/7.0 (GraphPad Software) was used for data analysis.
Study Approval

All animal experiments were approved by the Animal Center for Comparative Medicine at Baylor College of Medicine (BCM). Human tissue samples were obtained from the Human Tissue Acquisition and Pathology Core of the Dan L. Duncan Cancer Center and were collected from fresh radical prostatectomy specimens after obtaining informed consent under an Institutional Review Board approved protocol.

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Author Contributions:

R.S., B.W.O., and P.Y. conceived and designed the experiments. R.S. and D.K. performed cell studies. R.S., J.S. and S.D. performed animal studies. S.H. and D.P.E. performed RPPA analysis. M.M.I. provided human prostate tumor samples. R.S. B.W.O. and P.Y. interpreted the data and wrote the manuscript. All the authors discussed the results and commented on the manuscript.
References


Figure legends:

Figure 1. TRAF4 is overexpressed in prostate cancers. (A) TRAF4 mRNA is upregulated in prostate tumors (n=39) as compared with normal tissue (n=9) in a prostate cancer tissue scan array (OriGene) as analyzed by qPCR. Left panel, box plot of relative TRAF4 mRNA expression in normal and tumor samples as normalized against β-actin. *P <0.05 by Mann-whitney Test. Right panel, average fold induction of TRAF4 expression in tumor samples. *P <0.05 by two tailed Student’s t test. (B) TRAF4 protein is upregulated in human prostate tumors (T) compared to matched normal tissue (N) samples (n=10 each) as analyzed by Western blot. *P < 0.05 by two tailed Student’s t test. (C-F) TRAF4 is highly expressed in metastatic prostate cancers, The expression of TRAF4 was analyzed in 4 different prostate cancer datasets. β-Actin was used as an internal control. *P < 0.05 by one way ANOVA with multiple comparisons test. Data are represented as the mean ± SEM.

Figure 2. TRAF4 plays a role in prostate cancer cell migration, invasion and metastasis. Knockdown of TRAF4 in PC3 cell inhibited cell migration (A) and invasion (B). Left panels, Cells migrated through migration chamber (A) or matrigel (B) (n=3). Images were obtained at 100X magnification. Middle panels, quantitation of cell numbers per field. Right panel, the levels of TRAF4 in control or TRAF4 knockdown cells as assessed by qRT-PCR. *P <0.05 by Student’s t test versus si ctrl or siTRAF4 (A) and *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. Data are represented as the mean ± SEM (B). (C) TRAF4 knockdown reduced prostate cancer cell colonization and metastasis in vivo. Upper panel, representative bioluminescence images of SCID mice at different time periods after tail-vein injection of PC3 shcontrol or shTRAF4 cells. Lower panel, quantitation of the bioluminescence signals at the lung area in PC3 shcontrol or shTRAF4 injected mice (n=5). Right panel, the levels of TRAF4 in control or TRAF4 stable knockdown cells as assessed by qRT-PCR. *P <0.05 by two tailed Student’s t test. (D) TRAF4 knockdown reduced lung metastatic nodules. H&E stained lung sections and graph showing the number of lung metastasis nodules after tail vein injection (n=10). Data are represented as the mean ± SEM.
*P <0.05 by two tailed Student’s t test. (E) H&E stained bone sections (left panel) and immunohistochemistry using an anti-luciferase antibody staining (right panel) confirming the bone metastasis in control group but not in shTRAF4 knockdown group. Images were obtained at 40X and 100X (inset) magnification.

Figure 3. TRAF4-mediated TrkA ubiquitination is critical for cell invasion. (A) Overexpression of TRAF4 wild type but not the RING domain deletion mutant promoted LNCaP cell invasion. Left panel, LNCaP cells invaded through matrigel were stained with crystal violet (n=3). Images were obtained at 100X magnification. Middle panel, quantitation of invaded cells per field. Right panel, Western blot analysis of the expression of TRAF4 wild type or its mutant in LNCaP stable cells using a TRAF4-specific antibody. *P <0.05 by one way ANOVA. (B) TRAF4 overexpression enhanced TrkA ubiquitination. The human ubiquitin array kit was used to identify TRAF4 ubiquitination targets. Cell lysate from GFP (control) or TRAF4 expressing adenovirus infected PC3 cells were used on each array. Each dot represents the ubiquitination level of a target protein recognized by an anti-ubiquitin antibody. *P <0.05 by two tailed Student’s t test. (C) Knockdown of TrkA using specific siRNAs decreased DU145 cell invasion (n=3). Images were obtained at 100X magnification. Right panel, the levels of TrkA in control or TrkA knockdown cells as assessed by qRT-PCR. *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (D) TrKA inhibition by GW441756 reduced prostate cancer cell colonization and metastasis in vivo. Left panel, bioluminescence images of SCID mice at 0 or 9 weeks after tail-vein injection of PC3 luciferase cells with or without GW441756 treatment. Right panel, quantitation of the bioluminescence signals at the metastasis area in control or GW441756 injected mice 9 weeks after injection (n=5 per group). Data are represented as the mean ± SEM. *P <0.05 by two tailed Student’s t test.

Figure 4. TRAF4 interacted with TrkA and promoted its ubiquitination (A) HA-TRAF4 interacted with flag-TrkA in transiently transfected 293T cells. Shown is a co-IP experiment using an anti-flag antibody for immunoprecipitation. (B) Wild type TRAF4 but not the RING domain deletion mutant promoted TrkA ubiquitination. 293T cells were co-transfected with
constructs as indicated. Flag-TrkA was immunoprecipitated with an anti-Flag antibody and the ubiquitinated TrkA was visualized by Western blot analysis using an anti-HA antibody.

(C) Endogenous TrkA interacted with endogenous TRAF4 in DU145 cells. Shown is a co-IP experiment using a TrkA-specific antibody or IgG control for immunoprecipitation. (D) TRAF4 knockdown abolished NGF-induced TrkA ubiquitination. DU145 were transfected with control siRNA or siTRAF4 and HA-Ubiquitin. Cells were then treated with 50 ng/ml NGF for 15 min before harvest. Ubiquitinated TrkA was detected using an anti-ubiquitin antibody in a Western blot analysis from cell lysates immunoprecipitated with an anti-TrkA antibody. (E) TRAF4 overexpression promoted TrkA ubiquitination at the cell membrane. 293T cells were co-transfected with TrkA and HA-Ubiquitin. Cytosolic and membrane fraction were isolated and subjected to immunoprecipitation using an anti-Flag antibody and the ubiquitinated TrkA was visualized by Western blot analysis using an anti-HA antibody. (F) TRAF4 mediated TrkA polyubiquitination through K27 or K29-linked ubiquitin chain. K6-K63 represents the ubiquitin mutant with all lysine mutations except the depicted number of lysine. TRAF4 interacted with TrkA and promoted its ubiquitination (A) HA-TRAF4 interacted with flag-TrkA in transiently transfected 293T cells. Shown is a co-IP experiment using an anti-Flag antibody for immunoprecipitation. (B) Wild type TRAF4 but not the RING domain deletion mutant promoted TrkA ubiquitination. 293T cells were co-transfected with constructs as indicated. Flag-TrkA was immunoprecipitated with an anti-Flag antibody and the ubiquitinated TrkA was visualized by Western blot analysis using an anti-HA antibody. (C) Endogenous TrkA interacted with endogenous TRAF4 in DU145 cells. Shown is a co-IP experiment using a TrkA-specific antibody or IgG control for immunoprecipitation. (D) TRAF4 knockdown abolished NGF-induced TrkA ubiquitination. DU145 were transfected with control siRNA or siTRAF4 and HA-Ubiquitin. Cells were then treated with 50 ng/ml NGF for 15 min before harvest. Ubiquitinated TrkA was detected using an anti-ubiquitin antibody in a Western blot analysis from cell lysates immunoprecipitated with an anti-TrkA antibody. (E) TRAF4 overexpression promoted TrkA ubiquitination at the cell membrane. 293T cells were co-transfected with TrkA and HA-Ub...
the absence or presence of TRAF4 co-transfection. Cytosolic and membrane fraction were isolated and subjected to immunoprecipitation using an anti-Flag antibody and the ubiquitinated TrkA was visualized by Western blot analysis using an anti-HA antibody. (F) TRAF4 mediated TrkA polyubiquitination through K27 or K29-linked ubiquitin chain. K6-K63 represents the ubiquitin mutant with all lysine mutations except the depicted number of lysine.

Figure 5. TrkA plays an important role in TRAF4-promoted cell invasion. (A) Validation of the RPPA data for some of the invasion related genes after TRAF4 knockdown using the real time PCR. Lower panel, the levels of TRAF4 in control or TRAF4 knockdown cells as assessed by qRT-PCR. *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (n=3) (B) Expression of TRAF4-regulated genes at different time points following NGF stimulation as assessed by real time PCR. (n=3) (C) TrkA knockdown also downregulates TRAF4 regulated invasion related genes. *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (n=3) (D) Knockdown of TRAF4 inhibited NGF-stimulated gene expression. (n=3) (E) TrkA selective inhibitor treatment abolished TRAF4 stimulated cell invasion (n=3). Images were obtained at 100X magnification. PC3 cells were infected with GFP or TRAF4 adenovirus and then treated with or without 0.5mM GW441756 for two days before seeding in an invasion chamber. *P <0.05 by one way ANOVA with Tukey’s multiple comparisons test. Data are represented as the mean ± SEM.

Figure 6. TRAF4 ubiquitinated three lysine residues present in the kinase domain of TrkA. (A) Deletion of the tyrosine kinase domain (TK) of TrkA abolished its ubiquitination. Upper panel, schematic representation of TrkA and its deletion mutants. Lower panel, the ubiquitination levels of different TrkA deletion mutants. Flag-TrkA and the mutants were co-transfected with TRAF4 and HA-Ub into 293T cells. The ubiquitinated TrkA was immunoprecipitated using a flag antibody and then detected using an anti-HA antibody in the Western blot. (B) Mutation of K523, K544 and K547 residues at the TK domain abolished TrkA ubiquitination. (C) TRAF4 hyperactivated TrkA wild type but not the K523_544_547R
mutant in an in vitro kinase assay. Left panel, purified flag-TrkA in vitro kinase activity using a poly (Glu4, Tyr1) synthetic peptide as a substrate. The activity was measured through an ADP-Glo Kinase assay. Right panel, the protein levels of purified TrkA, its mutant and PKCd used in the kinase assay with or without TRAF4 overexpression as demonstrated by Western blotting using an anti-flag antibody. Data are represented as the mean ± SEM. (n=3) *P <0.05 by one way ANOVA.

Figure 7. TRAF4-mediated TrkA ubiquitination regulated NGF-stimulated TrkA signaling cascade. (A) TRAF4 overexpression increased the phosphorylation level of wild typeTrkA but not the K523_544_547R mutant in the presence of NGF. Specific tyrosine phosphorylation antibodies were used in the Western blot analyzing in cells treated with 50 ng/ml NGF for 15 min. (B) Mutation of the K523, K544 and K547 residues reduced the association of shc protein with TrkA. Flag-TrkA wild type or its mutant was transfected into DU145 cells and the interaction between shc protein and TrkA was determined in a co-IP experiment using a flag antibody for immunoprecipitation. (C) TRAF4 knockdown significantly reduced NGF-induced p38 MAPK phosphorylation (pT180/Y182). PC3 cells were treated with and without NGF (50µg/ml) for 10 min after 18h serum starvation (D) The level of TRAF4 expression correlated with the Slug gene expression in prostate cancer patient cDNA array. (E) The level of TRAF4 expression correlated with the IL-6 gene expression in prostate cancer patient cDNA array. Pearson's correlations for (D-E) in fold change (log2) of gene expression as determined by qRT-PCR, (r=0.852, p<0.0001 and r=0.89, p= 2.53e-14, respectively). (F) A working model of the role of TRAF4 in TrkA signaling and prostate cancer cell invasion. In low TRAF4 expressing cells, NGF-induced limited TrkA ubiquitination, resulting in low levels of TrkA kinase signaling. In TRAF4 overexpressing cells, high levels of TRAF4 significantly increase TrkA K27 and K29-linked ubiquitination at the K523, K544 and K547 in the kinase domain upon NGF stimulation. This ubiquitination enhances TrkA kinase activity, its tyrosine phosphorylation levels and the recruitment of downstream adaptor proteins, resulting in hyperactivated TrkA signaling.
cascade. Consequently, NGF-responsive invasion associated targeted gene transcription are upregulated to promote cell migration and invasion.

**Table legend:**

Table 1. TRAF4 regulates genes involved in cell migration/invasion. (A) Representative cell migration/invasion related proteins/protein phosphorylation are regulated by TRAF4 in a RPPA study comparing their levels in TRAF4 knockdown and non-targeting control PC3 cells (n=12). The median of the triplicate experimental values (normalized signal intensity) is taken for each sample for statistical analysis using Student’s t-test (significant for p<0.05).

**Supplementary Figure legend**

Supplemental Figure 1. (A) Knockdown of TRAF4 using specific siRNAs decreased DU145 cell invasion. Left panel, crystal violet staining of invaded cells (n=3). Images were obtained at 100X magnification. Middle panel, quantitation of invaded cells per field. Right panel, the levels of TRAF4 in control or TRAF4 knockdown cells as assessed by qRT-PCR. *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (n=3) (B) PC3 shTRAF4 cells have higher luminescence intensity than shcontrol cells. (C) Comparable levels of Ki-67 staining in shctrl and shTARF4 tumor samples as determined by IHC analysis. Images were obtained at 100X magnification. Data are represented as the mean ± SEM.

Supplemental Figure 2. (A) Knockdown of TrkA using specific siRNAs decreased PC3 cell invasion (n=3). Images were obtained at 100X magnification. Right panel, the levels of TrkA in control or TrkA knockdown cells as assessed by qRT-PCR. (n=3). *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (B) TrkA knockdown has an inhibitory effect on PC3 cell invasion similar to the effect of TRAF4 knockdown (n=3). Images were obtained at 100X magnification. Right panel, quantitation of TRAF4 or TrkA siRNA knockdown invaded cells per field after invasion. *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (C) TrkA inhibitor treatment did not change mouse
weights. Data are represented as the mean ± SEM. (n=3). *P <0.05 by two tailed Student’s t test.

Supplemental Figure 3. (A) TRAF4 promoted TrkA ubiquitination in vitro. Flag-TrkA was transiently transfected into 293T cells and purified using an anti-flag antibody. The purified protein was then incubated with HA-ubiquitin, UBE1, UbcH5a in the absence or presence of purified TRAF4. The levels of TrkA ubiquitination was determined by immunoprecipitation using an anti-flag antibody followed by Western blot analysis using an anti-HA antibody. (B) TrkA inhibitor, GW441756, did not significantly affect cell growth in PC3 cells for specified dose and time treatment in PC3 cells. (n=3). (C) Lysine residues K523, K544 and K547 (shown in red) are located close proximity to the DFG motif (shown in green) in the kinase activation loop. Show is crystal structure of apo-TrkA kinase domain (PDB ID: 4F0I (44)). (D) Mutation of K523 and K544 to R abolished TRAF4-mediated TrkA activation in vitro. The upper panel shows the in vitro kinase activity of TrkA and its mutants in the absence or presence of TRAF4 overexpression. The bottom panel shows that comparable amount of TrkA proteins were used in the in vitro kinase assay as assessed by the Western blot analysis. Data are represented as the mean ± SEM. (n=3). *P <0.05 by one way ANOVA.

Supplemental Figure 4. (A) TRAF4 overexpression did not have a major effect on TrkA cellular distribution. DU145 cells were serum starved and transfected with or without TRAF4 followed by NGF stimulation. Cytosolic and membrane fraction were then isolated and the levels of TrkA and TRAF4 in each fraction were visualized by Western blot analysis using specific antibodies. Right panel, total TrkA level in DU145 whole cell lysates with NGF treatment. Na-K ATPase was used as an internal control for membrane fraction and GAPDH for cytoplasmic and total protein. (B) K523_44R mutation abolished TrkA tyrosine phosphorylation. (C) K547R single mutation did not abolish TrkA tyrosine phosphorylation. (D) TRAF4 overexpression did not alter the interaction between TrkA and SHP-1 phosphatase. Flag-TrkA was transfected into 293T cells with or without TRAF4 co-transfection. Shown is a co-IP experiment using a flag-specific antibody. (E) The levels of p-
p38 (pT180/Y182) and p-TrKA (pY785) were significantly reduced in TRAF4 knockdown metastatic tumors compared to tumors derived from control cells. Shown are representative immunohistochemistry images at 40X and 100X (inset) magnification from lung tumors derived from PC3 cell tail-vein injection (n=3 per group). (F) TRAF4 overexpression increased ubiquitination of several receptor tyrosine kinases. Equal amount of cell lysates from GFP (control) or TRAF4 expressing adenovirus infected PC3 cells were incubated with a RTK array. Each duplicate dot represents the ubiquitination level of a target protein recognized by an anti-ubiquitin antibody.

**Supplementary Table legend**

Supplemental Table 1,2. RT-qPCR primer sequence and cloning sequence used in the study. 5.1 denotes forward and 3.1 denotes reverse primer.
Figure 1. TRAF4 is overexpressed in prostate cancers. (A) TRAF4 mRNA is upregulated in prostate tumors (n=39) as compared with normal tissue (n=9) in a prostate cancer tissue scan array (OriGene) as analyzed by qPCR. Left panel, box plot of relative TRAF4 mRNA expression in normal and tumor samples as normalized against β-actin. *P <0.05 by Mann-whitney Test. Right panel, average fold induction of TRAF4 expression in tumor samples. *P <0.05 by two tailed Student’s t test. (B) TRAF4 protein is upregulated in human prostate tumors (T) compared to matched normal tissue (N) samples (n=10 each) as analyzed by Western blot. *P < 0.05 by two tailed Student’s t test. (C-F) TRAF4 is highly expressed in metastatic prostate cancers, The expression of TRAF4 was analyzed in 4 different prostate cancer datasets. β-Actin was used as an internal control. *P < 0.05 by one way ANOVA with multiple comparisons test. Data are represented as the mean ± SEM.
Figure 2. TRAF4 plays a role in prostate cancer cell migration, invasion and metastasis. Knockdown of TRAF4 in PC3 cell inhibited cell migration (A) and invasion (B). Left panels, Cells migrated through migration chamber (A) or matrigel (B) (n=5). Images were obtained at 100X magnification. Middle panels, quantitation of cell numbers per field. Right panel, the levels of TRAF4 in control or TRAF4 knockdown cells as assessed by qRT-PCR. *P < 0.05 by Student’s t test versus si ctrl or siTRAF4 (A) and *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. Data are represented as the mean ± SEM (B). (C) TRAF4 knockdown reduced prostate cancer cell colonization and metastasis in vivo. Upper panel, representative bioluminescence images of SCID mice at different time periods after tail-vein injection of PC3 shcontrol or shTRAF4 cells. Lower panel, quantitation of the bioluminescence signals at the lung area in PC3 shcontrol or shTRAF4 injected mice (n=5). Right panel, the levels of TRAF4 in control or TRAF4 stable knockdown cells as assessed by qRT-PCR. *P < 0.05 by two tailed Student’s t test. (D) TRAF4 knockdown reduced lung metastatic nodules. H&E stained lung sections and graph showing the number of lung metastasis nodules after tail vein injection (n=10). Data are represented as the mean ± SEM. *P <0.05 by two tailed Student’s t test. (E) H&E stained bone sections (left panel) and immunohistochemistry using an anti-luciferase antibody staining (right panel) confirming the bone metastasis in control group but not in shTRAF4 knockdown group. Images were obtained at 40X and 100X (inset) magnification.
Figure 3. TRAF4-mediated TrkA ubiquitination is critical for cell invasion. (A) Overexpression of TRAF4 wild type but not the RING domain deletion mutant promoted LNCaP cell invasion. Left panel, LNCaP cells invaded through matrigel were stained with crystal violet (n=3). Images were obtained at 100X magnification. Middle panel, quantitation of invaded cells per field. Right panel, Western blot analysis of the expression of TRAF4 wild type or its mutant in LNCaP stable cells using a TRAF4-specific antibody. *P <0.05 by one way ANOVA. (B) TRAF4 overexpression enhanced TrkA ubiquitination. The human ubiquitin array kit was used to identify TRAF4 ubiquitination targets. Cell lysate from GFP (control) or TRAF4 expressing adenovirus infected PC3 cells were used on each array. Each dot represents the ubiquitination level of a target protein recognized by an anti-ubiquitin antibody. *P <0.05 by two tailed Student’s t test. (C) Knockdown of TrkA using specific siRNAs decreased DU145 cell invasion (n=3). Images were obtained at 100X magnification. Right panel, the levels of TrkA in control or TrkA knockdown cells as assessed by qRT-PCR. *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (D) TrkA inhibition by GW441756 reduced prostate cancer cell colonization and metastasis in vivo. Left panel, bioluminescence images of SCID mice at 0 or 9 weeks after tail-vein injection of PC3 luciferase cells with or without GW441756 treatment. Right panel, quantitation of the bioluminescence signals at the metastasis area in control or GW441756 injected mice 9 weeks after injection (n=5 per group). Data are represented as the mean ± SEM. *P <0.05 by two tailed Student’s t test.
Figure 4. TRAF4 interacted with TrkA and promoted its ubiquitination (A) HA-TRAF4 interacted with flag-TrkA in transiently transfected 293T cells. Shown is a co-IP experiment using an anti-flag antibody for immunoprecipitation. (B) Wild type TRAF4 but not the RING domain deletion mutant promoted TrkA ubiquitination. 293T cells were co-transfected with constructs as indicated. Flag-TrkA was immunoprecipitated with an anti-Flag antibody and the ubiquitinated TrkA was visualized by Western blot analysis using an anti-HA antibody. (C) Endogenous TrkA interacted with endogenous TRAF4 in DU145 cells. Shown is a co-IP experiment using a TrkA-specific antibody or IgG control for immunoprecipitation. (D) TRAF4 knockdown abolished NGF-induced TrkA ubiquitination. DU145 were transfected with control siRNA or siTRAF4 and HA-Ubiquitin. Cells were then treated with 50 ng/ml NGF for 15 min before harvest. Ubiquitinated TrkA was detected using an anti-ubiquitin antibody in a Western blot analysis from cell lysates immunoprecipitated with an anti-TrkA antibody. (E) TRAF4 overexpression promoted TrkA ubiquitination at the cell membrane. 293T cells were co-transfected with TrkA and HA-Ub in the absence or presence of TRAF4 co-transfection. Cytosolic and membrane fraction were isolated and subjected to immunoprecipitation using an anti-Flag antibody and the ubiquitinated TrkA was visualized by Western blot analysis using an anti-HA antibody. (F) TRAF4 mediated TrkA polyubiquitination through K27 or K29-linked ubiquitin chain. K6-K63 represents the ubiquitin mutant with all lysine mutations except the depicted number of lysine.
Figure 5. TrkA plays an important role in TRAF4-promoted cell invasion. (A) Validation of the RPPA data for some of the invasion related genes after TRAF4 knockdown using the real time PCR. Lower panel, the levels of TRAF4 in control or TRAF4 knockdown cells as assessed by qRT-PCR. *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (n=3) (B) Expression of TRAF4-regulated genes at different time points following NGF stimulation as assessed by real time PCR. (n=3) (C) TrkA knockdown also downregulates TRAF4 regulated invasion related genes. *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (D) Knockdown of TRAF4 inhibited NGF-stimulated gene expression. (n=3) (E) TrkA selective inhibitor treatment abolished TRAF4 stimulated cell invasion (n=3). Images were obtained at 100X magnification. PC3 cells were infected with GFP or TRAF4 adenovirus and then treated with or without 0.5mM GW441756 for two days before seeding in an invasion chamber. *P <0.05 by one way ANOVA with Tukey’s multiple comparisons test. Data are represented as the mean ± SEM.
Figure 6. TRAF4 ubiquitinated three lysine residues present in the kinase domain of TrkA. (A) Deletion of the tyrosine kinase domain (TK) of TrkA abolished its ubiquitination. Upper panel, schematic representation of TrkA and its deletion mutants. Lower panel, the ubiquitination levels of different TrkA deletion mutants. Flag-TrkA and the mutants were co-transfected with TRAF4 and HA-Ub into 293T cells. The ubiquitinated TrkA was immunoprecipitated using a flag antibody and then detected using an anti-HA antibody in the Western blot. (B) Mutation of K523, K544 and K547 residues at the TK domain abolished TrkA ubiquitination. (C) TRAF4 hyperactivated TrkA wild type but not the K523_544_547R mutant in an in vitro kinase assay. Left panel, purified flag-TrkA in vitro kinase activity using a poly (Glu, Tyr) synthetic peptide as a substrate. The activity was measured through an ADP-Glo Kinase assay. Right panel, the protein levels of purified TrkA, its mutant and PKCd used in the kinase assay with or without TRAF4 overexpression as demonstrated by Western blotting using an anti-flag antibody. Data are represented as the mean ± SEM. (n=3) *P <0.05 by one way ANOVA.
Figure 7. TRAF4-mediated TrkA ubiquitination regulated NGF-stimulated TrkA signaling cascade. (A) TRAF4 overexpression increased the phosphorylation level of wild type TrkA but not the K523_44_547R mutant in the presence of NGF. Specific tyrosine phosphorylation antibodies were used in the Western blot analyzing in cells treated with 50 ng/ml NGF for 15 min. (B) Mutation of the K523, K544 and K547 residues reduced the association of shc protein with TrkA. Flag-TrkA wild type or its mutant was transfected into DU145 cells and the interaction between shc protein and TrkA was determined in a co-IP experiment using a flag antibody for immunoprecipitation. (C) TRAF4 knockdown significantly reduced NGF-induced p38 MAPK phosphorylation (pT180/Y182). PC3 cells were treated with and without NGF (50µg/ml) for 10 min after 18h serum starvation (D) The level of TRAF4 expression correlated with the Slug gene expression in prostate cancer patient cDNA array. (E) The level of TRAF4 expression correlated with the IL-6 gene expression in prostate cancer patient cDNA array. Pearson’s correlations for (D-E) in fold change (log2) of gene expression as determined by qRT-PCR, (r=0.852, p<0.0001 and r=0.890, p= 2.53e-14, respectively). (F) A working model of the role of TRAF4 in TrkA signaling and prostate cancer cell invasion. In low TRAF4 expressing cells, NGF-induced limited TrkA ubiquitination, resulting in low levels of TrkA kinase signaling. In TRAF4 overexpressing cells, high levels of TRAF4 significantly increase TrkA K27 and K29-linked ubiquitination at the K523, K544 and K547 in the kinase domain upon NGF stimulation. This ubiquitination enhances TrkA kinase activity, its tyrosine phosphorylation levels and the recruitment of downstream adaptor proteins, resulting in hyperactivated TrkA signaling cascade. Consequently, NGF-responsive invasion associated targeted gene transcription are upregulated to promote cell migration and invasion.
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**Table 1. TRAF4 regulates genes involved in cell migration/invasion.** (A) Representative cell migration/invasion related proteins/protein phosphorylation are regulated by TRAF4 in a RPPA study comparing their levels in TRAF4 knockdown and non-targeting control PC3 cells (n=12). The median of the triplicate experimental values (normalized signal intensity) is taken for each sample for statistical analysis using Student's t-test (significant for p<0.05).
Supplemental Figure 1. (A) Knockdown of TRAF4 using specific siRNAs decreased DU145 cell invasion. Left panel, crystal violet staining of invaded cells (n=3). Images were obtained at 100X magnification. Middle panel, quantitation of invaded cells per field. Right panel, the levels of TRAF4 in control or TRAF4 knockdown cells as assessed by qRT-PCR. *P < 0.05 by one way ANOVA with Dunnett's multiple comparisons test. (n=3) (B) PC3 shTRAF4 cells have higher luminescence intensity than shcontrol cells. (C) Comparable levels of Ki-67 staining in shctrl and shTRAF4 tumor samples as determined by IHC analysis. Images were obtained at 100X magnification. Data are represented as the mean ± SEM.
Supplemental Figure 2. (A) Knockdown of TrkA using specific siRNAs decreased PC3 cell invasion (n=3). Images were obtained at 100X magnification. Right panel, the levels of TrkA in control or TrkA knockdown cells as assessed by qRT-PCR. (n=3). *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (B) TrkA knockdown has an inhibitory effect on PC3 cell invasion similar to the effect of TRAF4 knockdown (n=3). Images were obtained at 100X magnification. Right panel, quantitation of TRAF4 or TrkA siRNA knockdown invaded cells per field after invasion. *P < 0.05 by one way ANOVA with Dunnett’s multiple comparisons test. (C) TrkA inhibitor treatment did not change mouse weights. Data are represented as the mean ± SEM. (n=3). *P <0.05 by two tailed Student’s t test.
Supplemental Figure 3. (A) TRAF4 promoted TrkA ubiquitination in vitro. Flag-TrkA was transiently transfected into 293T cells and purified using an anti-flag antibody. The purified protein was then incubated with HA-ubiquitin, UBE1, UbcH5a in the absence or presence of purified TRAF4. The levels of TrkA ubiquitination was determined by immunoprecipitation using an anti-flag antibody followed by Western blot analysis using an anti-HA antibody. (B) TrkA inhibitor, GW441756, did not significantly affect cell growth in PC3 cells for specified dose and time treatment in PC3 cells. (n=3). (C) Lysine residues K523, K544 and K547 (shown in red) are located close proximity to the DFG motif (shown in green) in the kinase activation loop. Show is crystal structure of apo-TrkA kinase domain (PDB ID: 4F0I [44]). (D) Mutation of K523 and K544 to R abolished TRAF4-mediated TrkA activation in vitro. The upper panel shows the in vitro kinase activity of TrkA and its mutants in the absence or presence of TRAF4 overexpression. The bottom panel shows that comparable amount of TrkA proteins were used in the in vitro kinase assay as assessed by the Western blot analysis. Data are represented as the mean ± SEM. (n=3). *P <0.05 by one way ANOVA.

**Figure A:**
- IP: Flag
- WB: HA (HA-Ub)
- Flag-TrkA
- TRAF4

**Figure B:**
- PC3 cells
- 0 µM
- 0.1 µM
- 0.5 µM
- 1.0 µM
- 5 µM
- 10 µM

**Figure C:**
- Apo-TrkA structure (4F0I)
- K523
- K547
- DFG motif

**Figure D:**
- TRAF4
- TrkA
- +
- -
- K523_44R
- K523_44_47R
- Relative kinase activity (%)
Supplemental Figure 4. (A) TRAF4 overexpression did not have a major effect on TrkA cellular distribution. DU145 cells were serum starved and transfected with or without TRAF4 followed by NGF stimulation. Cytosolic and membrane fraction were then isolated and the levels of TrkA and TRAF4 in each fraction were visualized by Western blot analysis using specific antibodies. Right panel, total TrkA level in DU145 whole cell lysates with NGF treatment. Na-K ATPase was used as an internal control for membrane fraction and GAPDH for cytoplasmic and total protein. (B) K523_44R mutation abolished TrkA tyrosine phosphorylation. (C) K547R single mutation did not abolish TrkA tyrosine phosphorylation. (D) TRAF4 overexpression did not alter the interaction between TrkA and SHP-1 phosphatase. Flag-TrkA was transfected into 293T cells with or without TRAF4 co-transfection. Shown is a co-IP experiment using a flag-specific antibody. (E) The levels of p-p38 (pT180/Y182) and p-TrkA (pY785) were significantly reduced in TRAF4 knockdown metastatic tumors compared to tumors derived from control cells. Shown are representative immunohistochemistry images at 40X and 100X (inset) magnification from lung tumors derived from PC3 cell tail-vein injection (n=3 per group). (F) TRAF4 overexpression increased ubiquitination of several receptor tyrosine kinases. Equal amount of cell lysates from GFP (control) or TRAF4 expressing adenovirus infected PC3 cells were incubated with a RTK array. Each duplicate dot represents the ubiquitination level of a target protein recognized by an anti-ubiquitin antibody.
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**Supplemental Table T2**

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**Supplemental Table 1, 2.** RT-qPCR primer sequence and cloning sequence used in the study. 5.1 denotes forward and 3.1 denotes reverse primer.