Ror1 is a pseudokinase that is crucial for Met-driven tumorigenesis

UNIVERSITÀ DEGLI STUDI DI TORINO

Ror1 Is a Pseudokinase That Is Crucial for Met-Driven Tumorigenesis.

Alessandra Gentile, Luca Lazzari, Silvia Benvenuti, Livio Trusolino, and Paolo M. Comoglio.

Author Affiliations

Authors' Affiliations: 1Exploratory Research Laboratory and 2Molecular Pharmacology Laboratory, Institute for Cancer Research and Treatment, University of Turin Medical School, Candiolo, Turin, Italy

Corresponding Author:

Paolo M. Comoglio or Livio Trusolino, Institute for Cancer Research and Treatment, University of Turin, 10060 Candiolo, Turin, Italy. Phone: 39-011-9933601; Fax: 39-011-9621525; E-mail: pcomoglio@gmail.com or livio.trusolino@ircc.it

Abstract

The human kinome includes Ror1, a poorly characterized orphan receptor. Here we report the findings of an investigation of Ror1 contributions to cancer, undertaken through an integrated screening of 43 cancer cell lines where we measured protein expression, tyrosine phosphorylation, and growth response following RNAi-mediated Ror1 suppression. Ror1 was expressed in approximately 75% of the cancer cell lines without apparent histotype distribution. Gastric carcinoma cells (HS746T) and non–small cell lung carcinoma cells (NCI-H1993) exhibited high levels of Ror1 tyrosine phosphorylation, and Ror1 suppression caused growth inhibition. Biochemical assays revealed unexpectedly that Ror1 is a pseudokinase that is devoid of catalytic activity. Intriguingly, the two cell lines featuring tyrosine-phosphorylated Ror1 both exhibited amplification and activation of the Met oncogene. Ror1 phosphorylation was abrogated by Met inhibition, indicating Met-dependent transphosphorylation of Ror1. Conversely, Ror1 was not transphosphorylated by other constitutively active tyrosine kinases, including EGFR and ErbB2. Constitutive silencing of Ror1 in HS746T and NCI-H1993 carcinoma cells impaired proliferation in vitro and induced a dramatic inhibition of tumorigenesis in vivo. Together, our findings suggest a critical role for Ror1 in malignant phenotypes sustained by the Met oncogene. Cancer Res; 71(8); 3132–41. ©2011 AACR.

1. Introduction

Receptor tyrosine kinases (RTK) are transmembrane proteins with ligand-controlled intracellular kinase activity. They play central roles in several cellular processes as diverse as differentiation, proliferation, migration, angiogenesis, survival, and communication between cells. It has been largely shown that deregulation of RTKs (due to gene amplification, mutations, transcriptional overexpression, or autocrine stimulation) is causally linked to the initiation and progression of human cancers (1). Ror1 belongs to the evolutionarily conserved RTK family of Ror, which also includes Ror2 (2). The 2 receptors were originally identified by PCR cloning in a human neuroblastoma cell line (3). For a long time, their ligands remained elusive and both receptors were catalogued as “orphans.” It is now established that Wnt5A acts a ligand for Ror2 (4, 5), whereas the Ror1 ligand remains unknown.

The Ror1 extracellular region contains an immunoglobulin domain, a cysteine-rich domain, and a kringle domain; the intracellular region includes several tyrosines, a putative tyrosine kinase domain, and a proline-rich stretch flanked by 2 serine-threonine–rich domains (2). The tyrosine kinase domain of Ror1 is similar to that of Trk and MuSK; however, several key amino acids differ from the canonical consensus sequence of active kinases, shedding doubts on the actual enzymatic function of the receptor.

On the physiologic ground, the Ror1 protein plays essential roles during mouse development (6); it is expressed in the face, limbs, heart, and lungs. Ror1 knockout mice are viable, but exhibit respiratory defects and die within 24 hours after birth. In humans, Ror1 expression is prevalent in heart, lung, and kidney (7). The role of Ror1 in disease is still obscure; mutations in the Ror1 gene have not been linked to any pathologic condition, and only recently Ror1 has been found overexpressed in a subset of chronic lymphocytic leukemias (8–10). To get insight into the potential role of Ror1 in solid

human cancers, we undertook an RNA interference (RNAi) screen to analyze the effects of Ror1 silencing on cell growth. Unexpectedly, we found that Ror1 is a pseudokinase acting as a substrate for the oncogenic tyrosine kinase Met; by this function, Ror1 sustains the Met-driven transformed phenotype.

2. Material and methods

Cell cultures and cellular transfection

Cell lines were obtained from American Type Culture Collection, National Cancer Institute Division of Cancer Treatment and Diagnosis Tumor/Cell line Repository (NCI-Frederick Cancer Research and Development Center), or Japan Health Sciences Foundation, and cultured according to the instructions from cell banks by using the appropriate medium, 10% FBS (Sigma Aldrich), penicillin/streptomycin solution (Sigma Aldrich), and 2 mmol/L L-glutamine (Sigma Aldrich). Transient transfection of cell lines was carried out by using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions.

Reagents, vectors, and antibodies

Lentiviral shRNA_A, shRNA_B, and nontargeting short hairpin RNA (NT_shRNA) are pLKO.1-puro vectors from MISSION TRC shRNA Plasmid DNA (product number TRCN0000002024, TRCN0000002025, and SHC002, respectively). shMET_A has already been described (11); shMET_B is a pGIPZ lentiviral vector from Open Biosystems (product number V2LHS_76544). Human full-length Ror1 cDNA (NM_005012.1) was purchased from Origene and was subsequently cloned in the pRRL2 lentiviral vector (12). Ror1 cDNA insensitive to shRNA_A was produced by insertion of 3 point mutations (A2757G, C2769G, and T2772C) by QuikChange II XL Site-directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions.

The following primers were used (nucleotide mismatch is underlined) for subsequent complete cycles of mutagenesis:

Mut1_fw: CAAAGCAAGCATCTTTGCTAGGAGACGCCAATATTC

Mut1_rev: GAATATTGGCGTCTCCTAGCAAAGATGCTTGCTTTG

Mut2_fw: CAAAGCAAGCATCTTTGCTAGGAGACGCGAACATTCATGGACAC

Mut2_rev: GTGTCCATGAATGTTGGCGTCTCCTAGCAAAGATGCTTGCTTTG

Mut3_fw: GCATCTTTGCTAGGAGACGCGAACATTCATGGACAC

Mut3_rev: GTGTCCATGAATGTTCGCGTCTCCTAGCAAAGATGC

All mutations were verified by DNA sequencing.

The Met inhibitors were from Tocris Bioscience (PHA-665752) and Ortho-Biotech (JNJ-38877605); stock solutions of the drugs were prepared in dimethylsulfoxide and stored at −20°C. Primary antibodies were goat polyclonal anti-Ror1 (R&D Systems); mouse monoclonal anti–phospho-tyrosine (anti-pTyr; Upstate Biotechnology); rabbit polyclonal anti–phospho-Met Y1234/Y1235 (Cell Signaling Technology); mouse monoclonal anti-Met DQ13 and DL21, produced in our laboratory (13, 14); rabbit polyclonal anti-Met (c-12; Santa Cruz Biotechnology); mouse monoclonal anti-EGFR (Upstate Biotechnology); monoclonal trastuzumab anti-ErbB2 (Roche); goat polyclonal anti–α-actin (I-19; Santa Cruz Biotechnology). Secondary antibodies were horseradish peroxidase (HRP)-conjugated rabbit anti-goat immunoglobulin G (IgG; Dako); HRP-conjugated anti-mouse and anti-rabbit IgGs (GE Healthcare Bio-Sciences).

RNA extraction and real-time PCR

RNA was extracted by using RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. cDNA was retrotranscribed by using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), following the manufacturer's instructions.

Quantitative real-time PCR with Sybr Green assay (Applera, Applied Biosystems) was used to measure the relative amount of Ror1 cDNA with respect to the amount of a housekeeping gene (PGK). Primers were designed by the Primer Express software (Applied Biosystems):

Ror1_fw: TGCCAGCCCAGTGAGTAATCT

Ror1_rev: GCCAATGAAACCAGCAATCTG

PGK_fw: CTTATGAGCCACCTAGGCCG

PGK_rev: CATCCTTGCCCAGCAGAGAT

PCR reactions were carried out by a 7900HT sequence detection system (Applera), according to standard protocols.

Virus preparation and cell transduction

Lentiviral vectors were produced as described (15). Concentration of viral particles was assessed by determination of the viral p24 antigen concentration by using the HIV-1 p24 Core profile ELISA kit (Perkin-Elmer Life Science). Cells were infected with proper dilutions (1:10 or 1:20) of virus stocks in the presence of polybrene (8 mg/mL, Sigma), for at least 6 hours.

Western blotting analysis and immunoprecipitation

Total cellular proteins were extracted by solubilizing the cells in boiling Laemmli Buffer (50 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; and 1% SDS). Immunoprecipitation was carried out following cell lysis in an Extraction Buffer containing 50 mmol/L Hepes (pH 7.4), 5 mmol/L EDTA, 2 mmol/L EGTA, 150 mmol/L NaCl, 10% glycerol, and 1% Triton X-100 in the presence of protease and phosphatase inhibitors. Extracts were clarified at 12,000 rpm for 15 minutes, normalized with the BCA Protein Assay Reagent kit (Thermo), and incubated with different monoclonal antibodies for 2 hours at 4°C. Immune complexes were collected with either protein G-Sepharose or protein A-Sepharose, washed in lysis buffer, and eluted. Extracts were electrophoresed on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond; GE Healthcare). Nitrocellulose-bound antibodies were detected by HRP-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

In vitro kinase assay

For in vitro kinase assays, Ror1, ErbB2, and ErbB3 proteins were produced by transient transfection of the corresponding cDNA plasmids in COS-7 cells, extracted in Extraction Buffer and then immunoprecipitated as described earlier in the text. Immunoprecipitates were washed twice with Extraction Buffer and 3 times with kinase buffer (20 mmol/L Hepes, pH 7.4; 5 mmol/L MnCl2; 5 mmol/L MgCl2; 100 mmol/L NaCl). In the case of autophosphorylation assays, the reaction was carried out by using 50 μL of kinase buffer containing radiolabeled [γ32P]ATP (5 μCi/sample) and 40 μmol/L of unlabeled ATP at 37°C. Reactions were stopped after different incubation times (0, 5, 15, 40 minutes) by placing samples on ice and adding stop solution (Extraction Buffer + 10 mmol/L EDTA). Supernatants were discarded and precipitated proteins were eluted by using boiling denaturing Loading Buffer and resolved by SDS-PAGE. The polyacrylamide gels were dried and analyzed by autoradiography. The same amounts of immunoprecipitates were resolved by SDS-PAGE and protein expression and loading were analyzed by immunoblotting with anti-Ror1 and anti-ErbB2 antibodies. For kinase assays on exogenous substrates, the immune complexes were incubated in kinase buffer containing 40 μg poly[(Glu:Tyr),(4:1)] (Sigma) and 500 μmol/L ATP at 37°C for 20 minutes. The reaction was stopped by adding boiling denaturing Loading Buffer and resolved by SDS-PAGE and detected by immunoblotting with anti-pTyr antibody.

Proliferation screening and cell proliferation assay

Cells cultured in complete medium supplemented with 10% serum were plated in 96-well plates (2,000 cells/well). Twenty-four hours after seeding, cells were infected with lentiviral vectors (day 0). At days 0, 3, 6, and 9, cell quantification was done by using the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega), according to the manufacturer's instructions. Cell quantity was determined by measuring the absorbance at 485 nm by Victor X Multilabel Plate Readers (Perkin Elmer). Each point was carried out in triplicate.

Migration assay

To evaluate migration ability, 5 × 104 cells were seeded on the upper side of a Transwell chamber (Corning) on a porous polycarbonate membrane (8.0-μm pore size). The lower chamber of the Transwell was filled with Dulbecco's modified Eagle's medium/RPMI containing 10% FBS. After 16 hours of incubation, cells on the upper side of the filters were mechanically removed and cells migrated to the lower side were fixed, stained, and counted.

Soft-agar assay

A total of 3,000 cells were resuspended in complete medium containing 0.5% Seaplaque agar. Cells were seeded in 24-well plates containing a 1% agar underlay and supplemented twice a week with complete medium. Colonies were quantified by using AlamarBlue stain (AbD Serotec), according to the manufacturer's instruction. Representative colonies were photographed by a Leica microscope with a ×10 objective. Each point was carried out in quadruplicate.

Tumorigenesis assay

Lentiviral vector–transduced cells (3 × 106 cells/mouse) in 0.2 mL of serum-free medium were subcutaneously injected into the right posterior flank of 6-week-old immunodeficient nu−/− female mice on Swiss CD-1 background (12 mice/group; Charles River Laboratories). Tumor size was evaluated every 3 days by a caliper. Tumor volume was calculated by the formula: $V = 4/3\pi \times y/2 \times (x/2)2$, where x is the minor tumor axis and y the major tumor axis. A mass of 15 mm3 was chosen as a threshold for tumor positivity. Mice with tumors below this threshold were considered tumor-free. All the animal procedures were approved by the Ethical Commission of the University of Turin (Italy) and by the Italian Ministry of Health.

3. Results

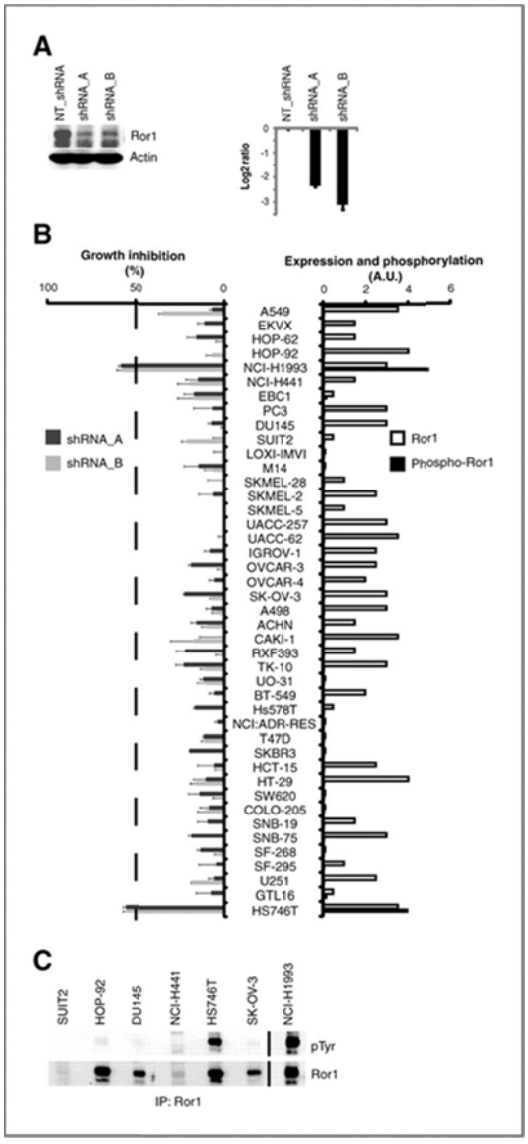
Ror1 expression, phosphorylation, and functional activity in cancer cells

A screening was undertaken in a panel of 43 cancer cell lines to asses: (i) the effect of Ror1 silencing on cell growth, (ii) Ror1 expression, and (iii) Ror1 tyrosine phosphorylation. First, we applied an RNAi-based approach to identify human tumors that rely on Ror1 for growth. The 43 cell lines were infected with lentiviral vectors containing shRNAs targeting the Ror1 gene: 2 shRNAs matching different Ror1 sequences (referred to as Ror1 shRNA_A and Ror1 shRNA_B) were used to minimize potential off-target effects; an NT_shRNA was used for mock transfectants. When tested on PC3 prostate carcinoma cells (a representative cell line known to express Ror1 according to existing databases; ref. 16), both shRNAs effectively reduced Ror1 mRNA and protein levels (Fig. 1A). Six days postinfection, cell viability was measured and cells featuring at least 50% growth inhibition over control (NT_shRNA) were scored as positive hits. Two cell lines, a gastric (HS746T) and a lung (NCI-H1993) carcinoma, were identified (Fig. 1B).

Figure 1.

Integrated screening. A, PC3 cells were infected with 2 specific shRNAs targeting Ror1 (shRNA_A and shRNA_B) and a control nontargeting shRNA (NT_shRNA). Left, immunoblotting analysis shows that both Ror1-specific shRNAs induced an almost complete protein downregulation (80%–90% as assessed by densitometry); actin was used as a loading

control. Right, total RNAs were isolated and the relative levels of Ror1 were analyzed by quantitative RT-PCR analysis. Ror1 levels were normalized against the Pgk housekeeping gene. Both shRNAs downregulated Ror1 mRNAs by approximately 8-fold. Error bars report SD of 3 experiments carried out in triplicate. B, left, proliferation screening in a panel of 43 human cancer cell lines on infection with Ror1-specific shRNAs (shRNA_A, dark gray bars; and shRNA_B, light gray bars). Growth inhibition is represented as a percentage of viable shRNA_A and shRNA_B cells 6 days postinfection, normalized versus NT_shRNA cells. Decrease of viability of at least 50% was defined as a threshold for sensitivity to Ror1 downregulation. Cell viability was measured by colorimetric determination of MTS reduction. NCI-H1993 and HS746T cells were sensitive to Ror1 silencing. Error bars report SD of 2 experiments carried out in triplicate. Right, Ror1 protein expression (white bars) and phosphorylation levels (black bars) in the panel of 43 human cancer cell lines subjected to the proliferation screening. Cells were cultured in complete medium supplemented with 10% serum. Protein lysates were immunoprecipitated with anti-Ror1 antibody; membranes were probed with anti-pTyr antibody and subsequently reprobed with anti-Ror1 antibody. Densitometric analysis of the bands was conducted. Ror1 was broadly expressed in 32 out of 43 cell lines. Phosphorylation of Ror1 was detected only in NCI-H1993 and HS746T. C, immunoblotting analysis of Ror1 expression and phosphorylation in 7 representative cancer cell lines (including NCI-H1993 and HS746T).



We reasoned that the response to Ror1 inactivation could be correlated with (i) Ror1 expression or (ii) Ror1 tyrosine phosphorylation. Therefore, we proceeded with a second screening, by densitometric measurement of the protein and by detection of Ror1 tyrosine phosphorylation (assessed by phospho-tyrosine content). Ror1 protein was broadly

distributed and expressed, at variable levels, in most of the cancer cell lines examined (32 of 43; Fig. 1B; Supplementary Fig. S1).

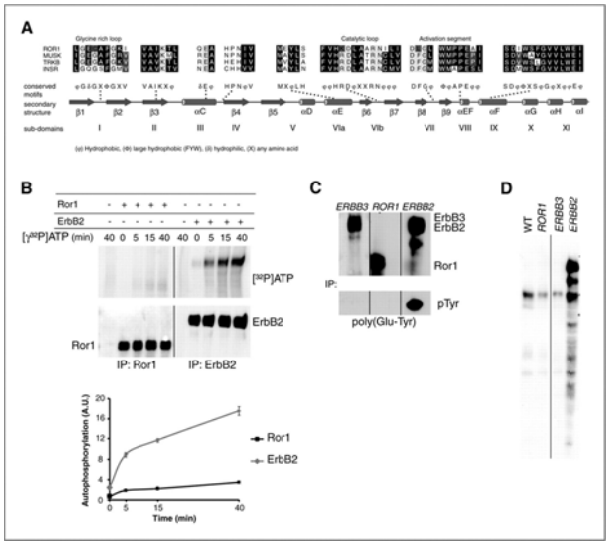
We then evaluated Ror1 tyrosine phosphorylation as a proxy of protein function. The receptor was found tyrosine phosphorylated only in 2 cell lines, namely HS746T and NCI-H1993 (Fig. 1B and C). This analysis revealed a clear connection between the biological response to Ror1 knockdown and Ror1 tyrosine phosphorylation, rather than protein expression.

Ror1 lacks kinase activity

Biochemical analyses indicated that Ror1 is a bona fide pseudokinase, devoid of intrinsic catalytic activity. This was initially suggested by the Ror1 amino acid sequence, which displays 6 deviations from the canonical tyrosine kinase consensus. At least 3 amino acid substitutions fall in regions essential for catalytic activity, specifically, C482G, K614R, and L634F (refs. 2, 17, 18; Fig. 2A).

Figure 2.

Ror1 is a pseudokinase. A, alignment of the peptide sequence of the Ror1 kinase domain with that of other tyrosine kinase receptors that feature the highest degree of homology with Ror1, namely, MuSK, TrkB (all catalytically active), and InsR, a prototype of catalytically active receptor. The amino acids conserved among the different tyrosine kinases are indicated by letters below. Highlighted in yellow are key residues that are deemed to be essential for enzymatic activity in protein kinases. The deviations of Ror1 sequence from the consensus sequence are highlighted in red. The secondary structure is schematized and the consensus sequences of common motifs and key conserved loops are given. B, top, COS-7 cells were transfected with Ror1 and an active tyrosine kinase receptor (ErbB2), used as a positive control. WT COS-7 was used as a sham control. Ror1 or ErbB2 immunoprecipitates were incubated with [γ-32P]ATP for the indicated times at 37°C and run on SDS-PAGE. Receptor autophosphorylation was determined by autoradiography. Protein expression and loading was checked by immunoblotting with anti-Ror1 and anti-ErbB2 antibodies. Bottom, the densitometric analysis of bands from the autoradiogram was normalized versus the protein content and plotted against times of reaction. Error bars indicate the range of densitometric values in 2 experiments. C, ErbB3, Ror1, or ErbB2 were overexpressed in COS-7 cells, immunoprecipitated, incubated with poly(Glu:Tyr) peptide in the presence of ATP at 37°C for 20 minutes, and run on SDS-PAGE. The peptide phosphorylation was evaluated with anti-pTyr antibody. Protein expression and loading were checked by immunoblotting with anti-ErbB3, anti-Ror1, and anti-ErbB2 antibodies. Immunopurified Ror1 was unable to phosphorylate exogenous substrates. D, Ror1, ErbB3, and ErbB2 were overexpressed in COS-7 cells. Cells were serum-starved for 48 hours. Total cell lysates were run and blots were decorated with anti-pTyr antibody. Ror1 overexpression did not modify the overall tyrosine phosphorylation status of endogenous proteins in whole cell lysates.



The intrinsic catalytic activity of Ror1 was tested by using kinase assays that assessed Ror1 autophosphorylation as well as heterologous phosphorylation of exogenous substrates. Ror1 was expressed by transfection in COS-7 cells and the autocatalytic activity was evaluated in the presence of radiolabeled-ATP after different incubation times. Comparison with the active form of a reference tyrosine kinase (ErbB2) showed that the extent of Ror1 autophosphorylation is negligible (Fig. 2B). Immunopurified Ror1 was also unable to phosphorylate the exogenous peptide poly(Glu:Tyr); this behavior was different from that of ErbB2 and similar to that of ErbB3, which is catalytically inactive (Fig. 2C). Finally, overexpression of Ror1 in COS-7 cells did not affect the tyrosine phosphorylation pattern of endogenous proteins in whole cell extracts, again in accordance with the results from ErbB3 overexpression; in contrast, and as expected, ErbB2 overexpression produced substantial changes in the tyrosine phosphorylation status of several proteins (Fig. 2D).

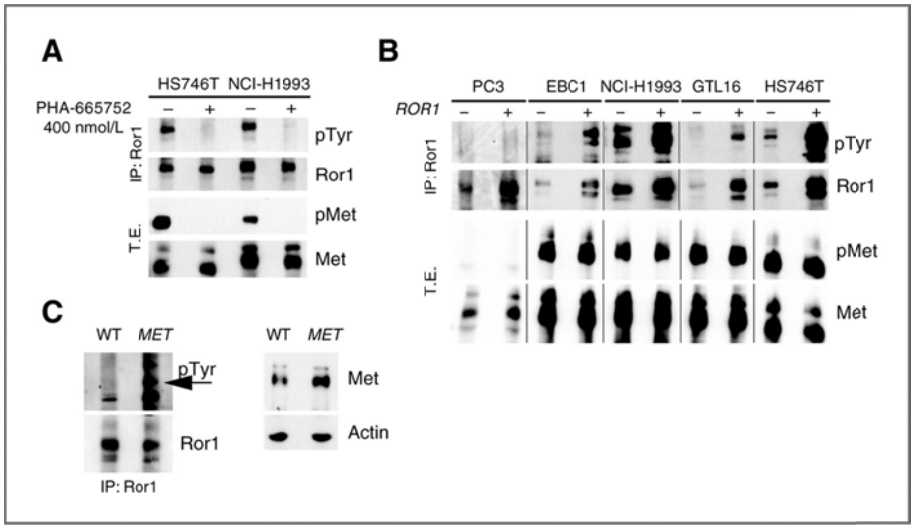
Ror1 is transphosphorylated by the Met oncogene

The observation that Ror1 lacks intrinsic catalytic activity suggests that its tyrosine phosphorylation might be due to transphosphorylation by another kinase. To pinpoint the tyrosine kinase(s) that may act as upstream regulator(s) of Ror1 in HS746T and NCI-H1993 cells, we carried out an in silico analysis of existing databases and literature (19–21). Interestingly, we found that both cell lines share an uncommon genetic alteration in that they harbor focal and high-grade amplification of the Met oncogene–with an aberrant gene copy number of 6.35 and 8.66, respectively–that results in Met constitutive activation (22).

To test whether constitutively active Met transphosphorylates Ror1, we treated HS746T and NCI-H1993 cells with the Met-specific inhibitor PHA-665752 at nanomolar concentrations. Pharmacologic blockade of Met led to complete abrogation of Ror1 phosphorylation in both cell lines (Fig. 3A). Similar results were obtained when Met neutralization was achieved by JNJ-38877605 (another Met-specific inhibitor) and by RNAi by using 2 different shRNAs (Supplementary Fig. S2A). In contrast, downregulation of Ror1 did not affect expression or phosphorylation of Met (Supplementary Fig. S2B).

Figure 3.

Ror1 phosphorylation depends on Met constitutive activation. A, immunoblotting analysis of Ror1 phosphorylation in HS746T and NCI-H1993 cells cultured in complete medium supplemented with 10% serum and treated for 2 hours with the Met selective inhibitor PHA-665752 (400 nmol/L). Lysates were run as Ror1 immunoprecipitates or as total extracts. Receptor phosphorylation status was checked by probing the membranes with anti-pTyr or anti-pMet antibodies. PHA-665752 caused concomitant reduction of Met and Ror1 phosphorylation. B, immunoblotting analysis of Ror1 expression and tyrosine phosphorylation upon Ror1 lentiviral transduction (+) in 4 cancer cell lines displaying constitutive Met phosphorylation (EBC1, NCI-H1993, GTL16, and HS746T) and in a cancer cell line (PC3) displaying basally unphosphorylated Met (see also Supplementary Fig. S2). Cells were cultured in complete medium supplemented with 10% serum and lysates were run as Ror1 immunoprecipitates. Ror1 expression was monitored by anti-Ror1 antibody and receptor phosphorylation was checked with anti-pTyr antibody. Input controls for Met and phospho-Met are represented by total cell extract (T.E.) used for each immunoprecipitation. When overexpressed, Ror1 was phosphorylated only in cancer cells expressing basally phosphorylated Met. C, immunoblotting analysis of Ror1 phosphorylation in WT and Met-transfected HT29 cells (expressing high levels of Ror1). Cells were cultured in complete medium supplemented with 10% serum. Ror1 immunoprecipitates were probed first with anti-pTyr antibody (phospho-Ror1 is indicated by an arrow) and then with anti-Ror1 antibody. Met expression was checked by anti-Met antibody on total lysates; actin was used as a loading control. Ectopic expression of Met in HT29 induced Ror1 phosphorylation.



To analyze whether transphosphorylation of Ror1 is a general occurrence in cells exhibiting constitutively active forms of Met, we extended the analysis to other cell lines featuring gene amplification and abnormal kinase activity of Met. In the panel of 43 cancer cell lines used for the functional and expression screenings, we found 2 other lines displaying Met amplification: GTL16 (gastric carcinoma) and EBC1 (non–small cell lung carcinoma), carrying a Met copy number gain of 6.10 and 5.80, respectively (22). These 2 cell lines, however, escaped the screening because they do not express detectable levels of endogenous Ror1 (Fig. 1B). We thus ectopically introduced Ror1 by lentiviral gene transfer. As a control, the gene was transferred in PC3, expressing physiologic levels of Met. Although all transduced cells expressed high levels of exogenous Ror1, the receptor was phosphorylated only in cells displaying Met amplification and constitutive activation (Fig. 3B; Supplementary Fig. S3). Finally, we overexpressed Met in HT29, a cell line that features high levels of Ror1 but expresses normal levels of basally inactive Met: in this setting, exogenous overproduction of Met resulted in a discernible, albeit modest, phosphorylation of Ror1 (Fig. 3C). Together, these results indicate that Ror1 tyrosine phosphorylation occurs in contexts in which Met is overexpressed and constitutively active.

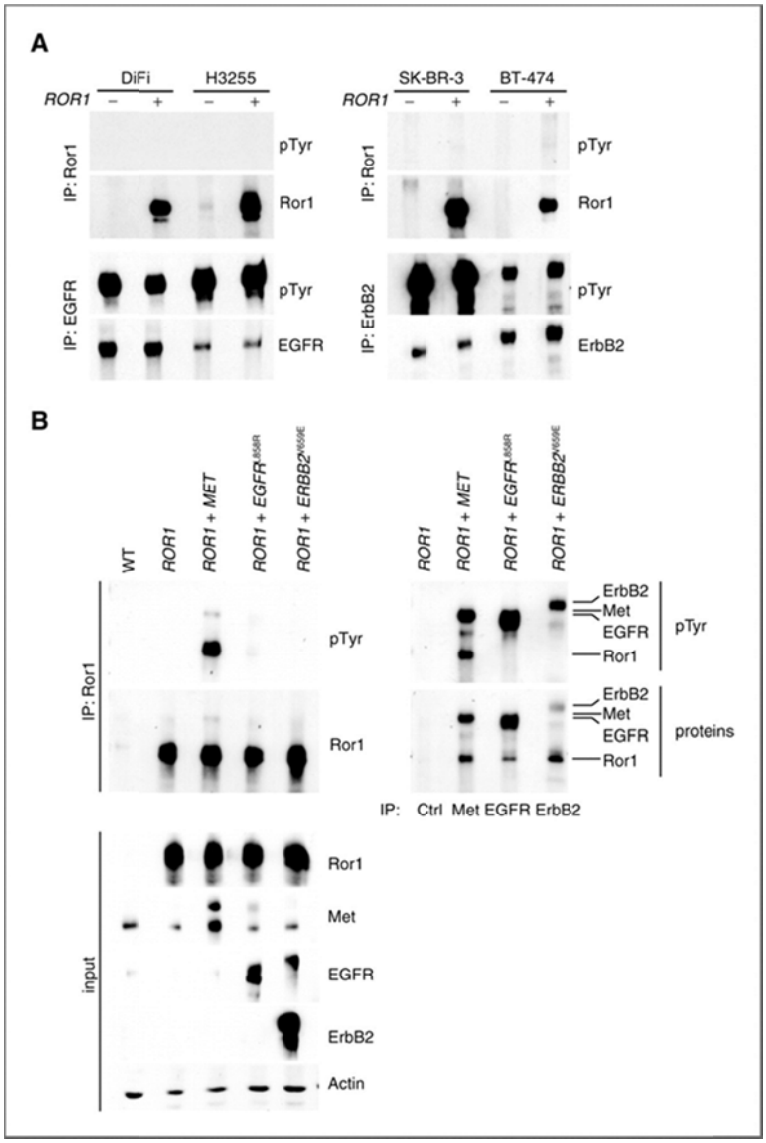
Met-dependent transphosphorylation of Ror1 is kinase specific

To assess whether Ror1 is selectively phosphorylated by Met, or may act as a promiscuous substrate for different tyrosine kinases, we overexpressed Ror1 in cells exhibiting constitutive activation of other tyrosine kinases and analyzed the extent of phosphorylation. To this end, we chose 4 cancer cell lines with deregulated activity of EGFR or ErbB2: (i) H3255, a non–small cell lung carcinoma cell line encompassing an activating point mutation of the EGFR gene (23); (ii) DiFi, a colon carcinoma cell line displaying EGFR amplification (24); (iii) BT474 and SKBR3, 2 mammary carcinoma cell lines with ErbB2 amplification (25). Unlike that observed in cells with Met amplification, Ror1 overexpression in these cell lines did not result in its tyrosine phosphorylation (Fig. 4A).

Figure 4.

Ror1 phosphorylation is specifically regulated by Met. A, immunoblotting analysis of Ror1 following ectopic overexpression (+) in 4 cancer cell lines: DiFi and H3255 (which display constitutively phosphorylated EGFR), and SK-BR-3 and BT-474 (which harbor basally activated ErbB2). Cells were cultured in complete medium supplemented with 10% serum and lysates were immunoprecipitated with anti-Ror1, anti-EGFR, and anti-ErbB2 antibodies. Receptor phosphorylation was checked by anti-pTyr antibody; receptor expression was checked by anti-Ror1, anti-EGFR, and anti-ErbB2 antibodies. Expression of Ror1, even at high levels, was not accompanied by Ror1 phosphorylation. B, COS-7 cells were transiently transfected with Ror1 alone or in combination with Met or in combination with active forms of EGFR (EGFRL858R) or ErbB2 (ErbB2V659E). The different receptors were immunoprecipitated with their specific antibodies. Preimmune mouse serum was used as a negative control for the coimmunoprecipitation experiments (Ctrl). Protein phosphorylation was checked by anti-pTyr antibody and expression and loading was checked by anti-Ror1, anti-ErbB2, and anti-ErbB3 antibodies. Input controls showing the exogenous expression levels of each transfected gene

(Ror1, Met, EGFR, and ErbB2) are shown below the immunoprecipitates. Overexpression of Met, EGFR, and ErbB2 led to their constitutive activation. Despite coimmunoprecipitation with all 3 receptors, Ror1 was phosphorylated only when coexpressed with Met.



To further validate this observation, we tested the effects of acute coexpression of Ror1 with constitutively active forms of Met, EGFR, and ErbB2. Transient cotransfection experiments were conducted in COS-7 cells with the following constructs: (i) Ror1 and wild-type (WT) Met (which becomes phosphorylated because of protein overexpression); (ii) Ror1 and EGFRL858R (an active mutant of EGFR); and (iii) Ror1 and ErbB2V659E (an active mutant of ErbB2). Transfection of Ror1 alone was performed for control purposes. Exogenous introduction of Met, EGFRL858R, and ErbB2V659E led to overexpression of highly phosphorylated (hence, active) receptor moieties in all 3 instances. However, Ror1 was phosphorylated only when coexpressed with Met (Fig. 4B). Interestingly, Ror1 coimmunoprecipitated with all 3 receptors, independent of its phosphorylation status. These data indicate that Ror1 behaves as a rather specific substrate for the Met kinase, and not for other receptors.

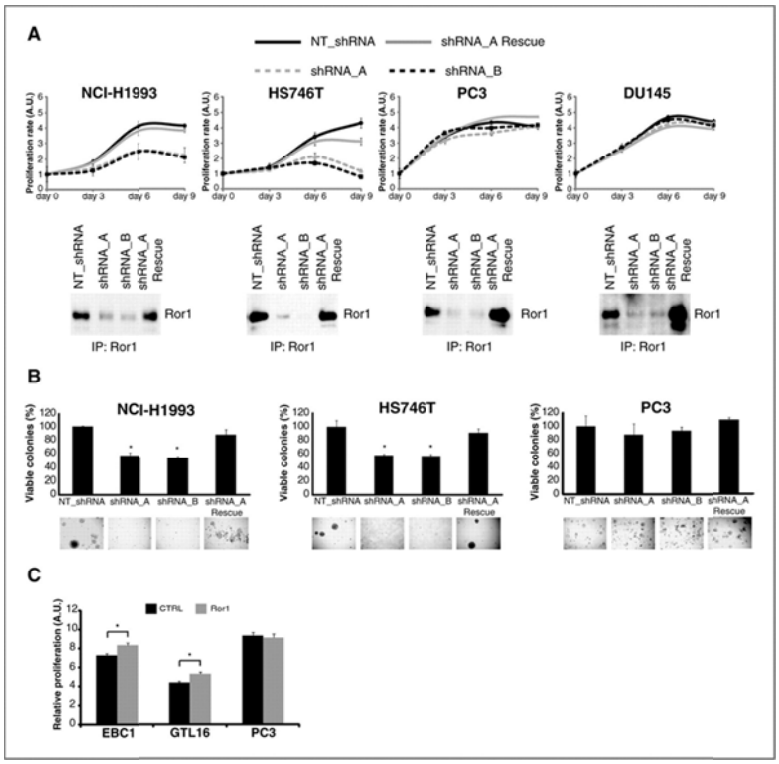
Ror1 sustains cell growth in vitro and tumorigenicity in vivo

The biological effects of Ror1 knockdown were studied in vitro and in vivo. HS746T and NCI-H1993 carcinomas were separately transduced with the 2 specific lentiviral shRNAs used in the initial screening. Cancer cell lines expressing intermediate levels of unphosphorylated Ror1 (PC3 or DU145) were used as controls, and a rescue experiment was done by expressing a Ror1 cDNA (harboring 3 silent mutations) refractory to the specific shRNA_A. Ror1 knockdown

and rescue were achieved in all cell lines. As expected from the results of the screening, silencing Ror1 slowed down proliferation of HS746T and NCI-H1993, but not that of PC3 and DU145 (Fig. 5A). A soft agar assays was conducted to verify whether shRNA-mediated Ror1 knockdown affects clonogenic potential. Indeed, formation of colonies was impaired in NCI-H1993 and HS746T. Again, Ror1 downregulation was ineffective in control cells (Fig. 5B). Reestablishment of Ror1 expression in Ror1-deficient cells was followed by restoration of cell proliferation and anchorage-independent growth (Fig. 5A and B). Similar results were obtained in a Transwell migration assay (Supplementary Fig. S4). In an opposite but complementary gain-of-function approach, ectopic introduction of Ror1 in Ror1-negative, Met-addicted GTL16, and EBC1 cells led to a statistically significant (although mild) growth advantage (Fig. 5C).

Figure 5.

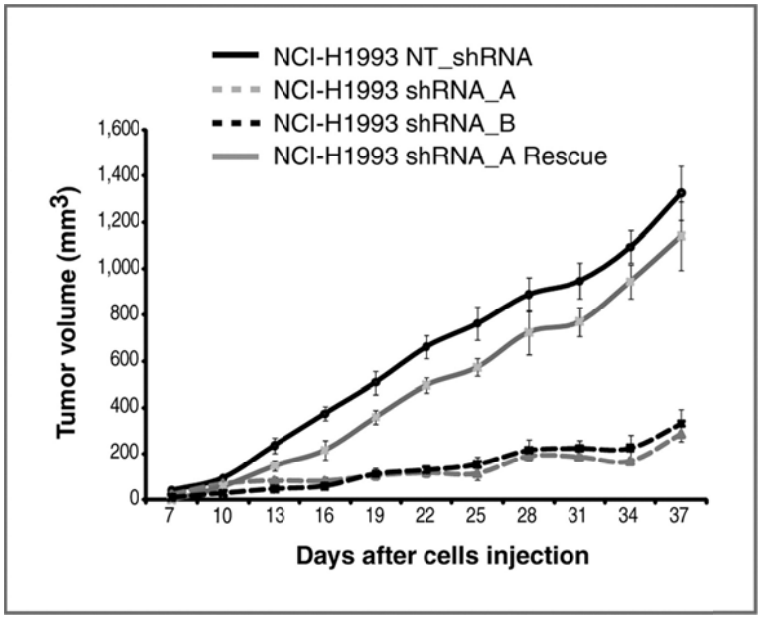
Effects of Ror1 silencing or overexpression in vitro. A, growth curves of NCI-H1993, HS746T, PC3, and DU145 cells (cultured in complete medium supplemented with 10% serum) upon infection with Ror1-specific shRNAs (shRNA_A, dashed gray lines; shRNA_B, dashed black lines), NT_shRNA (continuous black line), or shRNA_A Rescue (continuous gray line). Viable cells were estimated at days 3, 6, and 9. Curves were normalized versus cell numbers on day 0 (day of infection). Cell viability was measured by colorimetric determination of MTS reduction. Ror1 silencing decreased proliferation rates in NCI-H1993 and HS746T cells, whereas Ror1 rescue restored cell proliferation. PC3 and DU145 cells, which display unphosphorylated Ror1, were insensitive to Ror1 silencing. Error bars report SD of 2 experiments carried out in triplicate. Immunoblots showing Ror1 expression levels (following RNAi-mediated knockdown and rescue) are displayed below the graphs. B, anchorage-independent growth assay. NCI-H1993, HS746T, and PC3 cells infected with shRNA_A, shRNA_B, NT_shRNA, and shRNA_A Rescue were plated in soft agar and grown for 2 weeks. Upon Ror1 silencing, growth in soft agar was strongly impaired in NCI-H1993 and HS746T cells (approximately 50%); rescue of Ror1 expression restored their capacity to form colonies in soft agar. Growth in soft agar of PC3 and DU145 was not influenced by Ror1 silencing. Histograms represent viable colonies measured with Alamar Blue staining. Error bars report SD of 2 experiments carried out in quadruplicate. Representative images are shown below. *, $P < 0.01$ by Student's t test. C, endpoint MTS cell proliferation assay in control (CTRL) and Ror1-overexpressing GTL16 and EBC1. Six days after seeding, ectopic expression of Ror1 produced a statistically significant growth advantage. Error bars report SD of 2 experiments carried out in triplicate. *, $P < 0.01$ by Student's t test.



The involvement of Ror1 in tumorigenicity was further evaluated in vivo by xenograft models. NCI-H1993 cell lines featuring shRNA-mediated knockdown of Ror1 were injected into CD-1 nu−/− mice. Tumor growth was monitored twice a week for more then 1 month. Xenografts carrying Ror1 shRNA_A (n = 12) and shRNA_B (n = 12) formed subcutaneous masses at much slower rates than NT_shRNA controls (n = 12), with an end-of-study 80% reduction of tumor volume. Of note, Ror1 restoration in Ror1-deficient cells (n = 12) rescued tumor growth at levels comparable with those of control xenografts. These data strengthen the finding that Ror1 contributes to the tumorigenic phenotype of cancer cells featuring Met amplification (Fig. 6).

Figure 6.

Effects of Ror1 silencing in vivo. NCI-H1993 cells transduced with shRNA_A (dashed gray line), shRNA_B (dashed black line), NT_shRNA (continuous black line), and shRNA_A Rescue (continuous gray line) were implanted subcutaneously in nude mice (n = 12 per group). Tumor growth, as measured by tumor volume, was monitored at the indicated days. Error bars report SE. Ror1 silencing induced approximately 80% tumor growth inhibition compared with controls; rescue of Ror1 expression restored tumor growth.



4. Discussion

Increasing evidence points to a role for receptor pseudokinases in regulating functional processes in human cancers, despite their lack of catalytic activity (17). The case of ErbB3 is a paradigm: this kinase-defective, nonautonomous receptor binds 4 different EGF-like ligands and forms 3 functional heterodimers with other ErbB family members, among which ErbB2 is prominent (26). Following heterodimerization, the cytoplasmic domain of ErbB3 becomes a substrate for the catalytically active partners, and provides a platform for the recruitment of downstream transducers (27). ErbB3-dependent signals that emanate from ErbB2/ErbB3 heterodimers contribute to proliferation, invasion, and metastasis of ErbB2-overexpressing tumors (28, 29). Something similar may occur in the case of the Met/Ror1 couple. Although we did not investigate in detail whether Met and Ror1 can form heterodimers, here we show that Ror1 is a defective kinase that acts as a substrate for the Met tyrosine kinase receptor, an oncogene product that plays a relevant role in human cancer (30–32).

We cannot formally exclude, based on the intrinsic limits of detectability of ATP-based kinase assays, that Ror1 maintains a residual (but negligible) degree of enzymatic activity, as recently shown for ErbB3 (33). Although high-resolution structural data of the kinase-like domain of Ror1 are warranted to unequivocally address this issue, several lines of evidence indicate that Ror1 acts indeed as a pseudokinase: (i) receptor overexpression does not lead to kinase autophosphorylation; (ii) immunopurified Ror1 is unable to phosphorylate exogenous substrates; (iii) Ror1 overexpression does not modify the overall tyrosine phosphorylation status of endogenous proteins in whole cell lysates; (iv) the catalytic domain of Ror1 contains amino acid substitutions in critical residues that are evolutionarily conserved and that are known to regulate the enzymatic function of tyrosine kinases.

Met-dependent transphosphorylation of Ror1 is not observed in normal epithelial cells expressing physiologic levels of Met, nor is it induced by acute Met activation in response to HGF exogenous stimulation (Supplementary Fig. S5); conversely, it specifically occurs in cancer cells that overexpress chronically active forms of Met and rely on deregulated Met activity for continuous growth and survival (oncogene addiction). In these cells, Ror1 transphosphorylation seems to be necessary to fully sustain "Met addiction": in vitro, RNAi-mediated knockdown of Ror1 impairs cell proliferation and reduces anchorage-independent growth; in vivo, Ror1 silencing goes along with a delay in xenograft formation and progression. All these tumorigenic properties are rescued by overexpression of a shRNA-resistant Ror1 cDNA. The mechanistic explanation for these "enhancer" functions of Ror1 is largely unknown. On the basis of in silico analysis of the cytoplasmic domain, we can identify 3 tyrosines (Y641, Y645, and Y646) embedded in consensus sequences for Met-specific transphosphorylation (34). In turn, some of these tyrosines are predicted to bind, on phosphorylation, SH2-containing transducers such as Src and Stat-3 (35, 36).

It should be noted that not all the Met-addicted cell lines examined in this study express Ror1; we can speculate that, in these cells, the function of Ror1 as an expansion platform for signal transduction is surrogated by other unidentified kinases or pseudokinases. An example is the GTL16 gastric carcinoma, in which the function of signal transduction amplifier is exerted by Ron, a tyrosine kinase receptor endowed with weak catalytic activity (37).

As a result of an oncogenic alteration, cancer cells may also develop secondary dependencies on genes that are themselves not oncogenes. Perturbation of these genes can result in oncogene-specific "synthetic lethal" interactions that could provide new therapeutic opportunities. Here we show that genetic inactivation of the Ror1 pseudokinase constitutes synthetic lethality with genomic amplification of Met. Therefore, Met-addicted tumors also display a "nononcogene" addiction to Ror1. The findings reported in this article highlight the complexity of signaling networks regulated by addictive oncoproteins and, in the meantime, reveal their fragility. Interfering with one single component seems to be sufficient to neutralize, or at least attenuate, the transformed phenotype sustained by altered oncogenes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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