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The ROR1 pseudokinase diversifies signaling outputs in MET-addicted cancer cells

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Abstract

MET is a master gene controlling a genetic program driving proliferation, apoptosis protection and invasion. The ROR1 pseudokinase acts as a MET substrate. However, its contribution to MET signaling and MET-dependent biological outcomes remains to be elucidated. By structure-function analysis of ROR1 mutants, we show that ROR1 encompasses two major substrate regions: one is located in the proline-rich domain and is directly phosphorylated by MET; the other resides in the pseudokinase domain and is phosphorylated through intermediate activation of SRC. Differential phosphorylation of these two regions dictates the execution of specific responses: phosphorylation of the ROR1 proline-rich domain by MET—but not phosphorylation of the pseudokinase domain by SRC—is necessary and sufficient to control MET-driven proliferation and protection from apoptosis. Differently, both the proline-rich and the pseudokinase domains mediate cell invasion. Consistent with the role of ROR1 in specifying the functional consequences of MET-dependent signals, ROR1 silencing leads to selective attenuation of only some of the signal transduction pathways sustained by MET. These data enlighten the so far elusive function(s) of pseudokinases and identify a mechanism of biological diversification, based on substrate specificity of oncogenic kinases.

Signals from receptor tyrosine kinases (RTKs) proceed through stereotyped pathways that can be tuned—and diversified—by surface coreceptors acting as adaptor substrates. This mode of information transfer is epitomized by the ERBB RTK network, which comprises four structurally homologous surface proteins (ERBB1–4). Within this family, some receptors (ERBB1, ERBB2 and ERBB4) are catalytically proficient enzymes that can autonomously trigger mitogenic cascades, whereas ERBB3 is a kinase defective receptor that acts only when associated with the other ERBB members. The formation of ERBB/ERBB3 heterodimers and the ensuing transphosphorylation of ERBB3 provide consensus sites for privileged activation of PI3K, which in turn encourages preferential execution of antiapoptotic responses.[1]

Another example of biochemical cross-talk between RTKs and companion substrates, which regulates quality and quantity of signaling outputs, is offered by the MET oncogene product. In this case, MET association with the $\alpha 6\beta 4$ integrin results in tyrosine phosphorylation of the $\beta 4$ cytoplasmic domain and the consequent SH2-mediated recruitment of a number of downstream transducers: Shc and PI3K add on analogous moieties directly associated with MET, thus providing a “mass effect” that sustains the strength and duration of RAS-dependent pathways and fosters cell

proliferation[2, 3]; the Shp2 tyrosine phosphatase mainly mediates antiapoptotic effects *via* intermediate activation of SRC.[4]

Recently, we identified the ROR1 protein as a novel partner of MET that undergoes transphosphorylation as a consequence of MET hyperactivation.[5] In a functional screening of a panel of 43 cancer cell lines derived from various solid tumors, ROR1 was found to be expressed in 32 cell types; however, ROR1 proved to be tyrosine-phosphorylated only in cancer cells that exhibit high-grade amplification, overexpression and constitutive activation of the MET gene and rely on deregulated MET activity for continuous growth and survival ("oncogene addiction"[6]). In these cells, ROR1 transphosphorylation appeared to be essential to sustain MET "addiction," as its genetic silencing impaired the transformed phenotype.[5]

ROR1 encodes a 130 kDa transmembrane protein with an extracellular portion containing an immunoglobulin-like domain, a cysteine-rich domain and a "kringle" domain homologous to the MET ligand hepatocyte growth factor (HGF). The intracellular region features a kinase-like domain and a proline-rich domain, flanked by two similar serine-threonine-rich domains.[7] The intrinsic kinase activity of ROR1 is controversial.[8, 9] The canonical consensus sequence of the putative kinase domain displays six deviations, three of which result in changes of amino acids essential for RTK catalytic activity.[10] Accordingly, we and others observed that immunoprecipitated ROR1 does not incorporate, nor does it transfer to exogenous substrates, radiolabelled phosphate from ATP.[5][11-13]

In this article, we elucidated the function of ROR1 as a MET substrate. By systematic structure-function studies, we mapped the domains of MET required for ROR1 transphosphorylation and the domains of ROR1 that are phosphorylated by MET, as well as the tyrosine residues along the ROR1 cytoplasmic domain that act as major MET-dependent phosphorylation sites. We explored the functional consequences of ROR1 inactivation and linked them to the pattern of ROR1 phosphorylation. Finally, we dissected the signaling pathways specifically contributed by phosphorylated ROR1 using antibody-based phosphoproteomics. Through these combined approaches, we found that ROR1 diversifies MET signals and specifies MET biological outcomes by dedicated activation of specific pathways.

Material and Methods

Cell culture and cellular transfection

COS-7 cells and HS746T cells were purchased from American Type Culture Collection (ATCC); EBC-1 cells were procured from Japan Health Sciences Foundation. These cells were cultured according to the instructions from cell banks using the appropriate medium, supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), penicillin/streptomycin solution (Sigma Aldrich) and 2 mM L-glutamine (Sigma Aldrich). Transient transfection of cell lines was performed using Lipofectamine 2000 reagent (Invitrogen), according to manufacturer's instructions.

Reagents, vectors and antibodies

Lentiviral pLKO.1-puro vectors containing ROR1 shRNA (TRC number: TRCN0000002024), SRC shRNA (TRC number: TRCN0000038149) and Non Targeting shRNA Control (product number: SHC002; referred as NT_shRNA) were purchased from Sigma Aldrich - MISSION TRC shRNA Plasmid DNA.

The lentiviral vector expressing ROR1 cDNA, the constructs encoding for wild-type MET, MET K1110A kinase dead, MET Y1349F/Y1356F, TPR-MET, TPR-juxta-MET, Trk-MET and SRC have been previously described.[5][14-16]

ROR1 mutant constructs were produced by site-directed mutagenesis using QuikChange II XL Site-directed Mutagenesis Kit (Agilent Technologies), according to manufacturer's instructions and all inserted mutations were verified by DNA sequencing.

Each ROR1 tyrosine to phenylalanine mutant was produced using the following primer pairs to introduce a single point mutation at a time (nucleotide mismatch is underlined) on shRNA-untargetable ROR1 cDNA:

Y641F_fw: GGGCTTCCAGAGAAATTTCTCCGCTGATTACTACAG
Y641F_rev: CTGTAGTAATCAGCGGAGAAAATTTCTCTGGAAAGCCC
Y645F_fw: GAAATTTACTCCGCTGATTCTACAGGGTCCAGAG
Y645F_rev: CTCTGGACCCTGTAGAAATCAGCGGAGTAAATTC
Y646F_fw: CTCCGCTGATTACTTCAGGGTCCAGAG
Y646F_rev: CTCTGGACCCTGAAGTAATCAGCGGAG
Y786F_fw: CTCAGTAACCCAGATTTCTAATTACATGTTCCCG
Y786F_rev: CGGGAACATGTAATTAGGAAATCTGGGGTTACTGAG
Y789F_fw: CCCAGATATCCTAATTTTCATGTTCCCGAGCCAG
Y789F_rev: CTGGCTCGGGAACATGAAATTAGGATATCTGGGG
Y822F_fw: CATTCCCATCAATGGATTCCCAATACCTCCTGGATATG
Y822F_rev: CATATCCAGGAGGTATTGGGAATCCATTGATGGGAATG
Y828F_fw: CCAATACCTCCTGGATTTGCAGCGTTTCCAGC
Y828F_rev: GCTGGAAACGCTGCAAATCCAGGAGGTATTGG
Y836F_fw: CCAGCTGCCCACTTCCAGCCAACAGGTC
Y836F_rev: GACCTGTTGGCTGGAAGTGGGCAGCTGG

ROR1 truncated mutants were produced using the following primer pairs to create novel in-frame stop codon (nucleotide mismatch is underlined) in the sequence of shRNA nontargetable ROR1 cDNA corresponding to the end of each intracellular domain:

Δ cyto_fw: GGAATAACCAGAAGTAATCGTCGGCACCAGTCC
 Δ cyto_rev: GGACTGGTGCCGACGATTACTTCTGGTTATTCC
 Δ tail_fw: CGGTCCTGGGAGTGACTCTCAAGTCAC
 Δ tail_rev: GTGACTTGAGAGTCACTCCCAGGACCG
 Δ PRD/ST2_fw: CTCAGTAACCCAGATAACCTAATTACATGTTCCCG
 Δ PRD/ST2_rev: CGGGAACATGTAATTAGGTTATCTGGGGTTACTGAG
 Δ ST2_fw: CCACCTCCCTAAGAGTCGGTCCCCAAG
 Δ ST2_rev: CTTGGGGACCGACTCTTAGGGAGGTGG
 Δ C-term_fw: GCCCTCATCAGGATCCTAATCAGGAAGCAAATATTCC
 Δ C-term_rev: GGAATATTTGCTTCCTGATTAGGATCCTGATGAGGGC

The c-Src inhibitor Saracatinib was purchased from Astra Zeneca.

Apoptotic agents used are: etoposide (Teva) and staurosporine (Sigma Aldrich). Human Phospho-kinase Profiler Array was purchased from R&D Systems, according to the manufacturer's protocol. Quantification of each spot pixel density was performed by densitometric analysis using ImageJ software (<http://rsb.info.nih.gov/ij/>).

Ramig (rabbit anti-mouse IgG + IgM antibody) was purchased from Thermo. Protein G-Sepharose 4 Fast Flow and Protein A-Sepharose were purchased from GE Healthcare. The following primary antibodies were used: goat polyclonal anti-ROR1 (R&D Systems); mouse monoclonal anti-phosphotyrosine (Upstate Biotechnology); rabbit polyclonal anti-phosphoMET Y1234/Y1235 (Cell Signaling Technology); mouse monoclonal anti-MET DQ13 and DL21, produced in our laboratory[17, 18]; rabbit polyclonal anti-MET (c-12, Santa Cruz Biotechnology); rabbit polyclonal anti-SRC (SRC2; Santa Cruz Biotechnology); rabbit polyclonal anti-phosphoSRC Y416 (Cell Signaling Technology); HRP-conjugated anti-spreptavidin (GE Healthcare). The secondary antibodies were HRP-conjugated donkey anti-goat IgGs (Promega); HRP-conjugated anti-mouse and anti-rabbit IgGs (GE Healthcare).

Virus preparation and cell transduction

Lentiviral vectors were produced as previously described.[19] Virus stocks of transfer vectors containing puromycin-resistance cassette were titrated on A549 cells (ATCC) in presence of 1 µg/ml puromycin to estimate the proper dilution to use; for other non selectable transfer vectors, the titration and proper infection capability of each virus preparations was validated by western blot analysis. Concentration of viral particles was also assessed by determination of the viral p24 antigen concentration using the HIV-1 p24 Core profile ELISA kit (Perkin-Elmer). Cells were infected with proper dilutions of virus stocks in the presence of polybrene (8 mg/ml, Sigma), for at least 6 hr.

Western blotting analysis and immunoprecipitation

Protein extracts were obtained by lysing cells in EB buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA and 2 mM EGTA) in the presence of 1 mM sodium orthovanadate, 100 mM sodium fluoride and a mixture of protease inhibitors (pepstatin, leupeptin, aprotinin, STI and phenylmethylsulfonyl fluoride). Extracts were clarified by centrifugation and protein concentration was determined using BCA protein assay reagent kit (Thermo). Equal amounts of total protein extract were resolved by SDS-PAGE on a 8% polyacrylamide gel and transferred to Hybond-C Extra nitrocellulose membrane (Amersham Biosciences). After saturation in PBS-BSA 10%, membranes were immunoblotted using the appropriate antibody (see above) for at least 2 hr. Bound antibodies onto membranes were detected using the appropriated peroxidase conjugated secondary antibodies (see above) and an ECL kit (Promega).

For immunoprecipitation analysis, equal amounts of cellular total protein extract were immunoprecipitated using appropriate antibody adsorbed on Sepharose-protein G or protein A beads. Immunoprecipitated proteins were washed five times with EB buffer and then were eluted using boiling denaturing Laemmli buffer, resolved by SDS-PAGE and analyzed by western blotting using the appropriate primary and secondary antibodies.

Biotinylation assay

Proteins on cell surface were labeled with biotin using the Amersham ECL protein biotinylation module (GE Healthcare), according to manufacturer's instructions. Briefly, cells were washed twice with cold PBS and then labeled with the biotinylation reagent diluted in bicarbonate buffer. After incubation of cell plates on an orbital shaker for 30 min at 4°C, the biotinylation buffer was removed and cells were washed twice with cold PBS. Cells were lysed using EB buffer and cellular extracts were subjected to immunoprecipitation using appropriate antibodies adsorbed on Sepharose beads. Eluted immunoprecipitated proteins were resolved by SDS-PAGE and then analyzed by western blotting using HRP-conjugated antistreptavidin antibody.

Proliferation assay

Cells cultured in complete medium supplemented with 10% serum were plated in 96-well plates (2000 cells/well). After 5 days, cells quantification was performed using the CellTiter-GLO Luminescent Cell Viability Assay (Promega), according to manufacturer's instructions. Cell quantity was determined by measuring the absorbance at 485 nm using Victor X Multilabel Plate Readers (Perkin Elmer). Each experimental point was performed in triplicate.

Apoptosis assays

Cell apoptosis was evaluated by luminescent assay and FACS analysis. Regarding luminescent assay, cells cultured in complete medium supplemented with 10% serum were plated in duplicate 96-well plates (2000 cells/well), to measure apoptosis induction and cellular quantification. After 24 hr, cells were treated with different concentrations of etoposide for 48 hr or control medium. Each experimental point was performed in triplicate. Cell apoptosis was evaluated using Caspase-GLO 3/7 Assay (Promega), according to manufacturer's instructions. Caspase 3/7 activity was detected by measuring the luminescence using Glomax 96 microplate luminometer (Promega). Cellular quantitation was evaluated using the CellTiter-GLO Luminescent Cell Viability Assay (Promega). Relative apoptotic index was determined by normalization of caspase activity data on cell quantification data.

Regarding Annexin-V/PI apoptosis assay by FACS analysis, cells cultured in complete medium supplemented with 10% serum were treated with different concentrations of staurosporine. After 48 hr of treatment at 37°C, cells were collected, washed once with cold PBS and resuspended in binding buffer (Bender MedSystems). After 30 min of incubation at 4°C, cells were spinned, resuspended in binding buffer adding 2.5µl of Annexin-V-APC (Bender MedSystems) and 2 µl of 50 µg/ml PI (propidium iodide; BD Biosciences), vortexed and incubated at RT in the dark for 1 hr. Several apoptotic parameters were simultaneously assessed by FACS analysis, as described.[20]

Invasion assay

To evaluate invasion ability, 5×10^4 cells were seeded on the upper side of a Transwell chamber (Corning), on a porous polycarbonate membrane (8.0-µm pore size) pre-coated with 10 µg of Matrigel (BD Biosciences). The lower chamber of the Transwell was filled with proper complete medium containing 10% FBS. After 16 hr of incubation, cells on the upper side of the filters were mechanically removed and cells migrated to the lower side were fixed with 11% glutaraldehyde and stained with crystal violet. Photographs of each Transwell were done and quantification of migrated cells through the membrane was performed by analysis of each picture, using the ImageJ software (<http://rsb.info.nih.gov/ij/>).

Statistical analysis

Results are means of at least two independent experiments performed in triplicate. Standard deviation or standard error mean (SEM) of each result was reported. The differences of significance among groups were compared using two-tailed Student's *t* test. All statistical analysis were performed using Excel software (Microsoft Office 2010).

Results

MET domains required for ROR1 transphosphorylation

To elucidate how MET transphosphorylates ROR1 we used a number of MET constructs and chimeras bearing amino acid substitutions or lacking functional domains (Fig. 1a). These constructs were exogenously coexpressed in COS-7 cells together with wild-type ROR1 and the tyrosine phosphorylation levels of ROR1 were assessed in each of the cotransfection settings.

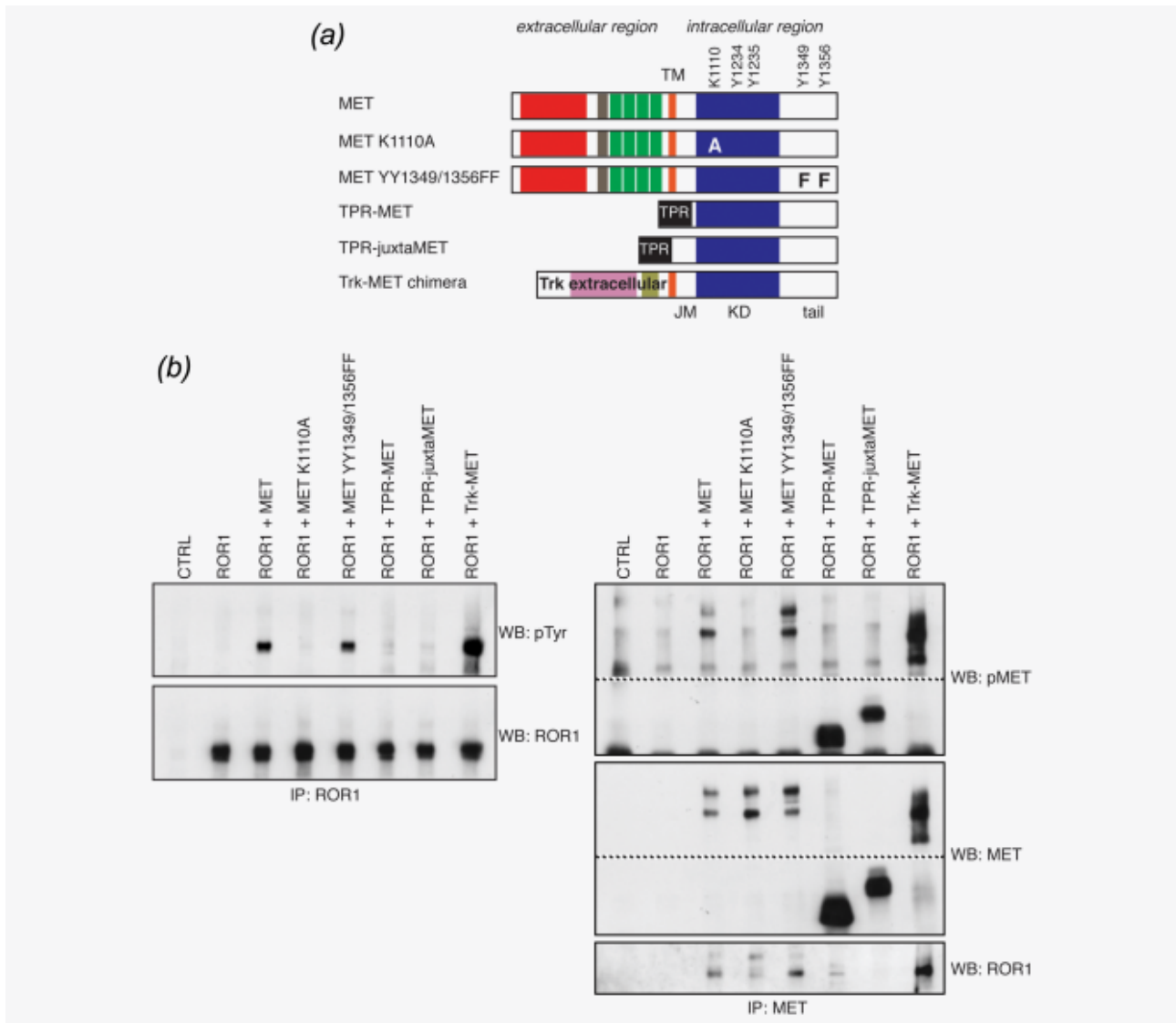


Figure 1. Mapping of MET domains involved in ROR1 transphosphorylation. (a) Schematic representation of wild-type MET and MET mutants and chimeras used in cotransfection experiments with ROR1. (b) Western blot analysis showing phosphorylation and expression of ROR1 (left panel) and MET (right panel) following cotransfection with wild-type MET and MET mutants and chimeras in COS-7 cells. The dashed line indicates cropping of the membrane, which was outsized.

As expected, ROR1 transphosphorylation was induced by wild-type, full-length MET but not by the MET K1110A kinase-dead variant [21-24] (Fig. 1b). The MET mutant Y1349F/Y1356F, which lacks the docking tyrosines for recruitment of downstream transducers, was as active as wild-type MET in phosphorylating ROR1 (Fig. 1b), suggesting that MET-associated signaling intermediates are not required for ROR1 transphosphorylation.

The TPR-MET chimera is a cytosolic oncogenic form of the MET receptor in which the extracellular domain of MET is replaced with TPR sequences, which provide two strong dimerization motifs that mimic the effect of the ligand [25]; this variant was unable to phosphorylate ROR1 despite strong constitutive kinase activity, as displayed by intense autophosphorylation (Fig. 1b). An analogous lack of activity was observed for TPR-juxta-MET, again a catalytically hyperactive cytosolic fusion variant of MET that differs from TPR-MET by containing an extra amino-terminal fragment, corresponding to the cytoplasmic juxta-membrane domain of full-length MET, immediately downstream from TPR (Figs. 1a and 1b). The fact that TPR-MET and TPR-juxta-MET – both exhibiting cytoplasmic localization [14] – were unable to phosphorylate ROR1 indicates that MET and ROR1 need to be localized to the plasma membrane for MET-dependent transphosphorylation of ROR1 to occur.

Finally, ectopic expression of a Trk-MET chimera, composed of the extracellular domain of Trk and the intracellular domain of MET[26], resulted in productive ROR1 phosphorylation (Fig. 1*b*). Collectively, these results show that the transmembrane and cytoplasmic domains of MET are necessary and sufficient to catalyze ROR1 transphosphorylation, whilst the MET extracellular portion is dispensable. It is worth-noting that transphosphorylation implies physical interaction between MET and ROR1; indeed, ROR1 coprecipitated only with MET mutants that were competent for phosphorylation (Fig. 1*b*).

“Primary” ROR1 phosphorylation sites

The intracellular domain of ROR1 contains 19 tyrosines, eight of which are predicted to be phosphorylatable by *in silico* analysis (www.phosphosite.org; <http://www.cbs.dtu.dk/services/NetPhos>).[27] Three tyrosines are located in the pseudokinase domain (Y641, Y645 and Y646) and five in the proline-rich domain (Y786, Y789, Y822, Y828 and Y836). By progressive removal of contiguous sections of the ROR1 cytoplasmic domain, we generated a panel of five deletion mutants (Fig. 2*a*). As assessed by biotinylation assays in transfected COS-7 cells, all these mutants were correctly expressed and exposed at the plasma membrane (Fig. 2*b*).

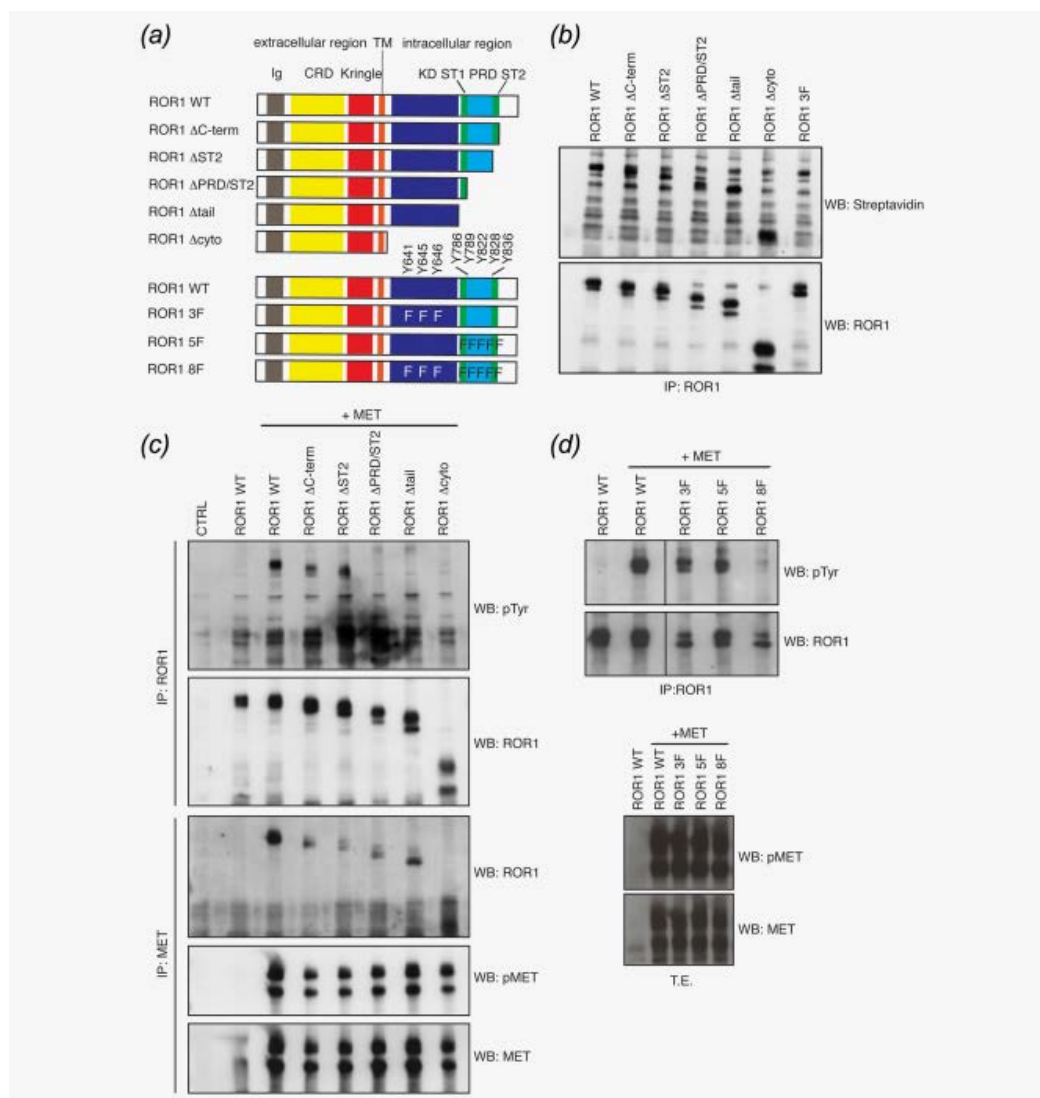


Figure 2. Identification of ROR1 phosphorylation sites. (a) Schematic representation of wild-type ROR1 (WT) and ROR1 mutants used in cotransfection experiments with MET. (b) Biotinylation assay. Wild-type ROR1 and ROR1 mutants were transfected in COS-7 cells and the corresponding proteins were immunoprecipitated to detect their expression at the cell surface. (c) COS-7 cells were transiently transfected with ROR1 wild-type or ROR1 deletion mutants in combination with MET. Western blot analysis showing

phosphorylation and expression of wild-type ROR1 and ROR1 deletion mutants in presence of MET (upper panels). Western blot analysis showing coimmunoprecipitation of wild-type ROR1 and ROR1 deletion mutants with MET (lower panels). Preimmune sera were used as a negative controls (CTRL). The formation of coimmunoprecipitates was evaluated with anti-ROR1 antibody and expression and loading of MET was checked by anti-pMET and anti-MET antibodies. (d) Western blot analysis showing phosphorylation and expression of wild-type ROR1 and ROR1 Y/F point mutants following cotransfection with wild-type MET in COS-7 cells. Phosphorylation and expression of MET were monitored in total extracts (T.E.). The vertical line indicates that lanes were not adjacent in the original blot.

First, we assessed the various ROR1 deletion mutants for their MET-dependent tyrosine phosphorylation content. Among the different variants tested, only two proved to be phosphorylated: ROR1 Δ C-term (lacking the carboxy-terminal tail, from amino acid 876) and ROR1 Δ ST2 (lacking the carboxy-terminal tail and the immediately upstream serine-threonine-rich domain, from amino acid 851). No phosphorylation was found in mutants ROR1 Δ PRD/STD (lacking also the proline-rich domain, from amino acid 785), ROR1 Δ tail (lacking the entire tail including the amino-terminal serine-threonine-rich domain, from amino acid 750) and ROR1 Δ cyto (lacking the whole cytoplasmic domain, from amino acid 433; Fig. 2c). Since lack of tyrosine phosphorylation occurs as soon as the proline-rich domain is removed, we conclude that MET phosphorylates ROR1 on one or more tyrosines embedded in this specific segment.

Then, we exploited such deletion mutants to dissect which domains in the ROR1 moiety are involved in MET binding. Similar to that described for MET variants, ROR1 deletion mutants were exogenously coexpressed in COS-7 cells and their association with wild-type MET was evaluated by coimmunoprecipitation experiments. All ROR1 mutants were found to combine with full-length MET (Fig. 2c). The presence of repeated interaction sites has been already documented for other oligomeric receptor complexes, such as the one formed by MET and Integrin β 4³ and that formed between Neuropilin-1 and Plexin 1.[28]

We then sought to identify which of the five tyrosines included in the ROR1 proline-rich domain are MET-dependent phosphorylation sites. To this aim, we mutagenized them into phenylalanines and coexpressed these mutants with MET in COS-7 cells (Fig. 2a). Surprisingly, however, mutation of all five (5F) was not sufficient to completely abolish tyrosine phosphorylation of ROR1. To achieve complete receptor dephosphorylation, mutation of the additional three tyrosines of the pseudokinase domain (8F) was necessary (Fig. 2d).

“Secondary” ROR1 phosphorylation sites

The above findings indicate that physical removal of the ROR1 proline-rich domain, while preserving the association with MET, fully abrogates MET-dependent transphosphorylation of ROR1; however, in the presence of this domain, phosphorylation of the pseudokinase region at Y641, Y645 and Y646 still occurs. We reasoned that a tyrosine kinase other than MET may interact with the ROR1 proline-rich domain and specifically catalyze phosphorylation of the pseudokinase sequence. We concentrated on SRC due to its well-known signaling cross-talk with MET[29] and because the proline-rich domain of ROR1 is known to be a consensus motif for SH3-mediated recruitment of SRC.[9] In line with this assumption, ROR1, MET and SRC formed a ternary complex when cotransfected in COS-7 cells (Fig. 3a).

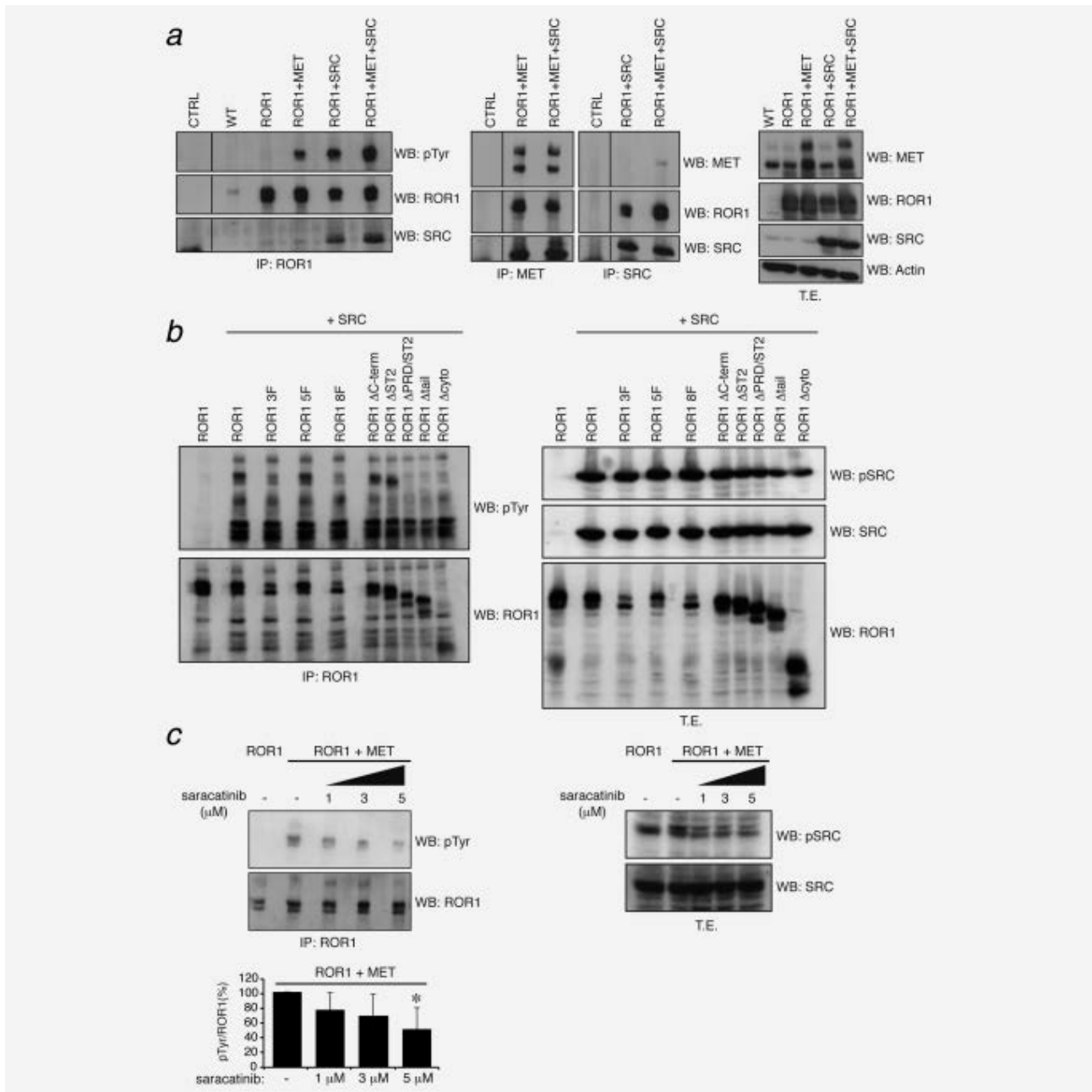


Figure 3. ROR1 indirect transphosphorylation by SRC. (a) Western blot analysis of ROR1-MET-SRC coimmunoprecipitation. COS-7 cells were transfected with ROR1 alone or in combination with MET, SRC or both. ROR1, MET and SRC immunoprecipitates were probed with anti-ROR1, anti-SRC and anti-MET antibodies to prove their interactions. Preimmune sera were used as negative controls (CTRL). Vertical lines indicate that lanes were not adjacent in original blots. Protein inputs are shown as total extracts (T.E.). (b) Western blot analysis showing phosphorylation and expression of wild-type ROR1, ROR1 Y/F point mutants and ROR1 deletion mutants following cotransfection with wild-type SRC in COS-7 cells. Phosphorylation and expression of SRC were monitored in total extracts (T.E.). (c) Western blot analysis showing phosphorylation and expression of ROR1 upon treatment with the SRC inhibitor saracatinib. ROR1 was immunoprecipitated from lysates of COS-7 cells transiently transfected with wild-type ROR1 and MET. Cells were pre-treated with the indicated concentrations of saracatinib for 2 hr. SRC phosphorylation and expression were evaluated in total extracts (T.E.). The western blot is representative of one of three independent experiments. Phospho-ROR1 bands were quantitated by densitometry and normalized against ROR1 total protein expression. The columns show the means of the three independent experiments \pm SEM. The densitometric value for wild-type phospho-ROR1 was set at 100% and the ROR1 mutants were represented as a percentage. * Represents $p < 0.05$ by Student's t test.

Coexpression of active SRC together with wild-type ROR1 in COS-7 cells resulted in ROR1 tyrosine phosphorylation, indicating that ROR1 is a substrate not only for MET but also for SRC. However, different from MET, phosphorylation of

the 5F ROR1 mutant lacking the tyrosines of the proline-rich domain was similar to that of wild-type ROR1, whilst phosphorylation of the 3F mutant lacking the tyrosines of the pseudokinase domain was reduced (Fig. 3*b*). An analogous reduction of phosphorylation was displayed by the 8F “null” variant (Fig. 3*b*). Collectively, these findings point to the pseudokinase domain as the major acceptor of SRC-dependent phosphorylation. SRC was unable to phosphorylate the ROR1 deletion mutant deprived of the proline-rich domain (Fig. 3*b*), consistent with the hypothesis that this domain is required for SRC recruitment and SRC-triggered transphosphorylation. In both ectopically transfected COS-7 cells and in HS746T cells with endogenous expression of MET and ROR1, inactivation of SRC by the SRC-specific small molecule inhibitor saracatinib (AZD0530) or RNAi-based knockdown impaired the overall phosphorylation of ROR1 mediated by MET (Fig. 3*c* and Supporting Information Fig. S1). This reinforces the notion that SRC is a signaling intermediate contributing to ROR1 phosphorylation downstream from MET.

ROR1 sustains different biological responses through the involvement of different kinases

Quenching ROR1 expression by shRNA induces significant growth inhibition of tumors obtained *in vivo* by transplantation of MET ‘addicted’ cells.[5] This impairment of the malignant phenotype may be due to increased cell death, lower proliferation rate and reduction of invasive properties. Cancer cell proliferation, protection from apoptosis and tumor dissemination are all capabilities governed by MET,[30] which suggests that ROR1 inactivation may negatively impact one or more of such MET-driven responses. We next dissected the role of ROR1 in mediating these activities using HS746T cells, which display a MET-addicted phenotype and feature constitutive ROR1 phosphorylation.

ROR1 silencing (Fig. 4*a*) potently sensitized HS746T cells to etoposide-induced apoptosis (Fig. 4*b*). This effect was specific, as it was rescued by transducing a non targetable ROR1 cDNA engineered to contain three silent mutations within the shRNA target sequence (Fig. 4*b*). In gain-of-function experiments, ROR1 overexpression in HS746T cells limited etoposide-mediated cytotoxicity (Fig. 4*b*). We also explored whether differential phosphorylation by MET and SRC could influence ROR1 pro-survival activity. To do this, we transduced wild-type ROR1 or the various ROR1 Y/F mutants in EBC-1, a cell line expressing constitutively active MET but not ROR1⁵ (Fig. 4*c*). Ectopic expression of the wild-type molecule, as well as the 3F mutant lacking the tyrosines of the pseudokinase domain, protected from etoposide-induced apoptosis (Figs. 4*d* and 4*e*). This effect was not retained by the 5F mutant lacking the tyrosines of the proline-rich tail and, as expected, was lost in the 8F “null” mutant (Figs. 4*d* and 4*e*). Similar data were obtained in proliferation assays: again, only exogenous expression of wild-type ROR1 and of the 3F ROR1 mutant increased proliferation of EBC-1 cells, whereas the 5F and 8F mutants were unproductive (Fig. 4*f*). Collectively, these data show that phosphorylation of the proline-rich domain of ROR1, which is likely driven by MET, contributes to protection from apoptosis and induction of proliferation. Conversely, phosphorylation of the pseudokinase domain, which is *bona fide* triggered by SRC, does not contribute to MET-driven mitogenic and survival responses.

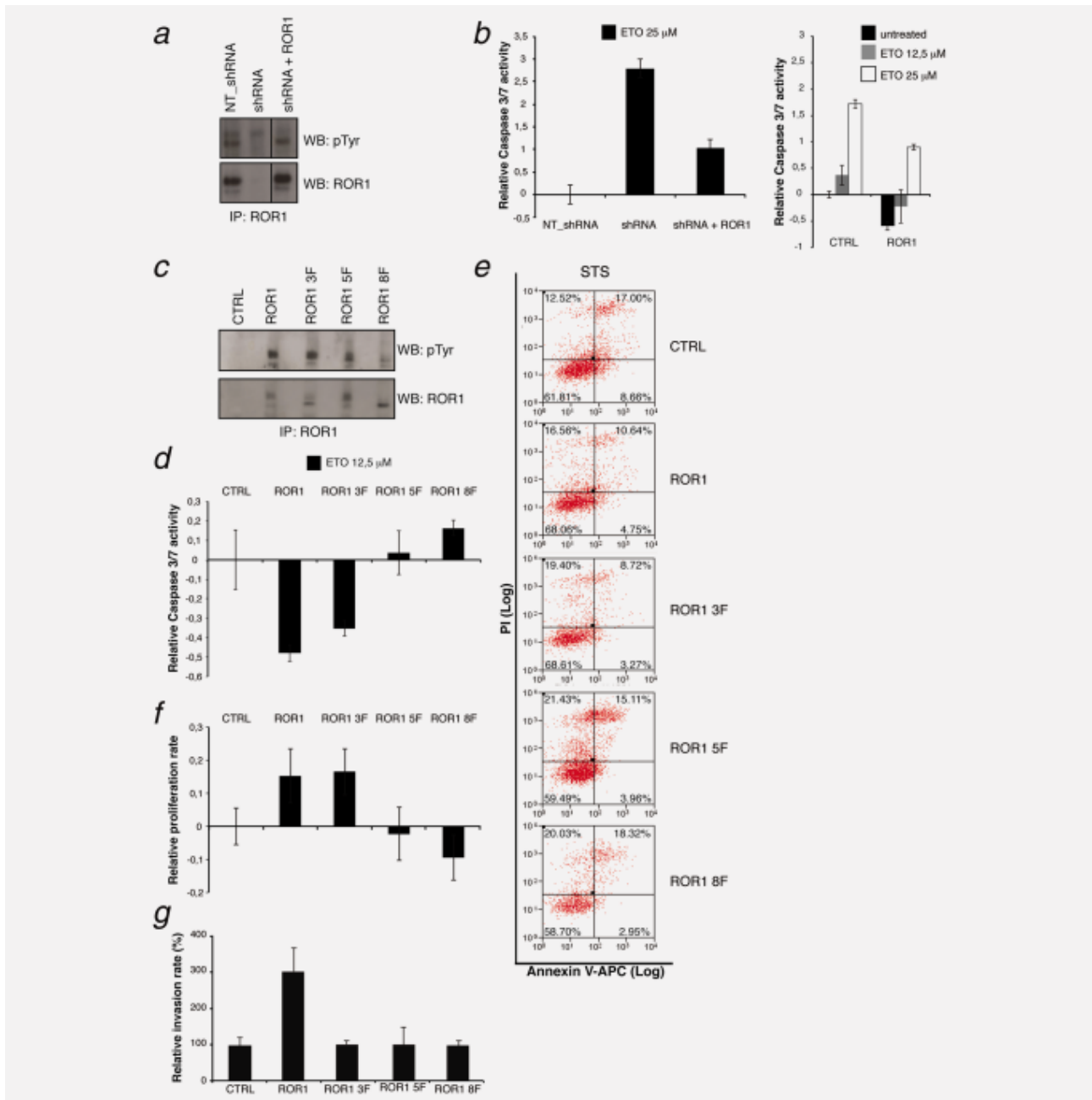


Figure 4. Biological effects of ROR1 mutants *via* different MET- or SRC-dependent pathways. (a) Immunoblot showing ROR1 phosphorylation and expression in HS746T used in the biological assays (the vertical line indicates that lanes were not adjacent in the original blot). (b) Apoptosis assay in HS746T cells. Induction of apoptosis was evaluated by luminometric methods as increased Caspase 3 and Caspase 7 activity in untreated or etoposide (ETO)-treated cells (48 hr) at the indicated concentrations. The graphs show the means \pm SEM from three independent experiments. Left panel: relative levels of apoptosis of HS746T cells transduced with control non targeting shRNA (NT_shRNA) or ROR1 shRNA (shRNA) as well as cells with re-expression of a shRNA non targetable form of ROR1. Right panel: relative levels of apoptosis of control HS746T (CTRL) or cells with ectopic overexpression of wild-type ROR1 (ROR1). (c) Immunoblot showing ROR1 phosphorylation and expression in EBC-1 used in the biological assays. (d) Apoptosis assay in EBC-1 cells. Relative levels of apoptosis in control (CTRL) EBC-1 cells and cells transduced with wild-type ROR1 (ROR1) and ROR1 mutants (ROR1 3F, ROR1 5F and ROR1 8F) upon treatment with 12.5 μ M etoposide (ETO) for 48 hr. Induction of apoptosis was evaluated by luminometric methods as increased Caspase 3 and Caspase 7 activity. The graph shows the means \pm SEM from three independent experiments. (e) Frequency (percentage) of apoptotic EBC-1 cells as assessed by Annexin-V/Propidium Iodide (PI) cytofluorimetric staining. Control (CTRL) EBC-1 cells and cells transduced with wild-type ROR1 (ROR1) or ROR1 mutants (ROR1 3F, ROR1 5F and ROR1 8F) were treated with 200 nM staurosporine (STS) for 16 hr. The percentage of plotted cells is shown in each quadrant of the dot plot diagrams. (f) End-point proliferation assay (5 days) of control (CTRL) EBC-1 cells and cells transduced with wild-type ROR1 (ROR1) or ROR1 mutants (ROR1 3F, ROR1 5F and ROR1 8F). The graph shows

the means \pm SEM from three independent experiments. (g) Invasion assay of control (CTRL) EBC-1 cells and cells transduced with wild-type ROR1 (ROR1) or ROR1 mutants (ROR1 3F, ROR1 5F and ROR1 8F). Cells migrated through the matrigel-coated Transwell filters were quantified, normalized on control cells and expressed as percentages. The graph shows the means \pm SEM from three independent experiments.

Results were different when evaluating invasiveness. In this setting, only wild-type ROR1 was able to enhance the invasive capability of EBC-1 cells. All the other mutants, including the 3F construct, proved to be ineffective (Fig. 4g). Since both the 5F and the 3F mutant were equally impaired in promoting cell motility, it was concluded that ROR1 controls cell invasion only when concomitantly phosphorylated by both MET and SRC. The contribution of SRC to the cell migratory phenotype was confirmed by treating HS746T cells with saracatinib; indeed, SRC inhibition impaired cell invasion without affecting cell proliferation (Supporting Information Fig. S2).

ROR1 silencing affects STAT and SRC-like kinase signaling

The observation that genetic inactivation of ROR1 impairs MET-dependent responses prompted us to analyze the signaling consequences of ROR1 knockdown in HS746T cells with antibody-based phosphoproteomics. We exploited phospho-kinase arrays for medium-scale assessment of a panel of phosphoproteins that include MET and SRC downstream transducers (the MEK/ERK cascade, the AKT/TOR pathway, STATs), SRC-like kinases (Src, Lyn, Lck, Fin, Yes, Hck), focal adhesion kinases and substrates (FAK, Pyk2 and paxillin) and apoptotic effectors (JNK, p38).

Computer-assisted densitometric analysis of protein spots revealed changes in the phosphorylation levels of most of the signals interrogated (Fig. 5a and 5b). In particular, when considering only signal modulations above or below a 20% threshold, we detected increased activation of the MAPKs of the JNK and p38 families as well as higher phosphorylation of the JNK canonical substrate c-Jun. Stimulation of the JNK and p38 MAPK pathways as a consequence of ROR1 silencing likely regulates sensitization to apoptosis, in line with the well-established role of JNK and p38 in transducing stress-induced death signals.[31]

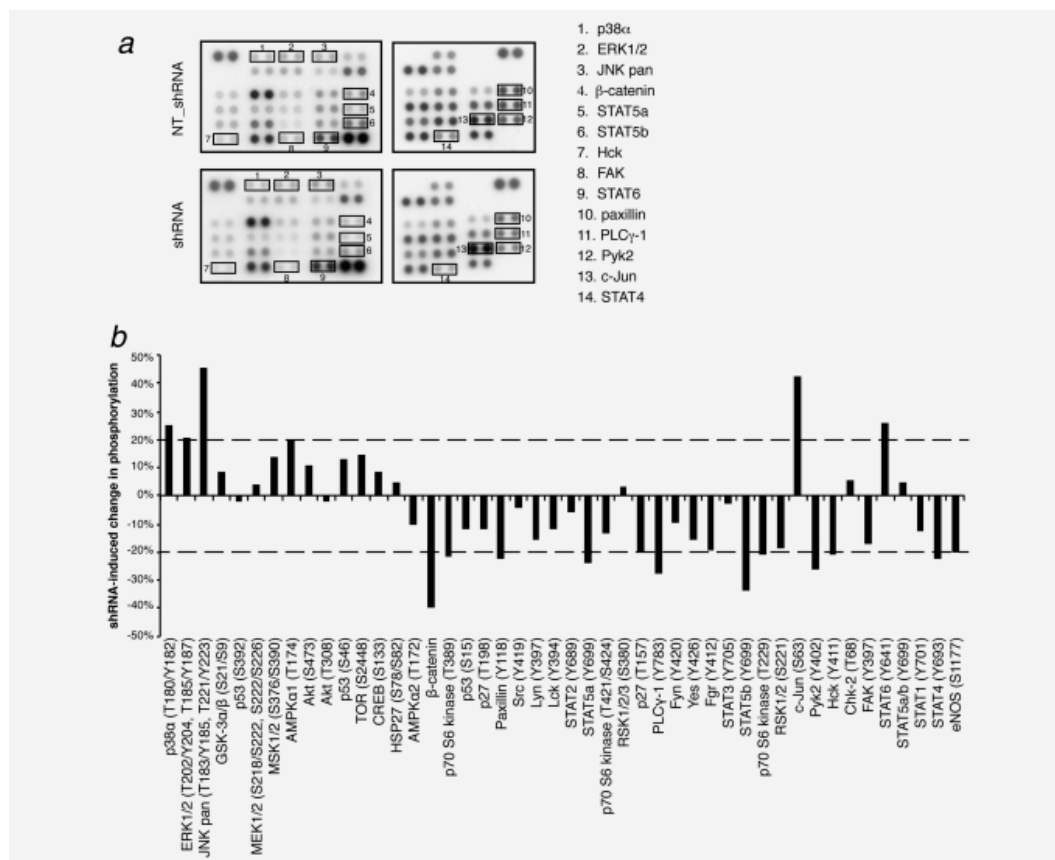


Figure 5. ROR1 modulates multiple signaling nodes. (a) Modulation of phosphorylation levels of 46 signaling proteins upon ROR1 knockdown in HS746T cells. Whole-cell lysates of cells transduced with non targeting shRNA (NT_shRNA) or ROR1 shRNA (shRNA) were subjected to human “phospho-kinase profiler array” and representative array images are presented. Rectangles indicate higher modulated proteins. (b) Densitometric analysis of “phospho-kinase profiler array” images represented in (a). The graph shows the relative shRNA/NT_shRNA values for each tested phosphoprotein, calculated as means of two spots for each antibody. The dashed line indicates the threshold of signal modulation, which was considered meaningful. A map of the phospho-kinase profiler array is shown in Supporting Information Fig. S3.

In the opposite direction, the major signal downmodulation was reduced phosphorylation of STAT5b and, to a lesser extent, of other STAT family members including STAT5a and STAT4 (but not STAT3 and STAT6). Notably, STAT5a/b is a substrate of SRC, while STAT3 is a substrate of MET.[32, 33] The fact that STAT5a/b was so extensively inhibited by ROR1 ablation whilst STAT3 exhibited scant changes suggests preferential involvement of SRC in encouraging STAT pathway activity in MET-addicted cancer cells. The contribution of SRC to MET/ROR1 cross-talk is further supported by the general reduction in the phosphorylation levels of many SRC-like kinases—including Lyn, Lck, Fyn, Yes and Hck—and by deactivation of phospholipase C- γ , a canonical downstream transducer of SRC.[34]

ROR1 silencing also negatively impacted the phosphorylation levels of several proinvasive signals—including FAK, the FAK-related kinase Pyk2 and the FAK and SRC substrate paxillin—and led to decreased expression of β -catenin. This overall deactivation of functional and structural molecules implicated in cell migration corroborates the contribution of ROR1 to MET-driven invasive growth.

Discussion

Cancer cells often displays phosphorylated pseudokinases whose role in the malignant phenotype is still obscure.[35] Because the repertoire of intracellular transduction pathways is limited, RTKs usually increase the diversity of biochemical outputs by installing signaling dialogues with other kinases. Such degenerate connectivities are further secured by interactions between RTKs and enzymatically mute substrate adaptors that, when phosphorylated, can tune the number and nature of signaling effectors and therefore modulate biological responses.[36, 37] Here, we report the mechanistic dissection of a novel functional unit composed of the MET RTK and the ROR1 pseudokinase receptor. Based on new experimental findings and consolidated data from us and others, we propose a two-step model of MET-ROR1 cross-talk. First, MET directly phosphorylates ROR1 on tyrosines of the intracellular proline-rich domain. This modification is a priming event that likely facilitates accessibility of tyrosines of the pseudokinase region for phosphorylation by SRC, which is activated by MET[16] and participates to the MET-ROR1 complex by SH3-mediated interaction with the ROR1 poly-proline sequence[9] (Supporting Information Fig. S4). The functional interaction between SRC and ROR1 is further supported by the genetic silencing of ROR1, resulting in reduced activation of several SRC-like kinases. Of note, ROR1 appears to be basally tyrosine-phosphorylated in cancer cell lines featuring high-grade MET gene amplification and constitutive MET kinase hyperactivation, but not in normal or cancer cells with physiological levels of MET, not even after HGF stimulation (Ref. 5 and our unpublished results). This suggests that the role of ROR1 as a MET substrate becomes operational only in conditions where MET activity is chronically fostered by genetic abnormalities.

The contribution of SRC to ROR1 phosphorylation is probably a “class effect”: indeed, SRC has been demonstrated to phosphorylate the structurally homologous ROR2 receptor upon Wnt5A stimulation.[38] More in general, the role of SRC as a signal intermediate between tyrosine kinases and substrates (and vice versa) is now well established. For example, activation of SRC by MET leads to SRC-mediated transphosphorylation of EGFR, which generates docking sites for EGFR interactors involved in downstream signaling. By this mechanism, the EGFR scaffolding function is preserved even when catalytic activity is abrogated, which is exploited by cancer cells to foster compensatory pathways in the presence of EGFR inhibitors.[39] Similarly, activation of SRC by the β 4 integrin results in transphosphorylation of ERBB2, which potentiates ERBB2 signaling capacity and exacerbates progression of mammary tumors.[40]

MET is a key mediator of “invasive growth,” a complex program in which cell proliferation combines with cell-cell dissociation and movement, matrix degradation and protection from apoptosis during organ development and regeneration and during tumor progression.[30, 41] ROR1 contributes to the whole program, as its silencing impairs mitogenesis, motogenesis and survival in MET-dependent cancer cell lines. Accordingly, ROR1 has been demonstrated to sustain the tumorigenic phenotype of B cell hematologic malignancies[42-47] and to promote epithelial-mesenchymal transition in breast cancer cells.[48] A major finding of this study is that the molecular anatomy of ROR1 appears to dictate the execution of specific biological outcomes: phosphorylation of the ROR1 proline-rich domain by MET—but not phosphorylation of the pseudokinase domain by SRC—is necessary and sufficient to control cell proliferation and protection from apoptosis. Differently, both the proline-rich and the pseudokinase domains mediate cell invasion. The fact that the pseudokinase domain, which only mediates ROR1 proinvasive activity, is a privileged SRC substrate is coherent with the well-documented role of SRC/FAK signaling in tumor invasion through cytoskeletal modulation of cell-cell and cell-matrix adhesion[49] and is consistent with the finding that ROR1 knockdown is accompanied by decreased expression of β -catenin and reduced phosphorylation of FAK and paxillin.

We show here that silencing of ROR1 in MET-addicted cancer cells attenuates a limited complement of signaling pathways, all revolving around the STAT axis and—not surprisingly—SRC. It is worth noting that MET inhibition in the same context decreases the activity of signals that are different from and complementary to those affected by ROR1 inactivation—including the RAS-RAF cascade and the PI3K-AKT pathway—but it does not intercept STATs and SRC.[50] It is tempting to speculate that the MET addicted phenotype is sustained by a dominant node of “high impact” signals, under the direct control of MET and by a more nuanced repertoire of transducers with ancillary functions, which are regulated by ROR1 and further tuned by ROR1 differential phosphorylation by MET or SRC.

In conclusion, we demonstrate that ROR1 modulates the MET signaling apparatus with the participation of the SRC kinase and through two substrate regions that specify distinct biological outcomes. How differential phosphorylation of the two regions by MET and SRC impinges on differential activation of downstream pathways remains ground for future mechanistic studies. At the translational level, the present data offer immediate insight into new therapeutic opportunities for more effective neutralization of oncogenic signals in those tumors that rely on MET hyperactivation for growth, survival and dissemination.

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