Tensin-4-Dependent MET Stabilization Is Essential for Survival and Proliferation in Carcinoma Cells

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http://dx.doi.org/10.1016/j.devcel.2014.03.024

SUMMARY

Inappropriate MET tyrosine kinase receptor signaling is detected in almost all types of human cancer and contributes to malignant growth and MET dependency via proliferative and antia apoptotic activities. Independently, Tensin-4 (TNS4) is emerging as a putative oncogene in many cancer types, but the mechanisms of TNS4 oncogenic activity are not well established. Here, we demonstrate that TNS4 directly interacts with phosphorylated MET via the TNS4 SH2-domain to positively regulate cell survival, proliferation, and migration, through increased MET protein stability. In addition, TNS4 interaction with β1-integrin cytoplasmic tail positively regulates β1-integrin stability. Loss of TNS4 or disruption of MET-TNS4 interaction triggers MET trafficking toward the lysosomal compartment that is associated with excessive degradation of MET and triggers MET-addicted carcinoma cell death in vitro and in vivo. Significant correlation between MET and TNS4 expression in human colon carcinoma and ovarian carcinoma suggests TNS4 plays a critical role in MET stability in cancer.

INTRODUCTION

The receptor tyrosine kinase (RTK) MET is activated by its ligand the hepatocyte growth factor (HGF) and is a potent regulator of morphogenesis and migration, during development and in response to tissue injury in the adult (Trusolino et al., 2010). MET activation induced by receptor overexpression, genetic amplification (Holdsworth et al., 1990), mutation (Peschard et al., 2001), or increased HGF secretion (Rong et al., 1994; Straussman et al., 2012) is frequently observed in cancer cells. HGF stimulation results in MET activation and subsequent phosphorylation of key tyrosine residues that regulate the recruitment of adaptor proteins (Trusolino et al., 2010), MET internalization (Peschard et al., 2001), transient endosomal signaling (Kermorgant et al., 2004), and MET receptor trafficking toward either degradation (Hammond et al., 2001) or recycling back to the membrane (Hammond et al., 2003; Parachoniak et al., 2011). In cancer, activating mutations in MET (Joffre et al., 2011) or gain-of-function mutants of p53 (Muller et al., 2013) induce sustained MET recycling, promoting tumorigenesis and invasion. Therefore, a better understanding of the mechanisms regulating MET turnover is critical.

RTK signaling is adhesion dependent under normal conditions, and crosstalk between integrin cell-adhesion receptors and RTKs, including MET, is well established (Ivaska and Heino, 2011; Lai et al., 2009). Tensins, a family of four scaffolding proteins (TNS1, TNS2, TNS3, and TNS4), are emerging as important regulators of cell motility and growth (Qian et al., 2009). Tensins 1–3 link integrins to actin via their PTB domains (Calderwood et al., 2003) and are important components of fibrillar adhesions (Clark et al., 2010; McCleverty et al., 2007). Interestingly, unlike other tensins, TNS4 expression is restricted within normal tissue (Chen et al., 2013; Lo and Lo, 2002). TNS4 promotes cell migration by triggering the uncoupling of integrins from the actin cytoskeleton (Katz et al., 2007) and is emerging as a putative oncogene in many cancer types (Albasri et al., 2009; Katz et al., 2007; Liao et al., 2009; Sakashita et al., 2008; Sasaki et al., 2003a, 2003b). However, the mechanisms underlying the oncogenicity of TNS4 are poorly described. All tensins are known to interact via their SH2 domains with tyrosine-phosphorylated cytoplasmic signaling molecules (Lo, 2007), such as FAK, PI3K, and p130Cas, but the functional relevance of these interactions is not fully elucidated (Cui et al., 2004; Defilippi et al., 2006; Mitra and Schlaepfer, 2006).

Here, we show a direct, tyrosine phosphorylation-dependent interaction between MET and TNS4 that occurs through the TNS4 SH2-domain and inhibits MET endocytosis and subsequent lysosomal degradation. TNS4 also regulates β1-integrin stability, MET-dependent cell migration, proliferation, and survival in vitro and functions as a critical determinant of “MET-addicted tumor” viability in vivo.
**Developmental Cell**

**TNS4 Inhibits MET Endocytosis**

| Gene Name | # of clones | SID   | /functional domains          |
|-----------|-------------|-------|-----------------------------|
| GAB1      | 20          | AA 460-516 | MBD (Met binding domain)    |
| GRB2      | 14          | AA 57-216 | /SH2 and C-terminal SH3     |
| PLCG1     | 19          | AA 616-662 | /SH2                        |
| PIK3R1 variant 1 | 21   | AA 411-498 | /SH2                        |
| TNS3      | 5           | AA 1135-1356 | /SH2                     |
| TNS4      | 4           | AA 416-624 | /SH2                        |

**B**

**Transfection**

**GFP IP**

| Transfection | MET | MET | MET | MET |
|--------------|-----|-----|-----|-----|
| TNS3         | MET | MET | MET | MET |
| TNS4         | MET | MET | MET | MET |

**C**

**Transfection**

**GFP IP**

| Transfection | MET | MET | MET | MET |
|--------------|-----|-----|-----|-----|
| TNS4         | MET | MET | MET | MET |

**D**

**Transfection**

**GFP IP**

| Transfection | TEM | TEM | TEM | TEM |
|--------------|-----|-----|-----|-----|
| TNS3         | TEM | TEM | TEM | TEM |
| TNS4         | TEM | TEM | TEM | TEM |

**E**

**Input 10%**

**GFP IP**

| Blot | MET | MET |
|------|-----|-----|
| TNS4 | MET | MET |

**F**

**TNS4-GFP**

**MET**

**Merge**

| Control | r = 0.11 ± 0.03 | **HGF** |
|---------|-----------------|---------|
|         | r = 0.27 ± 0.04 | **GFP** |

**G**

**Control**

**HGF**

**TNS4**

**Paxillin**

**MET**

**Merge**

(legend on next page)
RESULTS

Tensin Isoforms 3 and 4 Associate with Active MET

Yeast two-hybrid screens using a truncated intracellular version of MET (containing its kinase domain) as bait revealed an interaction between tensin isoforms 3 and 4 and MET (Figure 1A). Several of the well-defined MET-interacting proteins (PI3K, GAB1, and GRB2 protein isoforms) were also identified, thus validating the approach. TNS3-GFP and TNS4-GFP, but not GFP alone, coimmunoprecipitated with overexpressed MET in HEK293 cells (Figure 1B). This interaction did not require HGF stimulation as the transiently overexpressed MET is constitutively phosphorylated in HEK293 cells, due to high expression levels (Figure S1A available online; note that both bands detected in the overexpressing cells represent phosphorylated MET bands). TNS4, unlike TNS3, lacks an actin-binding domain (Figure S1B) and has been suggested to possess oncogenic functions in many cancer types. This notion was further validated by our analysis of publicly accessible microarray data for changes in TNS3 and TNS4 gene expression. In colorectal, lung, ovarian, and gastric cancers, TNS4 was significantly upregulated with concomitant downregulation of TNS3 levels (Figure S1C) compared to normal tissue. Focusing on TNS4, we further confirmed that the association with MET was dependent on MET kinase activity (Figures 1C and 1D). TNS4-GFP coimmunoprecipitated phosphorylated MET, whereas expression of a kinase-dead mutant of MET (MET WT) or wild-type (WT) MET (MET WT) in combination with three different specific MET kinase inhibitors abolished TNS4-MET association. Consistent with the recruitment of TNS4 to MET in a phosphorylation-dependent manner, coimmunoprecipitation of endogenous MET and TNS4 (Figure 1E) was observed in GTL-16 gastric carcinoma cells harboring MET amplification and high levels of constitutively active MET. In A549 lung carcinoma cells, endogenous MET overlapped with TNS3-GFP, confirming their ability to associate as shown above (Figure S1D). Endogenous MET and TNS4-GFP (Figure 1F) localized to the same membrane structures and exhibited a significant increase in colocalization following HGF stimulation (Figure 1F and GFP-transfected control cells Figure S1E). Furthermore, endogenous TNS4 and MET overlapped in paxillin-positive adhesion sites (Figure 1G).

Active MET Binding to TNS4 SH2 Domain Requires the R474 Residue

To better define the TNS4-MET association, we generated a TNS4 C-terminal construct containing only the TNS4 SH2 and PTB domains (Figure 2A) and expressed this as a recombinant glutathione S-transferase (GST) fusion protein (Figure S2A). In pull-downs, a GST-TNS4-SH2-PTB fragment (but not GST alone; Figure S2B) was sufficient to pull down MET from HGF-responsive A549 cells (Figures 2B and 2C) with low basal HGF secretion (Figure S2C) and MET activity and from GTL-16 cells with MET amplification and high levels of the constitutively phosphorylated MET receptor (Figures 2B and 2C). TNS4-MET binding was dependent on MET activity; binding was induced by HGF stimulation in A549 cells and reduced in GTL-16 cells upon MET kinase inhibition (Figure 2C).

Next, we sought to identify the TNS4 MET binding site, which according to our yeast two-hybrid screen resides in the SH2 domain (Figure 1A). SH2 domains that bind phosphotyrosine (pY) motifs include a conserved arginine residue corresponding to arginine-175 in v-Src (Huang et al., 2008). Homology modeling of TNS4 SH2 domain superimposed on the v-Src SH2 domain, identified arginine-474 as a potential putative MET pY binding site (Figure 2D). Consistent with this prediction, substitution of arginine-474 with alanine (R474A), in the context of full-length TNS4-GFP, significantly decreased the MET-TNS4 association in cells coexpressing MET (Figure 2E). Thus, the association between phosphorylated MET and TNS4 depends on R474 in the SH2 domain of TNS4.

The TNS4 SH2 Domain Is Required for Binding MET on Y1313 and the Docking Site

MET recruits binding partners via key pY residues within its cytoplasmic domain (Figure 2F). To test whether the TNS4 SH2 domain directly interacts with MET, and to map the MET pY residue(s) required for TNS4 association (Figure S2D), we employed an in vitro ELISA binding assay (Garcia-Echeverria et al., 1997). Biotinylated MET phosphopeptides encompassing the juxtamembrane Y1003, the kinase domain Y1234-Y1235 (in combination), Y1313, the docking-site Y1349 and Y1356 (separately or in combination in one peptide), and Y1365 were tested for their ability to interact with recombinant GST-TNS4-SH2-PTB. MET peptides containing Y1003 and Y1365 demonstrated only residual binding to GST-TNS4-SH2-PTB. MET peptides encompassing Y1234-Y1235 and Y1349 exhibited intermediate binding, whereas peptides containing Y1313, Y1356, and the combination of Y1349 and Y1356 presented the strongest interaction with GST-TNS4-SH2-PTB (Figures 2F and 2G). We next compared a consensus pY recognition sequence reported for TNS4 SH2 domain binding (Huang et al., 2008) with the MET phosphopeptide sequences and with a previously reported
Figure 2. TNS4 SH2 Domain Interacts with Several Phosphorylated Tyrosines in MET
(A) Schematic representation of recombinant GST fusion constructs containing TNS4 C terminus SH2 and PTB domains.
(B) A549 and GTL-16 cells treated as indicated with HGF (100 ng/ml, 15 min) or PHA (300 nM, 16 hr) and analyzed by western blot.
(C) Pull-down of MET with recombinant GST-TNS4-SH2-PTB from A549 or GTL-16 cell lysates treated as in (B).
(D) Model of TNS4-SH2 domain (blue) superimposed on the published Src SH2 domain crystal structure (yellow) in complex with a phosphotyrosine (pY) peptide (gray). The critical arginine in the Src SH2 domain required for pY-binding is indicated in green, and the predicted corresponding arginine residue in TNS4 (R474) is indicated in red.
(E) GFP-TRAP pull-down in HEK293 cells cotransfected with MET and TNS4-GFP or TNS4-GFP_R474A.
sequence of DLC-1 that also binds TNS4 SH2 domain (Liao et al., 2007). The results of our binding assays, i.e., the interaction affinity between MET and TNS4, correlated with the degree of sequence homology between the MET phosphopeptides and the consensus pY recognition motif reported for TNS4 SH2 domain (Figure 2H).

The TNS4-MET interaction was further validated in the cellular context. Transiently transfected TNS4-GFP associated with TRK-MET-WT (chimeric neurotrophin and MET receptor fusion) but failed to coimmunoprecipitate the TRK-MET mutant (Mut; bearing Y1313F, Y1349F, Y1356F, and Y1365F substitutions) (Figure 2I). The observed residual binding of TNS4 to the mutant receptor may be due to the intact MET kinase domain that is still present within the mutant construct that exhibited intermediate binding to TNS4 in our ELISA assays (Figures 2F and 2G). In correlation with the in vitro binding, phenylalanine substitution of Y1003 (TRK-MET-Y1003F) did not abolish the ability of TNS4-GFP to associate with the MET receptor, whereas mutation of Y1313 (TRK-MET-Y1003F,-Y1313F,-Y1365F) markedly reduced TNS4 binding to MET. Mutagenesis of the docking-site residues (TRK-MET-Y1349F,-Y1356F) also reduced TNS4 interaction (Figure 2J). Taken together, these data suggest that TNS4 binds preferentially to MET via the docking site and the less well-characterized Y1313 residue within MET.

**TNS4, MET, and β1-Integrin Form Complexes**

As TNS4-MET association requires the TNS4 SH2 domain (Figure 2), and as TNS4-β1-integrin interaction is known to occur through TNS4 PTB domain (adjacent to TNS4 SH2) (Calderwood et al., 2003; Katz et al., 2007), we hypothesized that these three proteins may form a complex to regulate cellular functions, such as cell migration. In A549 cells expressing TNS4-GFP, we observed overlap between TNS4-GFP, MET, and β1-integrin staining, particularly in adhesion sites (Figure 3A). In addition, endogenous MET, TNS4, and β1-integrin coimmunoprecipitated in HGF-stimulated A549 cells (Figure 3B) and recombinant GST-TNS4-SH2-PTB associated with both MET and β1-integrin (Figure 3C). The TNS4-β1-integrin complex was not dependent on growth factor stimulation, consistent with the ability of tensins to interact with both phosphorylated and nonphosphorylated β1-integrin tails (Legate and Fassler, 2009). However, as expected HGF stimulation increased the recruitment of MET to TNS4 (Figure 3C).

As both β1-integrin and MET are strongly implicated in cell motility, we tested if TNS4 is involved in cell migration. Analysis of A549 cell migration on cell-derived matrices revealed a modest effect of TNS4 silencing on the basal migration of nonstimulated cells. However, loss of TNS4 fully abolished the HGF-induced cell motility as compared to control HGF-treated cells (Figure 3D; control small interfering RNA [siRNA] cells in Movie S1 and TNS4 siRNA cells in Movie S2). Importantly, reintroduction of an siRNA-resistant TNS4-GFP, and not GFP alone (see Figure 4B for siRNA D efficiency), restored cell migration comparable to levels seen in control cells. Moreover, expression of TNS4-GFP alone was sufficient to increase cell migration in control siRNA-transfected cells (Figure 3E). Importantly, the ability of TNS4 to interact with MET was critical for cell migration as expression of the TNS4 MET-binding mutant (TNS4_R474A-GFP) significantly inhibited basal and more notably HGF-stimulated A549 cell migration (Figure 3F).

**TNS4 Positively Regulates MET Levels and Downstream Signaling**

Unexpectedly, we observed that MET and β1-integrin total protein levels were reduced upon TNS4 silencing (Figure 4A; TNS4 smartpool siRNA [four oligos] and a single independent 3′ UTR RNAi oligo), without significant reduction in the corresponding transcription levels (quantitative real-time PCR measurements; mean Rq values ± SD fold changes: 1.04 ± 0.33 and 1.03 ± 0.45, respectively, for β1 and MET in TNS4- versus control-silenced cells, n = 2), indicative of protein level regulation. TNS4 silencing reduced total β1-integrin levels by 26%–55% (Figure 4A) and cell-surface β1-integrin expression (Figure S3A). In contrast, β1-integrin silencing did not influence TNS4 expression (Figure S3B). Furthermore, this effect was specific to TNS4 as TNS3 downregulation did not markedly affect MET or β1-integrin levels (Figure S3B). The effect of TNS4 silencing on MET expression was even more pronounced. TNS4 smartpool siRNA (four oligos) or a single RNAi oligo (siD) (resulting in a 40%–52% and 41% reduction in TNS4 expression, respectively) led to a significant downregulation of MET protein expression (28% and 59%, respectively as compared to control cells) (Figure 4B). As expected, TNS4 silencing significantly reduced MET cell-surface expression (Figure 4C) and influenced MET downstream signaling. In correlation with decreased MET levels, ligand-induced MET activation (pMET levels, 30 min HGF) was impaired together with a 54% reduction in Akt activation compared to HGF-stimulated control cells (Figure 4D). TNS4-silencing reduced Akt activation also after 1 or 2 hr HGF stimulation (Figure S3C). Interestingly, HGF induced a modest activation of ERK with no significant difference in ERK activity between control and TNS4 siRNA cells (Figure S3C). Hence, these data demonstrate a requirement for TNS4 in the maintenance of β1-integrin and MET protein levels and HGF-induced signaling. However, the ability of TNS4 to support expression of both receptors is unlikely to be related to the formation of...
the MET-β1-integrin-TNS4 complex as β1-integrin silencing had no effect on MET levels (Figure S3B). Careful observation of the data also revealed a possible feedback loop between MET and TNS4 expression. HGF stimulation significantly increased TNS4 levels (approximately 40%) (Figure S3C), with a modest increase already detectable after 30 min (Figure 4D and S3D). Correspondingly, in MET overexpressing GTL-16 cells pharmacological inhibition of MET activity reduced endogenous TNS4 levels by 64% (Figure S3D), suggesting a reciprocal positive regulatory loop between MET and TNS4.

**TNS4 Regulates MET Stability**

Ligand-induced activation of MET and subsequent MET receptor internalization, degradation, and/or recycling is well established (Parachoniak et al., 2011). Recent studies have demonstrated that under basal, non-HGF-induced conditions, several mechanisms may regulate the half-life of the MET receptor independently of ligand binding (Lefebvre et al., 2012). We investigated whether TNS4 could contribute to the steady-state turnover of MET in cells. MET levels remained nearly unaltered in TNS4_WT-GFP-expressing cells treated with cycloheximide but were clearly reduced in GFP cells (Figure 4E). A similar pattern was detected in total MET levels analyzed by western blot (Figure S4A). The ability of TNS4 to stabilize MET was dependent on the TNS4-MET interaction as expression of the MET-binding-defective mutant, TNS4_R474A-GFP, significantly increased MET turnover compared to TNS4_WT-GFP (Figure 4F). In addition, the C terminus of TNS4 was sufficient for MET stabilization as
reintroduction of TNS4-SH2-PTB lacking the RNAi B target sequence was able to rescue cell-surface MET levels in TNS4-silenced cells (Figure 4G).

The difference in protein stability suggested a possible role in receptor trafficking, as ligand-activated MET is predominantly degraded following internalization. Using the well-established cell-surface biotinylation-based endocytosis assay (Mai et al., 2011; Roberts et al., 2001), we observed that in A549 cells, MET was constantly endocytosed, even in the absence of HGF (Figure 4H), most likely due to the low-level basal phosphorylation of MET present in these cells (Figure 2B). Silencing of TNS4 clearly increased MET endocytosis but had no significant effect on basal recycling of endocytosed MET (Figures 4H, S4B, and S4C; note that twice as much input lysate was used for siTNS4 cells to achieve comparable levels of MET immunoprecipitation). This was validated further by the finding that in cells overexpressing TNS4, the basal and HGF-induced endocytosis of MET was slower compared to control GFP cells (Figure 5A). The role of TNS4 in regulating MET internalization was validated further with another endocytosis assay employing antibody labeling and fluorescence-activated cell sorting (FACS) analysis. The data show that TNS4 WT-GFP expression significantly reduced the loss of cell-surface MET compared to GFP cells. Conversely, expression of the MET-binding mutant, TNS4_R474A-GFP, functioned in a dominant-negative fashion (similar to TNS4 siRNA) to augment MET uptake from the cell membrane (Figure 5B). Thus, TNS4 binding to MET functions to reduce MET endocytosis and retain MET on the membrane, both under basal, low MET activity and HGF-stimulated conditions. Recently, GGA3 was shown to be critical for MET stability by regulating MET recycling to the plasma membrane upon HGF stimulation (Parachoniak et al., 2011). In line with these data, we find that GGA3 silencing has no effect on MET levels under basal conditions but significantly increases HGF-induced MET down-regulation. In contrast, TNS4 silencing reduces MET levels, both in control and HGF-stimulated cells (Figures 5C and S4D), further confirming that TNS4 regulates MET traffic at the level of endocytosis and that receptor recycling is regulated by GGA3 as shown previously (Parachoniak et al., 2011).

We also observed a modest reduction in integrin endocytosis in TNS4 WT-GFP-expressing cells under basal conditions (Figure 5D). This could be linked to the obvious cotrafficking of cell-surface-derived active β1-integrin and MET that was observed upon HGF induction (Figure 5E).

**TNS4 Inhibits MET Trafficking toward a Lysosomal Compartment**

Next, we examined the localization of MET in the presence of TNS4 WT-GFP or the binding-incompetent TNS4_R474A-GFP mutant, both transiently expressed with lysosomal marker Lamp1-RFP (Figures 5F and S5A). We observed faster MET trafficking toward the lysosomal compartment in TNS4_R474A mutant-expressing cells compared to TNS4 WT cells, especially following 10 min of ligand stimulation. In line with this, after 30 min, very little MET remained detectable in the TNS4_R474A-expressing cells compared to the much higher MET signal residing in the Lamp1-vesicles of TNS4 WT cells (Figure S5A). The loss in intracellular MET staining upon TNS4 silencing was rescued with bafilomycin, a lysosomal inhibitor (Figure S5B). Interestingly, in control-silenced cells, with lower basal MET endocytosis, bafilomycin clearly increased focal adhesion-type localization of MET at the plasma membrane. However, this phenomenon remains to be investigated in future studies. Taken together, the presence of functional TNS4 protects MET from degradation under basal conditions and affects MET lysosomal targeting upon HGF stimulation.

**TNS4 and MET Expressions Correlate in Colorectal and Ovarian Tumors**

Human colorectal and ovarian cancers exhibit typically high MET expression (Birchmeier et al., 2003; Zhou et al., 2008). In addition, TNS4 has been identified as an oncogene in colorectal cancer (Albasri et al., 2009; Liao et al., 2009). Our data indicate that TNS4 levels correlate with MET protein stability in cell lines. To investigate whether this is applicable in vivo, we stained for TNS4 and MET in human tumor specimens. From a large cohort (Algars et al., 2011) of metastatic or locally advanced colorectal cancers, ten were selected because of their strong MET immunoreactivity in invasive areas and heterogeneous overall staining in the tumor. Independent scoring of staining intensity, in 80 areas of serial histology sections, demonstrated a significant correlation between MET and TNS4 staining within the same regions of the tumor (p < 0.0001) (Figure 6A).

Next, an ovarian tumor tissue microarray containing 196 tumor cores was stained for MET and TNS4 (Figure 6B). Whereas all tumor cores were immunoreactive for TNS4, 17 cases showed no staining for MET. TNS4 showed weak expression in 32 cases, moderate expression in 99 cases, and intense expression in 65 cases. The level of TNS4 and MET immunoreactivity correlated significantly (p < 0.0001) when all tumor cores were studied from adjacent histology slices from the same samples (Figure 6B). After exclusion of benign and borderline tumors from the analysis, the correlation remained significant (p < 0.0001). However, differences between histological subtypes of carcinomas were observed. In high-grade serous carcinomas (n = 49; p < 0.0001) and in mucinous carcinoma (n = 39; p = 0.012), TNS4 and MET staining correlated significantly. In endometrioid cystadenocarcinoma (n = 43; p = 0.13) such a correlation was not seen, suggesting that in some tumor types the regulatory activity of TNS4 on MET expression may be overcome by alternative pathways. Taken together, these data implicate TNS4 as a potential regulator of MET levels in human cancer.

**TNS4 Is Required for MET-Induced Survival In Vitro and In Vivo**

Both HGF/MET signaling and integrin-mediated cell adhesion regulate cell proliferation. By using the IncuCyte-automated incubator-microscope system (Haapa-Paananen et al., 2012), we observed a clear reduction in cell proliferation following TNS4 silencing (Figures 7A and S6A), correlating with reduced MET and β1-integrin levels in TNS4-silenced cells. However, the antiproliferative effect of TNS4 silencing is most likely linked to MET downregulation. Indeed, MET silencing abolished cell proliferation, whereas silencing of β1-integrin had no significant effect (Figures S6A and S6B). The effect of TNS4 silencing on cell proliferation (Figure 7B) was rescued with the concomitant
**DISCUSSION**

The MET interactome has been studied extensively in recent years and many proteins facilitating MET downstream signaling have been identified (Gherardi et al., 2012). In this study, we provide evidence for a previously unidentified direct interaction between MET and TNS4. We further dissect how TNS4 regulates the stability of MET at the protein level, thus promoting MET prosurvival activity both in vitro and in vivo (summarized in Figure 7H).

Although many of the details of HGF-induced MET trafficking are well known (Hammond et al., 2001; Peschard et al., 2001), the precise mechanisms regulating turnover of this receptor in human carcinomas remain unidentified. We find that MET protein stability is critically regulated by the ability of the receptor to couple to the intracellular scaffold protein TNS4, recently described as an oncogene in multiple cancer types. TNS4 expression inhibits MET endocytosis and correlates with increased MET levels and protein stability. Conversely, introduction of a defective MET-binding TNS4 mutant or TNS4 silencing increases MET endocytosis, lysosomal targeting, and degrada-
tion, thus significantly reducing MET levels. Consistent with these in vitro data, examination of human colon and ovarian tumors, where MET overexpression has been correlated with poor outcome (reviewed in Blumenschein et al., 2012), revealed a high degree of conformity between MET and TNS4 immunoreactivity, suggesting coregulation of MET and TNS4 in vivo.

A previous study described a critical role for the intact TNS4 SH2 domain in the developmental process of HGF-induced tubulogenesis. However, the critical molecular interactions mediated by the TNS4 SH2 domain were not investigated in detail in that study (Kwon et al., 2011). Interestingly, we demonstrate that TNS4 interacts with several phosphorylated tyrosines on MET, namely, Y1349 and Y1356, which form the multifunc-
tional docking site (Trusolino et al., 2010) and with the less well-characterized Y1313. As TNS4 plays a fundamental role in MET stabilization, the existence of multiple TNS4 binding sites is not surprising and would potentially allow TNS4 binding even if Y1313 or the docking site are monopolized by other MET-binding downstream effectors.
Our data demonstrate that in addition to increased MET gene expression, frequently detected in cancer (Zou et al., 2007), MET protein levels are controlled by TNS4. Hence, the ability of TNS4 to stabilize MET delineates an additional important level of MET regulation. Interestingly, we find a positive feedback loop between MET signaling and TNS4 through which HGF stimulation increases TNS4 levels and MET inhibition has the opposite effect. These data support the role of HGF in inducing TNS4 in MDCK cells (Kwon et al., 2011). We further observed that TNS4 levels are linked to increased β1-integrin stability and there are several cancer types, including breast cancer, in which elevated integrin expression is linked to poor prognosis (Goodman and Picard, 2012). The ability of TNS4 to regulate the endocytosis of and to stabilize MET and integrins is most likely one of the mechanisms whereby TNS4 mediates oncogenicity.

TNS4 was very recently implicated in stabilization of EGFR following EGF stimulation (Hong et al., 2013) and is transcriptionally regulated by EGFR signaling in mammary epithelial cells (Katz et al., 2007) and by BRAF in KRAS-mutated colorectal cancers (Al-Ghamdi et al., 2011). These data suggest that TNS4 could function not only as a regulator of several RTKs but also as an important node linking distinct oncogenic pathways in cancer cells. Here, we describe TNS4 as an important regulator of MET protein stability and MET-dependent cell survival. It is also possible that TNS4 couples EGFR or BRAF signaling to MET protein stability and MET-dependent cell survival. It is also possible that TNS4 couples EGFR or BRAF signaling to MET stability; however, this remains to be investigated.

In conclusion, our study demonstrates a direct interaction between MET and TNS4 that influences MET stability and regulates survival of MET-dependent carcinomas in vitro and in vivo. TNS4 supports prolonged MET plasma membrane localization, thus sustaining MET oncogenic signaling, and the significant coexpression of MET and TNS4 in ovarian and colon carcinomas suggests that this interaction is of putative clinical importance.

EXPERIMENTAL PROCEDURES

Cell Lines and DNA Constructs

For cell lines, culture conditions, and DNA constructs, see the Supplemental Experimental Procedures.

Antibodies and Reagents

Antibodies, RNAi oligomers, and phosphopeptides used are listed in the Supplemental Experimental Procedures. HGF, NGF, and cycloheximide were purchased from Sigma. PHA665752, SU1174, and bovine plasma fibronectin were purchased from Calbiochem, and INCB28060 was purchased from Selleckchem.

Yeast Two-Hybrid

The yeast two-hybrid screen was performed by Hybrigenics using human MET (amino acids 1003–1376) as bait on a human placenta cDNA library.

DNA and RNAi Transfections

DNA construct and siRNA transfections were carried out using Lipofectamine 2000 (Life Technologies) or HiPerFect (Qiagen) according to the manufacturer’s instructions.

Immunoprecipitation and Western Blotting

See Supplemental Experimental Procedures.

Phosphopeptide Binding Assay

The in vitro ELISA binding assay was adapted from Garcia-Echeverria et al. (1997). For further details and for modeling of TNS4 SH2 domain binding to phosphotyrosine peptide, see the Supplemental Experimental Procedures.

Microscopy and Image Analysis

See the Supplemental Experimental Procedures.

FACS Analysis

Cells were detached with HyQ Tase (HyClone) and fixed with PBS containing 2% PFA for 15 min at room temperature. Cell-surface MET was stained with MET-extracellular antibody (1/100 from R&D) and incubated for 1 hr at 4°C. After washing once with PBS, Alexa Fluor-conjugated secondary antibody was added and incubated for 1 hr at 4°C. Samples were analyzed with FACS array (Becton Dickinson). The mean fluorescent intensity was determined from 10,000 or 20,000 counted events. For the FACS-based endocytosis assay, adherent cells were labeled with nonstimulating extracellular domain-binding MET antibody (1:500, Ebioscience) on ice for 30 min. Unbound antibody was washed away, and the cells were chased in warm medium in the presence or absence of 30 ng/ml HGF. Cells were washed with cold PBS and carefully collected by scraping. Cells were fixed for 10 min at 4°C with 4% PFA, washed, and suspended in PBS followed by Alexa-647 conjugated secondary antibody staining and FACS analysis.

Biotin IP-Based Internalization and Recycling Assay

MET and β1-integrin endocytosis and MET recycling rates were measured using a cell-surface biotinylation-based assay as previously described (Arjonen et al., 2012). Briefly, cells were grown to 80% confluence, placed on ice, and washed once with cold PBS, and cell-surface proteins were labeled with 0.5 mg/ml of EZ-link cleavable sulfo-NHS-Ss-biotin (#21331; Thermo Scientific) in Hanks’ balanced salt solution (HBSS; Sigma) for 30 min at 4°C. Unbound biotin was removed, and cells were washed with cold media and allowed to internalize receptors in prewarmed 10% serum-containing medium at 37°C for the indicated times. Cells were then quickly placed back on ice with the addition of cold media. The remaining biotin at the cell surface was removed with 60 mM MesNa (63705; sodium 2-mercaptoethanesulfonate; Fluka) in MesNa buffer (50 mM Tris-HCl [pH 8.6, 100 mM NaCl) for 30 min at 4°C, followed by quenching with 100 mM iodoacetamide (IAA, Sigma) for 15 min on ice. To detect the total surface biotinylation, one of the cell dishes was left on ice after biotin labeling and did not undergo internalization or recycling.

Figure 5. TNS4-MET Interaction Attenuates MET Internalization and Lyosomal Targeting

(A) Endocytosis of biotinylated cell-surface MET in A549-TNS4_WT-GFP and control A549-GFP cells ± HGF (30 ng/ml) (mean ± SEM band intensity normalized to end point [20 min]; n = 3). Statistical differences at each time point between GFP- and TNS4_WT-GFP-overexpressing cells were analyzed by Student’s t test.

(B) FACS analysis of cell-surface MET endocytosis rate in A549-GFP, A549-TNS4_WT-GFP, or A549-TNS4_R474A-GFP cells ± HGF (30 ng/ml, 30 min) (mean ± SEM fluorescence intensity; n = 3).

(C) Total MET levels in control-, TNS4-, and GGA3-silenced A549 cells treated with DMSO or HGF (30 ng/ml, 1 hr) (mean ± SEM band intensity normalized to control-silenced cells; n = 3). Statistical significance was analyzed between control-silenced cells and the other conditions.

(D) Endocytosis rate of biotinylated cell-surface β1-integrin was measured as described in (A).

(E) MET and active β1-integrin coendocytosis upon HGF induction (30 ng/ml) in A549 cells labeled with cell-surface-bound MET (antibody directed against extracellular domain) and β1-integrin (12G10) antibodies. Scale bar, 10 μm.

(F) A549 cells cotransfected with TNS4_WT-GFP or TNS4_R474A-GFP mutant and Lamp1-RFP were stained for MET (antibody directed against extracellular domain) after HGF (30 ng/ml) induction. r: Pearson’s correlation coefficient between Lamp1-RFP and MET staining in cells (mean ± SEM; n = 7 cells per condition). Scale bar, 10 μm. *p < 0.05, **p < 0.005, ***p < 0.0005.

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MesNa treatment. Cells were then washed with PBS, scraped in lysis buffer (1.5% octylglucoside, 1% NP-40, 0.5% BSA, 1 mM EDTA with phosphatase and protease inhibitor cocktail tablets [Roche]) at 4°C for 20 min. In recycling experiments, following the first MesNa/IAA treatment, the cells were returned to prewarmed 10% serum-containing medium at 37°C for the indicated times and treated again with MesNa/IAA to remove biotin from the cell-surface recycled receptor. All cell extracts were cleared by centrifugation (14,000 x g, 10 min, 4°C), and biotinylated MET or integrin were immunoprecipitated.

Figure 6. TNS4 and MET Are Coexpressed in the Same Areas of Colorectal and Ovarian Epithelial Tumors
(A) MET and TNS4 staining of adjacent colorectal cancer sections from the same tumor (magnification ×20).
(B) MET and TNS4 staining of adjacent sections of ovarian tumor tissue microarray spots (magnification ×10). ***p < 0.0001.
Developmental Cell
TNS4 Inhibits MET Endocytosis

A

A549 cells

B

A549 cells

C

OVCAR3 cells

D

GTL-16 cells

E

GTL-16 cells

F

RNAi Control RNAi TNS4

G

RNAi Control IgG K877 TUNEL

RNAi TNS4 IgG K877 TUNEL

H

+ TNS4

- TNS4

(legend on next page)
from the supernatants with appropriate antibodies and protein G sepharose beads (17-0618-01; GE Healthcare).

Biotinylated internalized MET and β1-integrin receptors and total receptor levels were detected by immunoblotting with horseradish peroxidase (HRP)-conjugated anti-biotin antibody (#7075; Cell Signaling Technology) and receptor-specific antibodies, respectively.

Enhanced chemiluminescence-detected biotin and receptor signals were quantified as integrated densities of protein bands with ImageJ (v. 1.43u), and each biotin signal was normalized to the corresponding receptor and total biotin signal. The endocytosis rate of MET was similarly measured in control- or TNS4-silenced cells.

Animal Studies
Experimental procedures were approved by the Committee for Care and Use of Animals in Experiments at the University of Turku and the State Provincial office of Western Finland (authorization ESLM-2008-08600). TNS4- or control-silenced GTL-16 cells (2.10^6) were subcutaneously injected in one flank of ten athymic nude mice (5–6 weeks old females, Harlan) for each cell type. Mice were sacrificed 12 days after injection. Tumors were removed and fixed in formalin before paraffin inclusion. All tumor sections were stained for hematoxylin and eosin to quantify viable tumoral tissue. For Ki67 and TUNEL staining, tumor sections from three mice in each group were compared.

Immunohistochemical Staining of Human Tumor Samples
Tumor sections were formalin fixed, paraffin embedded, and stained with antibodies against TNS4 and MET using Ventana BenchMark XT (Ventana Medical Systems, Roche Diagnostics). See the Supplemental Experimental Procedures for detailed staining and scoring protocols.

In Vitro Proliferation and Survival Assay
Cells were imaged in an IncuCyteHD (Essen Instruments) automated incubator microscope, and cell confluence was calculated per well by associated software algorithm (Haapa-Paananen et al., 2012). WST-1 in vitro proliferation assay (Roche) was used to measure A549 and OVCAR3 cells proliferation. Apoptosis was measured using DEVD 488 Nucview substrate (1 μM, Essen BioScience) of caspase-3/7. For further details, see the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, one table, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.03.024.

Figure 7. TNS4 Silencing Induces MET Downregulation Associated with Reduced Cell Proliferation and Survival In Vitro and In Vivo
(A) Proliferation of control- or TNS4-silenced (SP) A549 cells analyzed by IncuCyteHD incubator microscope (mean confluence values were compared at 70 hr; n = 5). Corresponding cell lysates were analyzed by western blot.
(B) Proliferation of A549 cells transfected with either control or TNS4 siRNAs (SP or single TNS4 RNAi B) + TNS4-SH2-PTB-GFP rescue construct as analyzed by IncuCyteHD incubator microscope (mean confluence values were compared at 70 hr; n = 3). Western blot shows TNS4 silencing efficiency (lane 1: control-silenced cell extracts; lane 2: TNS4 SP; lane 3: TNS4 RNAi B).
(C) WST-1 proliferation assay of control- or TNS4 (SP)-silenced OVCAR3 cells ± HGF (5 ng/ml) (mean ± SEM; absorbance normalized to day 1 values; n = 3). Statistical significance was analyzed at each time point between untreated control- versus TNS4-silenced cells (nonsignificant) or HGF-stimulated control- versus HGF-treated TNS4-silenced cells at the indicated time point (*p < 0.05). Corresponding cell lysates were analyzed by western blot.
(D) Proliferation of control- or TNS4-silenced GTL-16 cells analyzed with IncuCyteHD (mean confluence values were compared at 70 hr; n = 3). Corresponding cell lysates were analyzed by western blot.
(E) Apoptosis of control- or TNS4-silenced GTL-16 and MKN-45 cells detected using DEVD-488 Nucview Caspase3/7 substrate in IncuCyteFLR incubator microscope. Shown are fluorescent apoptotic cell counts (normalized to cell confluence per well; mean ± SEM; n = 2–3).
(F) Quantification of tumor fluorescence in xenograft sections of transfected GTL-16 cells (n = 10 mice per group). Viable cells are outlined in representative images (magnification x10). *p < 0.05, **p < 0.005, ***p < 0.0001.
(G) Ki67, TUNEL, and IgG (negative control) staining of tumor sections used in (F).
(H) Proposed model: TNS4 interacts with β1-integrin and MET to inhibit internalization and subsequent degradation of both receptors. TNS4-dependent high MET expression correlates with sustained MET signaling and increased cancer cell survival, motility, and proliferation. Suppression of TNS4 expression (right panel) results in increased MET internalization and either subsequent MET recycling back to the cell surface in a HGF-activation-dependent manner (Parachoniak et al., 2011) via the previously described GGAs3 pathway or leads to increased lysosomal degradation associated with decreased MET levels and downstream signaling.

AUTHOR CONTRIBUTIONS
G.M. designed and performed experiments, analyzed data, and wrote the paper; P.S. and T.K. contributed equally; P.S. performed and analyzed the trafficking experiments, and T.K. performed and analyzed the histology staining. N.D.F. performed the sequence analysis and structural modeling. R.K. assisted with the cell-derived matrix experiments. K.C. and D.T. provided research tools, expertise, and help with the protein-interaction studies. The yeast two-hybrid experiments were carried out in D.T.’s laboratory. O.C. designed the experiments related to the clinical material, analyzed data, and contributed to writing the paper. J.J. designed and performed experiments, discussed data, and wrote the paper.

ACKNOWLEDGMENTS
The authors are very grateful for technical assistance from J. Nevo, E. Mattila, L. Lahtinen, J. Silvonen, and P. Laasola. Dr. H. Hamidii is acknowledged for scientific writing, and Professor J. Westermanck is acknowledged for critically reading the manuscript. Professor D. Critchley (University of Leicester) is acknowledged for support in generating Tensin constructs and TNS3 antibody. Dr. G. Serini (University of Torino) is acknowledged for providing Lamp1–RFP construct. K.C. has been funded by the Wellcome Trust. P.S. is funded by the Turku Doctoral program of Molecular Medicine (TuDMM). This study has been supported by the Academy of Finland, an ERC Starting Grant, an ERC Consolidator Grant, the Sigrid Juselius Foundation, and the Finnish Cancer Organizations.

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Tensin-4-Dependent MET Stabilization Is Essential for Survival and Proliferation in Carcinoma Cells

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Supplementary Figures

A

Transfection TNS4 GFP TNS4 MET -

Blot MET pMET Tubulin

Non-phospho-MET peptide

Phospho-MET peptide

Empty MET\(^{\text{K429}}\) MET\(^{\text{Y1245}}\) Empty MET\(^{\text{K429}}\) MET\(^{\text{Y1245}}\)

Blot pMET MET Tubulin

Figure S1

B

ABD = Actin Binding Domain
FAB = Focal Adhesion Binding site (C- or N-terminal)

Tensin3 Phosphatase C2 SH2 PTB

Tensin4

C

Fold Change

Colorectal Lung Ovarian Gastric

D

TNS3-GFP MET Merge

E

Transfection GFP MET Merge

Control

HGF
Figure S1 related to Figure 1.

(A) Control from experiment in Fig. 1B. HEK 293 cells were co-transfected with TNS4-GFP and MET, GFP and MET or TNS4-GFP alone and analyzed as indicated for total MET and pMET levels with Tubulin as loading control. In the right-hand panel cellular extracts of HEK 293 cells overexpressing wild-type (MET<sup>WT</sup>), a kinase dead MET (MET<sup>KD</sup>) or an empty-vector were pre-incubated with a phospho-MET-peptide (competing) or the same peptide in an unphosphorylated form (non-competing) spanning the MET kinase domain and analyzed by western blot for pMET, total MET and Tubulin.

(B) Schematic representation of TNS3 and TNS4 with known functional domains. Adapted from (Lo, 2004).

(C) TNS3 and TNS4 mRNA expression levels in tumor vs normal samples were determined using meta-data analysis Oncomine<sup>TM</sup> Research Edition (Rhodes et al., 2004): colorectal carcinoma, n = 681; lung carcinoma, n = 701; ovarian carcinoma, n = 153; and gastric carcinoma, n = 319. All of the expression data shown are significant relative to expression levels in normal tissue (p < 0.05).

(D) A549 cells transfected with TNS3-GFP were stained for MET (antibody directed against intracellular domain). Shown are representative single channel images and a merge. Scale bar 10 µm.

(E) Control from experiment in Fig. 1F. Control and HGF-treated (30 ng/ml, 15 min) A549 cells transfected with GFP were stained for MET. Shown are inverted single channel images and a merge. Arrowheads indicate MET re-localization from the cell surface to intracellular compartments upon HGF treatment. Scale bar 10 µm.
Figure S2 related to Figure 2.

(A) TNS4-SH2-PTB construct was expressed as a GST fusion protein in the Escherichia coli strain Rosetta BL21DE3 (lane 1). Protein expression was induced at OD600 = 0.5 with 250 µM IPTG overnight at RT (lane 2) and proteins were purified on glutathione-sepharose beads (GSH) at 4°C (lane 3) and blotted with anti-GST antibody.

(B) Pulldown of MET with recombinant GST-TNS4-SH2-PTB from A549 whole cell lysates treated with or without HGF (100 ng/ml, 15min).

(C) HGF secretion in the supernatant of the indicated cell types was measured by ELISA (mean ± SEM concentration; n = 3. RKO cells were used as a positive control hence were out of range of plot area values).

(D) Schematic representation highlighting key MET tyrosines with the sequences of the corresponding phosphopeptides used in the ELISA binding assay as illustrated in Fig. 2F and G.
Figure S3, related to Figure 4.

(A) FACS analysis of cell surface β1-integrin levels in A549 cells silenced for TNS4 (SP or oligo D RNAi, 100 nM).

(B) A549 cells were silenced for TNS4 (SP, 100 nM) TNS3 (SP, 100 nM) and β1-integrin (single oligo 20 nM) or control-silenced ± HGF (30 ng/ml, 15 min).

(C) Western blot analysis of TNS4, MET, pMET, pAkt/Akt, pErk/Erk and loading (Tubulin) from Control- or TNS4-silenced A549 cells treated with DMSO or HGF (30 ng/ml) for the indicated time points (mean ± SEM band intensity normalized to Tubulin and relative to Control-silenced DMSO treated cells at 0 h; n = 3; * p < 0.05, ** p < 0.005, *** p < 0.0005).

(D) Quantification of TNS4 levels from Fig 2B (mean ± SEM band intensity normalized to tubulin and relative to DMSO-treated cells in each type; n = 3; * p < 0.05, ** p < 0.005).
Figure S4, related to Figure 4.

(A) Total MET levels from A549-TNS4-GFP and control A549-GFP cell lysates after cycloheximide treatment (10 µg/ml). Shown is a representative western blot and quantifications (mean ± SEM band intensities, normalized to 0 h time-point; n = 3; * p < 0.05, ** p < 0.005).

(B) Cell lysates from the same experiment shown in Fig 4H were analyzed with western blot for TNS4 silencing efficiency, downregulation of MET, and loading (Tubulin).

(C) Control- or TNS4-silenced A549 cells were surface labelled on ice with Sulfo-NHS-SS-biotin and allowed to endocytose MET for 10 min at 37°C under non-HGF conditions. Remaining cell surface biotin was removed with MesNa at 4°C and receptors allowed to recycle at 37°C for the indicated times in the absence of HGF followed by a second reduction with MesNa. The amount of recycled MET is expressed as the percentage of the endocytosed pool after 10 min (mean ± SEM biotin band intensity normalized to total MET in the IP and relative to 10 min endocytosis in each siRNA-treated sample; n = 3; n.s. non-significant).

(D) Controls for Fig. 5C showing TNS4 (SP, 50 nM) or GGA3 (single oligo, 50 nM) silencing efficiency and the effect on MET levels analyzed by western blot.
Figure S5, related to Figure 5.

(A) HGF treatment (20 and 30 min time points) for trafficking assay shown in Figure 5F.

(B) Representative single plane images of TNS4 or MET in Control- or TNS4-silenced A549 cells (*shows a silenced cell next to a non-silenced cell) treated with DMSO or cycloheximide (5 µg/ml) + Bafilomycin (25 nM) for 2 h. Scale bar 10 µm.
Figure S6, related to Figure 7.

(A) Proliferation of Control-, TNS4- (SP) or MET-silenced A549 cells analyzed with IncuCyteHD™ incubator microscope (mean confluence values were compared at 70 h; n = 3; ** p < 0.001, *** p < 0.0005). Western blot shows TNS4 and MET silencing efficiency and loading (Tubulin).

(B) Proliferation of Control- or β1-integrin-silenced A549 cells analyzed with IncuCyteHD™ incubator microscope. n.s. = not significant.

(C) Western blot analysis of MET phosphorylation and total MET levels in control and 30 ng/ml 15 min HGF stimulated OVCAR3 cells.

(D) Proliferation of Control- or TNS4 (SP)-silenced MET amplified EBC-1 and MKN-45 cells, analyzed with IncuCyteHD™ incubator microscope (mean confluence values were compared at 70 h; n = 3; * p < 0.05). Western blot shows TNS4 silencing efficiency, subsequent MET downregulation and loading (Tubulin).

(E) Control, TNS4- or MET-silenced GTL-16 apoptotic cells were detected using DEVD-488 Nucview™ Caspase3/7 substrate in IncuCyteFLRTM incubator microscope.

(F) MET, TNS4 and pMET immunohistochemistry staining of tumor sections used in Figure 7F (magnification x10).
**Supplemental Experimental Procedures:**

**Cell Lines**
A549 and MKN-45 cells were cultivated in RPMI-1640; HEK 293, GTL-16, MDCK and OVCAR3 in DMEM, 4.5 g glucose; EBC-1 cells in MEM, 25 mM HEPES. All media were supplemented with 10% foetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin (Sigma). A549-GFP, A549-TNS4_WT-GFP and A549-TNS4_R474A-GFP stable cell lines were selected and maintained with G418 after initial GFP-signal-based FACS sorting (Becton-Dickinson). MDCK TRK-MET-WT or TRK-MET–Y13-65F stable cell lines were previously described (Tulasne et al., 1999).

**DNA constructs**
TNS4-GFP construct was generated as previously described for TNS3-GFP (Clark et al., 2010) after inserting SalI-digested sequence into pEGFP-C1 vector cut with XhoI and HindIII enzymes (Clontech). TNS4-GFP-R474→A site-directed point mutation was introduced with Quick Change XL mutagenesis kit (Stratagene). The GST-TNS4-SH2-PTB construct (corresponding to SH2-PTB domains at the C-terminus of TNS4 starting from position 1345) was cloned into pGEX-4T-1 vector (GE Healthcare) following PCR amplification (5’AACCGAATTCTGGTTTAAGCCAAAACATCACCCG3’ containing EcoRI restriction site and 5’AACTCGAGCTACATCCTTTCTGCGTCCTGC3’ containing XhoI restriction site) using TNS4-GFP as a template. TNS4-GFP resistant to RNAi oligo D was generated by introducing silent point mutations (GAG→GAA and CTG→CTC) encoding residues Glu626 and Leu631, respectively. Integrity of all constructs was verified with sequencing. Human MET WT and MET KD constructs (Foveau et al., 2009) and the TRK-MET chimera expressing vectors (Weidner et al., 1995) have been previously described.

**Phosphopeptide binding assay**
The in vitro ELISA binding assay was adapted from (Garcia-Echeverria et al., 1997). Briefly, 96-well Reacti-Bind streptavidin-coated plates (Thermo Scientific) were incubated overnight with biotinylated phosphopeptides (for sequences see Table S1 and Figure S2D) in buffer (Tris-HCl 50mM pH 7.5, DTT 1 mM) at 4°C. Plates were washed three times in TBS-T (Tris-HCL 50 mM pH 7.5, 150 mM NaCl, 0.1% Tween-20), blocked for 15 min at room temperature (RT) in blocking buffer (3% BSA, TBS-T). GST-TNS4-SH2-PTB or control GST constructs (1 µg/mL) were added and incubated for 2 h, at RT. Following three TBS-T washes, plates were incubated with anti-GST antibody (1/30,000 in blocking buffer) and incubated for 1 h, at RT. After three washes, secondary
HRP-conjugated antibody (1/5,000 in 0.1% BSA TBS-T) was added and incubated for 1 h, at RT. Finally, plates were washed three times in TBS-T and bound complexes were detected by a 5 min incubation with TMB substrate kit (Thermo Scientific) and addition of a 2 M sulfuric acid stop solution. Absorbance was read at 450 nm with a Wallac EnVision 2100 multilabel microplate reader (Perkin Elmer).

Modelling of TNS4 SH2 binding to phosphotyrosine peptide
TNS4 SH2 domain (Uniprot access code: Q8IZW8) model was obtained with SWISS-MODEL (Kiefer et al., 2009) server (automated mode; modelled residue range: 22 to 138; template PDB access code: 2KnoA; sequence identity: 56.41%; E-value: 5.20e-37). Superimposition with v-src:phosphotyrosine complex (PDB accession code: 1SHB) was accomplished using Protein3Dfit (Lessel and Schomburg, 1994) (matching Cα: 75; rmsd: 1.065370).

Immunoprecipitation
Cells were lysed in buffer (20 mM Tris-HCL, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1% vol/vol Triton X-100) freshly supplemented with protease and phosphatase inhibitor tablets (Roche), before clearing by centrifugation. Lysates were then either incubated with the indicated antibodies (see Table S1) for 1 h at 4ºC followed by precipitation with protein G Sepharose 4 Fast Flow beads (GE Healthcare) for 1 h at 4ºC or incubated directly with GFP-Trap-A beads (Chromotek) for 1 h at 4ºC. Immunoprecipitated complexes were washed three times in lysis buffer and once in PBS, eluted in reducing Laemmli buffer and denatured for 5 min at 95ºC.

HGF concentration determination
HGF was measured from cell supernatants using Human HGF ELISA Kit (RayBiotech) following manufacturer instruction as described in (Straussman et al., 2012). Briefly, cells were plated for 3 days and allowed to reach 75–90% confluence before medium was collected and filtered. The medium was added to the assay microplate in 1:1 ratio with diluent B, incubated at 4ºC and HGF was detected using a biotinylated primary antibody and streptavidin and absorbance was read at 450 nm.

Western Blotting
Reduced and heat-denatured lysates were separated on 7.5% SDS-PAGE gels by electrophoresis. After transferring onto 0.2 μm nitrocellulose membranes, proteins were detected using specific
primary antibodies (Table S1) associated with appropriate fluorophore-conjugated secondary antibodies. Membranes were scanned with Odyssey (LICOR) imaging system.

**Microscopy and image analysis**

For time-lapse videos, TNS4- or Control-silenced A549 cells were plated on coverslips coated with TIFF-cell-derived matrix ± HGF (10 ng/ml). Phase-contrast images were taken with an inverted widefield microscope (AxioCam MRm camera, EL Plan-Neofluar 20×/0.5 NA objective, pictures taken every 10 min [Carl Zeiss]) equipped with a heated chamber (37 °C) and CO2 controller (4.8 %). For cells overexpressing GFP, TNS4_WT-GFP or TNS4_R474A-GFP (Fig. 3E and 3F) a picture was taken in the 488 channel prior to the acquisition of phase-contrast images (collected every 10 min for 12 h), in order to identify and only track GFP-positive cells. Images were processed and cells were tracked with ImageJ software.

For live-cell trafficking assays (Arjonen et al., 2012) of MET and β1-integrin, A549 cells were incubated for 2 h on ice with medium containing primary and secondary antibodies, and then transferred to 37 °C for 30 min in the presence of HGF (30 ng/mL). Cells were then fixed before confocal microscopy.

For immunofluorescence, cells were fixed in 4 % PFA for 10 min at RT, permeabilised in PBS containing 30% horse serum (HS) and 0.1% Triton X-100 for 10 min at RT, washed in PBS and then blocked in PBS-30% HS for 1 h at RT, before addition of specific primary antibodies (Table S1) overnight at 4°C. Cells were washed three times over 15 min and incubated for 1 h at RT with AlexaFluor-conjugated secondary antibodies (Life Technologies) at a concentration of 1/400. After final washing, cells were mounted with Mowiol containing anti-fading reagent (Vectashield, Vector Labs) for 30 min at 37°C. Immunofluorescence samples were analyzed with an inverted widefield microscope (Carl Zeiss) with a confocal unit, Orca-ER camera (Hamamatsu Photonics), Plan-Neofluar 63× oil/1.4 NA objective (Carl Zeiss), and SlideBook 5.0 imaging software (Intelligent Imaging Innovations, Inc.). Pearson’s correlation coefficients were obtained with ImageJ software.

**Animal Studies**

Experimental procedures were approved by the Committee for Care and Use of Animals in Experiments at the University of Turku and the State Provincial office of Western Finland (authorization ESLM-2008-08600). TNS4- or Control-silenced GTL-16 cells (2.10⁶) were subcutaneously injected in one flank of 10 athymic nude mice (5-6 weeks old females, Harlan) for each cell type. Mice were sacrificed 12 days after injection. Tumors were removed and fixed in formalin before paraffin inclusion. All tumor sections were stained for hematoxylin and eosin to
quantify viable tumor tissue. For Ki67 and TUNEL staining tumor sections from three mice in each group were compared.

**Immunohistochemical staining of human tumor samples**

The collection of tumor specimens was approved by the Turku University Hospital ethics committee. Ovarian tumor tissue microarray (TMA) consisted of 248 tumor cores derived from 124 different tumors. In this collection, benign, borderline and malignant tumors of various histology (serous-, mucinous- and endometrioid) were represented (Myohanen et al., 2012). Colorectal cancer specimens (n = 10) were selected from a cohort (n = 64) of metastatic or locally advanced colorectal cancer cases (Algars et al., 2011).

Paraffin embedded formalin fixed tissue specimens were cut in to 3µm sections and serial sections were stained with antibodies against TNS4 (1/75 mouse mab, Abcam) and MET (rabbit mab, clone SP44, Ventana Medical Systems/Roche Diagnostics). Immunohistochemical staining was performed with Ventana BenchMark XT (Ventana Medical Systems, Roche Diagnostics). Intensity of the immunohistochemical staining was evaluated by two investigators (T.K., O.C.) to semi-quantitatively score tumor cores (ovarian cancer) and tumor areas (colorectal cancer) into 4 different classes: 0 negative, 1 weak, 2 moderate and 3 intense staining. Cores with no tumor cells and unstained cores were excluded. For colorectal cancer scoring, eight areas were selected from each slide based on MET staining intensity (1, 2 or 3). These areas were identified in the TNS4 slides, marked, and each area was separately scored for TNS4 intensity. The TNS4 staining was scored blind, without knowledge of the MET results. Statistical analyses were performed with the SAS Enterprise Guide 4.3 software (SAS institute inc). \( \chi^2 \) or Fisher’s exact test was used to evaluate the association of different categories on tumor staining intensities. Associations were tested with Fisher’s exact test when the requirements of the \( \chi^2 \) test were not achieved.

**Oncomine™ meta-data analysis**

The correlation between TNS4 and TNS3 gene expression in various cancers (colorectal, lung, ovarian and gastric) was established using the microarray meta-data analysis from Oncomine™ Research Edition (Rhodes et al., 2004) with p-value threshold of 0.05 and no threshold for fold-change and gene rank. The analysis was restricted to mRNA arrays and to datasets containing both TNS4 and TNS3 genes. Weighted mean mRNA expression Fold-Change (Tumor/Normal) for each gene was then calculated from the mRNA expression Fold-Change and the number of samples in each dataset. The datasets for each cancer type and their respective no. of samples are as follows: Colorectal Cancer - Gaedcke Colorectal n = 130 (Gaedcke et al., 2010), Sabates-Bellver Colon n = 64 (Sabates-Bellver et al., 2007), TCGA Colorectal n = 237 (TCGA (The Cancer Genome Atlas),
2011), Skrzypczak Colorectal n = 105 (Skrzypczak et al., 2010), Skrzypczak Colorectal 2 n = 40 (Skrzypczak et al., 2010), Kaiser Colon n = 105 (Kaiser et al., 2007); Gastric cancer - D'Errico gastric n = 69 (D'Errico et al., 2009), Cui Gastric n = 160 (Cui et al., 2011), Cho Gastric n = 90 (Cho et al., 2011); Lung Cancer - Hou Lung n = 156 (Hou et al., 2010), Okayama Lung n = 246 (Okayama et al., 2012), Su Lung n = 66 (Su et al., 2007), Wachi Lung n = 10 (Wachi et al., 2005), Landi Lung n = 107 (Landi et al., 2008), Selamat Lung n = 116 (Selamat et al., 2012); Ovarian cancer - Hendrix Ovarian n = 103 (Hendrix et al., 2006), Lu Ovarian n = 50 (Lu et al., 2004).

**In vitro Proliferation and Survival Assay**

Equal numbers of TNS4- or Control-silenced cells were seeded in 6- or 12 -well plates and placed in IncuCyteHD™ (Essen Instruments Inc.) automated incubator microscope. Images were taken every hour (12 and 9 images per well, respectively), and cell confluence was calculated per well by associated software algorithm (Haapa-Paananen et al., 2012). Proliferation curves represent mean confluence values ± SEM of triplicates for each condition normalized to values measured after the cells had adhered to the plates (depicted 0 h).

WST-1 in vitro proliferation assay (Roche) was also used to measure A549 and OVCAR3 cells proliferation. Control- or TNS4-silenced cells were counted and plated in 96-wells plates (0h, 24h, 48h and 72h). WST-1 reagent was directly added to each well following manufacturer instructions, incubated for 1h at 37°C and absorbance was measured at 450 nm.

Apoptosis was measured in IncuCyteFLR™ automated incubator microscope using DEVD 488 Nucview™ substrate (1 µM, Essen BioScience) of caspase-3/7.

### Table S1. Antibodies, RNAi sequences and Peptides used in this study

| Antibodies for immunofluorescence | Antibody                        | Dilution | Source          |
|----------------------------------|---------------------------------|----------|-----------------|
| anti-MET extracellular domain     | 1:100                           | R&D      |
| anti-MET intracellular domain (L41G3) | 1:250                         | Cell Signaling |
| anti-Tensin 4                    | 1:250                           | Abcam    |
| anti-integrin β1 (12G10)         | 1:100                           | Abcam    |
| anti-integrin β1 (9EG7)          | 1:250                           | BD Pharmingen |
| anti-Paxillin                    | 1:100                           | Santa Cruz Biotechnology |

| Antibodies for western blotting  | Antibody              | Dilution | Source      |
|----------------------------------|-----------------------|----------|-------------|
| anti-MET (L41G3)                 | 1:1000                | Cell Signaling |
| anti-phosphorylated MET (Y1234/Y1235) | 1:1000             | Cell Signaling |
| Antibody/Peptide | Dilution | Source |
|------------------|----------|--------|
| anti-Tubulin(12G10) | 1:1000 | Development Studies Hybridoma Bank |
| anti-GFP | 1:1000 | Life Technologies |
| anti-Tensin 4 (M01) | 1:500 | Abnova |
| anti-GST | 1:2000 | Life Technologies |
| anti-TRKA | 1:1000 | R&D |
| anti-integrin β1 tail (EP1041Y) | 1:1000 | Abcam |
| anti-Akt | 1:1000 | Cell Signaling |
| anti-phosphorylated Akt (S473) | 1:1000 | Cell Signaling |
| anti-Erk | 1:1000 | Cell Signaling |
| anti-phosphorylated Erk (Thr202/Tyr204) | 1:1000 | Cell Signaling |
| anti-GGA3 | 1:1000 | BD Transduction Laboratories |
| anti-Biotin/HRP conjugated | 1:1000 | Amersham Biosciences |

### RNAi sequences

| Sequence | Target | Source |
|----------|--------|--------|
| UUCCAAAGCUGGUAUCGUA | TNS4 3'UTR | Thermo Scientific |
| TNS4 SMARTpool | | |
| GAACGUAUGCCACCUCUUU | TNS4 oligo-A | Thermo Scientific |
| GACCUUGACUCCUAAUUG | TNS4 oligo-B | |
| GAUGUCAGCUAUAGUUUG | TNS4 oligo-C | |
| GAGCAGGGCAUCACUCUGA | TNS4 oligo-D | |
| **GGA3** | | |
| Hs_GGA3_1 FlexiTube siRNA | | Qiagen |
| **β1-integrin** | | |
| Hs_ITGB1_9 FlexiTube siRNA | | Qiagen |

### Peptides

| Sequence | Name | Source |
|----------|------|--------|
| EMVSNESVDY^RATF| pY1003 | Life Tein LLC |
| GLARDMYDKEY^S| pY1234-pY1235 | |
| QPEYCPDP^LVEMLK | pY1313 | |
| IFSTFIGEHY^VHVNAT | pY1349 | |
| VHVNAT^VNVK | pY1356 | |
| IFSTFIGEHY^VHVNAT^VNVK | pY1349-pY1356 | |
| VNVKCVAP^PSLLSSE | pY1365 | |
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