Synthetic Lethality Screens Reveal RPS6 and MST1R as Modifiers of Insulin-like Growth Factor-1 Receptor Inhibitor Activity in Childhood Sarcomas

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Abstract

The insulin-like growth factor-1 receptor (IGF1R) is emerging as a promising therapeutic target in human cancers. In the high-risk childhood sarcomas Ewing family tumor and rhabdomyosarcoma, IGF1R-blocking antibodies show impressive antitumor activity in some but not all patients, and acquired resistance is observed. Because tumor IGF1R mutations are not described, the basis of IGF1R inhibitor resistance remains unknown. We hypothesized that compensatory signaling cascades bypassing targeted IGF1R inhibition might be involved. To test this systematically, we performed small interfering RNA (siRNA) screens in sarcoma cell lines to identify IGF1R pathway components or related protein tyrosine kinase (PTK) networks that modulate the antitumor efficacy of the BMS-536924 IGF1R kinase inhibitor. This strategy revealed (*a*) that sarcoma cells are exquisitely sensitive to loss of distal rather than proximal IGF1R signaling components, such as ribosomal protein S6 (RPS6); (*b*) that BMS-536924 fails to block RPS6 activation in resistant sarcoma cell lines; and (*c*) that siRNA knockdown of the macrophage-stimulating 1 receptor tyrosine kinase (MST1R; also known as RON) restores BMS-536924 efficacy, even in highly drug-resistant cell lines. We confirmed MST1R expression across a broad panel of childhood sarcomas, and found that loss of MST1R by RNA interference blocks downstream RPS6 activation when combined with BMS-536924 *in vitro*. These findings underscore the importance of fully understanding PTK networks for successful clinical implementation of kinase inhibitor strategies.

Introduction

The insulin-like growth factor-1 receptor (IGF1R) is an important target for cancer drug development, with several humanized blocking antibodies currently in clinical trials (1–3). Binding of IGF1 or IGF2 to IGF1R initiates a myriad of signaling events, including activation of the Ras–extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)–AKT–mammalian target of rapamycin (mTOR) pathways, among others, leading to diverse cellular processes such as growth, survival, tumor angiogenesis, and drug resistance (4, 5). Deregulation of IGF1R via *IGF1R* amplification or enhanced activation by ligand overexpression is common in solid tumors, suggesting broad utility for inhibitors of this receptor.

In high-risk childhood sarcomas such as Ewing family tumor (EFT) and rhabdomyosarcoma (RMS), IGF1R is under intense scrutiny as a therapeutic target. Despite impressive progress with conventional chemotherapies, prognosis remains very poor for patients with relapsed and metastatic disease, underscoring the urgent need for new therapeutics (6–8). Numerous preclinical findings highlight IGF1R as a promising molecular target in childhood sarcomas: EWS-ETS fusion proteins of EFT and the congenital fibrosarcoma ETV6-NTRK3 chimeric kinase require a functional IGF1R axis to transform cells, and the PAX3-FKHR fusion protein of alveolar RMS acts synergistically with IGF1R (9–11). EWS-ETS fusion proteins induce IGF1 promoter activity, and an IGF1/IGF1R autocrine loop exists in EFT (12, 13), whereas *IGFBP3*, which negatively regulates IGF1R signaling, is repressed by EWS-FLI1 (14). RMS is associated with IGF2 overexpression and loss of *IGF2* imprinting, and PAX3-FKHR activates *IGF1R* transcription in alveolar

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RMS (15–17). Functionally, IGF1R seems to be the major activator of the PI3K-AKT cascade in childhood sarcoma cells (18). IGF1R links PI3K-AKT signaling to activation of the energy-sensitive mTOR complex 1 (mTORC1) in both RMS and EFT cells (19, 20). Signaling by the mTORC1 serine-threonine kinase drives cell growth and division when nutrient conditions are appropriate for proliferation (21). mTORC1 is essential for Cap-dependent translation via phosphorylation of S6 kinase, which phosphorylates and activates ribosomal protein S6 (RPS6), and eukaryotic initiating factor 4E-binding protein 1 (4EBP1), which releases 4EBP1 inhibition of the eIF4E translation factor (22).

Early clinical trials have shown remarkable responses to IGF1R-blocking antibodies in some patients with advanced EFT and RMS (1–3, 8, 23–25). In spite of this, disease progression and resistance is seen in many patients even during treatment. A key question therefore remains as to what determines resistance toward these agents, as tumor-associated genetic mutations within the IGF1R pathway have not been detected (2). Recently, Cao and colleagues (18) showed that IGF1R levels correlate with *in vivo* sensitivity to IGF1R-blocking antibodies in RMS xenografts. Other potential biomarkers include levels of IGF1 and IGF-binding proteins (IGFBP), or activation of effectors such as AKT (1, 3, 18, 26). Interestingly, although treatment of mice bearing human RMS xenografts with IGF1R antibodies initially reduced xenograft growth, tumors reemerged after extended treatment. This correlated with reactivation of AKT in tumor cells, although IGF1R phosphorylation remained blocked (18). In human epithelial tumors, coactivation of receptor protein tyrosine kinases (PTK) such as epidermal growth factor receptor (EGFR) and ERBB2 confers resistance to IGF1R antagonists and vice versa, and cotargeting these PTKs along with IGF1R enhances antitumor activity (26–28). This suggests that alternative PTKs may impinge on pathways downstream of IGF1R to maintain pro-oncogenic signaling in human tumors.

To address this in childhood sarcomas, we performed RNA interference screens in EFT cell lines combining a small-molecule IGF1R kinase inhibitor, BMS-536924 (29), which also blocks the insulin receptor (INSR), with pathway-specific small interfering RNA (siRNA) sublibraries to systematically study the IGF1R signaling cascade and its relation to other PTK networks. The goal of this functional approach was to identify PTKs or other signaling molecules that modify IGF1R inhibitor activity, as a means to elucidate potential resistance mechanisms and rational cotargeting strategies.

Materials and Methods

Additional information and detailed experimental procedures are provided in Supplementary Materials and Methods.

Cell lines

All sarcoma cell lines were previously described. MCF-7 and MDA-MB-231 breast cancer cell lines with stable firefly luciferase expression were a kind gift of Dr. Marcel Bally (BC Cancer Research Centre, Vancouver, British Columbia,

Canada). HCT116 and HEK293 were from the American Type Culture Collection.

Compounds and reagents

BMS-536924 was provided by Bristol-Myers Squibb Company αIR3 was from Calbiochem, and isotype-matched IgG control (MOPC-21) was from Abcam. Rapamycin was from Cell Signaling Technology. Recombinant human IGF1 and MST1 were from Sigma-Aldrich and R&D Systems, respectively.

siRNA screens

We screened 31 proteins of the insulin/IGF1 signaling pathway and all 88 known PTKs of the human genome using Dharmacon siRNA libraries in pools of four siRNA duplexes per target gene (SMARTpool; Dharmacon). For control siRNAs, we used a commercially available and validated, functional nontargeting siRNA (nontargeting siRNA #3; Dharmacon). Details on experimental procedures and data analysis are found in Supplementary Materials and Methods.

Cell survival and death assays

Relative cell viability and number (fractional survival) were determined by bioluminescence (D-luciferin; Caliper Life Sciences) or WST-1 assays (Roche Applied Science). Cell death was analyzed by ethidium homodimer-1 (EthD-1; Invitrogen) uptake.

Western blotting

Western blotting was performed as previously described (9). For description and dilutions of all antibodies used, see Supplementary Table S1.

Flow cytometry

Apoptotic cells and cell cycle distribution were determined after propidium iodine staining of nuclei using a FACScan analyzer (BD Biosciences; refs. 9, 30).

Tumor samples and gene expression analysis

Frozen tumor samples from patients enrolled in Children's Oncology Group D9802 and D9803 trials were obtained from the Pediatric Cooperative Human Tissue Network tumor bank (Columbus, OH). RMS samples were previously published by Davicioni and colleagues (31); additional tumor samples were obtained from the Children's Hospital Los Angeles tumor bank. All samples contained >80% tumor cells. Bone marrow mesenchymal stem cells (MSC) were provided by Dr. Darwin Prockop (Tulane University, New Orleans, LA). Microarray protocols (GeneChip U133A array; Affymetrix) were as described (31). Data sets are deposited on the National Cancer Institute Cancer Array Database at https://array.nci.nih.gov/caarray/project/trich-00099. Box plots were generated with Genomics Suite 6.5 (Partek, Inc.) and normalized using RobustMultichipAverage (RMA).

Immunohistochemistry

MST1R protein expression was analyzed in tissue microarrays by probing with a MST1R antibody (Ron β C-20; Santa Cruz Biotechnology) as previously described (32). Samples

were scored as follows: high positive, >50% of tumor cells with membranous/cytoplasmic positivity; medium positive, 10% to 50% positivity; and negative.

Results

EFT cell lines are highly sensitive to the BMS-536924 IGF1R kinase inhibitor

To explore IGF1R inhibitor efficacy in high-risk childhood sarcomas, we first evaluated the activity of the BMS-536924 IGF1R kinase inhibitor in well-established EFT cell lines, TC71 and TC32. Dose-response curves showed an effective dose 50 (ED_{50}; dose reducing viability to 50% of nontreated controls) of 100 and 175 nmol/L, respectively, and almost complete loss of cellular luminescence (loss of viable cells) at doses above 1 µmol/L (Fig. 1A). For comparison, the ED_{50}s of breast cancer cell lines MCF-7 and MDA-MB-231 were 330 nmol/L and 3.5 µmol/L, respectively (Supplementary Fig. S1A). In TC32 cells, BMS-536924 was more potent than the monoclonal anti-IGF1R antibody αIR3 (13, 33), whereas in TC71 cells potency was comparable with αIR3 (Supplementary Fig. S1B). DMSO vehicle or isotype-specific IgG control antibodies did not affect cell viability (data not shown). Western blotting confirmed BMS-536924 inhibition of IGF1R tyrosine phosphorylation at 100 nmol/L at both 1 and 24 hours in EFT lines, correlating with inhibition of AKT S473 phosphorylation (Fig. 1B). Ras-mitogen-activated protein kinase signaling (ERK1/2 phosphorylation) was not consistently influenced by BMS-536924. Flow cytometric cell cycle analysis revealed both cell death and cell cycle arrest, as indicated by increased sub-G_1 and G_1 and decreased S and G_2-M fractions, respectively (Fig. 1C). BMS-536924 induced cell death was furthermore confirmed by increased poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 1D). Therefore, TC71 and TC32 EFT cell lines are highly sensitive to the BMS-536924 IGF1R kinase inhibitor.

siRNAs targeting distal IGF1R signaling components block EFT cell survival

To explore which components of the IGF1R cascade mediate survival of EFT cells, we conducted an siRNA fractional survival screen using a siRNA library targeting 31 key proteins of the IGF1R/INSR signaling axis. Briefly, TC71 and TC32 cells stably expressing firefly luciferase were transfected with siRNA pools (4 duplexes/target gene) and analyzed 96 hours later for luciferase substrate-induced bioluminescence to measure relative cell viability (fractional survival). Thus, siRNAs reducing fractional survival likely target progrowth or survival proteins, whereas siRNAs that increase fractional survival potentially target tumor suppressors. Figure 2A (top) shows that, along with the *XPO1* positive control, siRNAs to *RPS6, FRAP1 (mTOR), AKT2, AKT3, FOXO1A, IRS-1*, and *CBLB* reduced fractional survival in both cell lines, whereas others such as *PDPK1* or *IGF1R* were only effective in individual lines. To better compare results between the two cell lines, we defined screening hits according to specific criteria (see Supplementary Materials and Methods) and generated heat maps for visualization (Fig. 2A, bottom). Using these definitions, only siRNAs to *mTOR* and *RPS6* reproducibly reduced survival, whereas only *TSC1* siRNAs increased survival in both cell lines. Protein knockdown by siRNAs was confirmed by Western blotting; representative results are shown in Fig. 2B and Supplementary Fig. S2A. Moreover, effects on cell survival of individual siRNA duplexes from the pools were confirmed (Supplementary Fig. S2B), and this correlated with protein knockdown (shown for IGF1R in Supplementary Fig. S2C).

Maximal loss of cell viability was observed for siRNAs to *RPS6*, the most distal element of the IGF1R pathway tested, with nearly complete cell death in both EFT cell lines (Fig. 2A). This was validated over a time course (Fig. 2C, left): *RPS6*-targeting siRNAs severely impaired survival and induced cell death within 96 hours, confirming RPS6 as a crucial element of IGF1R survival signaling. In contrast, time course experiments with siRNAs to *TSC1* confirmed sustained effects of *TSC1* knockdown, with survival increasing up to 7-fold compared with controls (Fig. 2C, right; absolute values in Supplementary Fig. S2D). These data point to crucial roles for distal rather than proximal IGF1R pathway elements in survival of EFT cells, such as RPS6 and TSC1.

Knockdown of TSC1/TSC2 tumor suppressors induces *in vitro* BMS-536924 resistance

Because EFT cells are highly sensitive to both BMS-536924 and distal IGF1R pathway blockade, we wondered whether BMS-536924 efficacy is modulated by levels of distal proteins such as TSC1/TSC2, RPS6, or mTOR. To test this, cells were transfected with siRNAs to the above genes and then treated with varying doses of BMS-536924. First, *RPS6-* or *mTOR*-targeting siRNAs did not further sensitize cells to BMS-536924 because, as shown in Fig. 2A and C, the vast majority were already dead from knockdown alone. Second, siRNAs to proximal pathway elements, such as *IGF1R* itself, *AKT, IRS-1*, or *PDPK1*, failed to consistently sensitize cells to low-dose BMS-536924 (data not shown). Possibly, functional redundancy or complex feedback loops exist that override knockdown of proximal pathway components. Third, TSC1 knockdown markedly shifted dose-response curves over a broad BMS-536924 dose range, increasing ED_{50}s by up to 10-fold (Fig. 2D). Moreover, knockdown of TSC2, which complexes with TSC1 (34), also dramatically desensitized TC32 and BMS-536924–responsive SK-N-MC cells (Fig. 2D). Therefore, loss of TSC1/TSC2 induces marked resistance to BMS-536924 in EFT cells.

Distal IGF1R pathway responses correlate with BMS-536924 sensitivity

To more rigorously test the link between BMS-536924 resistance and activation states of distal IGF1R signaling components, we assessed BMS-536924 sensitivity in six additional childhood sarcoma cell lines. From dose-response analyses (Fig. 3A), EFT cell lines SK-N-MC, TTC633, and TTC466 were designated BMS-536924 sensitive (ED_{50} ~200 nmol/L), the EFT cell line 5838 as intermediate (ED_{50} ~500 nmol/L), and the Rh18 and Rh30 RMS cell lines as resistant (ED_{50} >10 and 2 µmol/L, respectively). We then analyzed IGF1R signaling
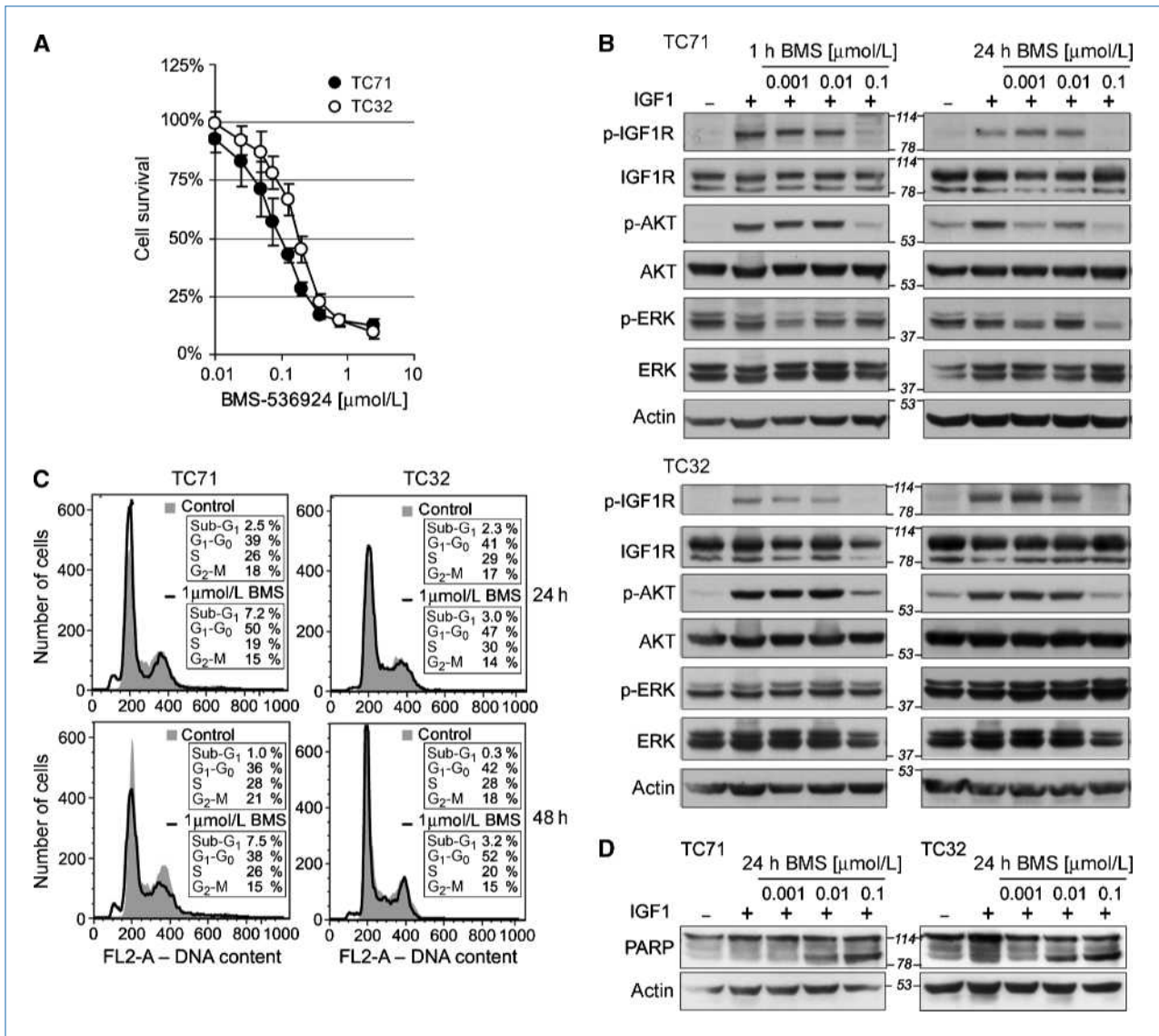
Figure 1. BMS-536924 is active in EFT cell lines *in vitro*. A, dose response to 72-h BMS-536924 treatment. Cell survival was analyzed by luminescence and graphed relative to nontreated control. Points, mean (*n* = 3); bars, SD. B, BMS-536924 effects on IGF1R signaling. Cells were treated with BMS-536924 or DMSO vehicle, IGF1 (100 ng/mL) was added for 15 min before lysis, and cell lysates were immunoblotted as specified; actin served as loading control. C, cell cycle distribution following BMS-536924 or DMSO vehicle treatment was analyzed by flow cytometry after propidium iodine staining of nuclei. Graphs show one representative experiment (*n* = 3). D, cell lysates from B, analyzed for induction of cell death by immunoblotting for cleaved PARP.

molecules in response to IGF1 stimulation. BMS-536924 sensitivity did not correlate with total IGF1R or AKT levels (Fig. 3B), and BMS-536924 inhibited phosphorylation of both IGF1R and AKT. Therefore, differential levels or activation of proximal molecules do not explain the distinct *in vitro* BMS-536924 sensitivities in these lines. In contrast, BMS-536924 sensitivity clearly correlated with its ability to block RPS6 and 4EBP1 phosphorylation. In fact, BMS-536924 failed to block RPS6 or 4EBP1 phosphorylation in both Rh18 and Rh30 cell lines, even at 1 and 10 µmol/L (Fig. 3B; Supplementary Fig. S3A). Moreover, RPS6 siRNA knockdown induced marked cell death in BMS-536924–resistant Rh18 and Rh30 cells (Supplementary

Fig. S3B). Because RPS6 and 4EBP1 are known mTORC1 targets (21), we tested effects of rapamycin on these cell lines. Rh18 and Rh30 cells are highly sensitive to rapamycin in cytotoxicity assays (19, 35), and we found that TC71 and TC32 cells were similarly sensitive (Supplementary Fig. S3C). Rapamycin potently inhibited phosphorylation of RPS6 both in BMS-536924–sensitive TC71 and TC32 cells and in BMS-536924–resistant Rh18 and Rh30 cells (Supplementary Fig. S3D). Therefore, although RPS6 and 4EBP1 phosphorylation are inhibited by BMS-536924 in sensitive sarcoma cells, these distal effectors are not affected by BMS-536924 treatment in resistant sarcoma cells (although they remain mTORC1 dependent).
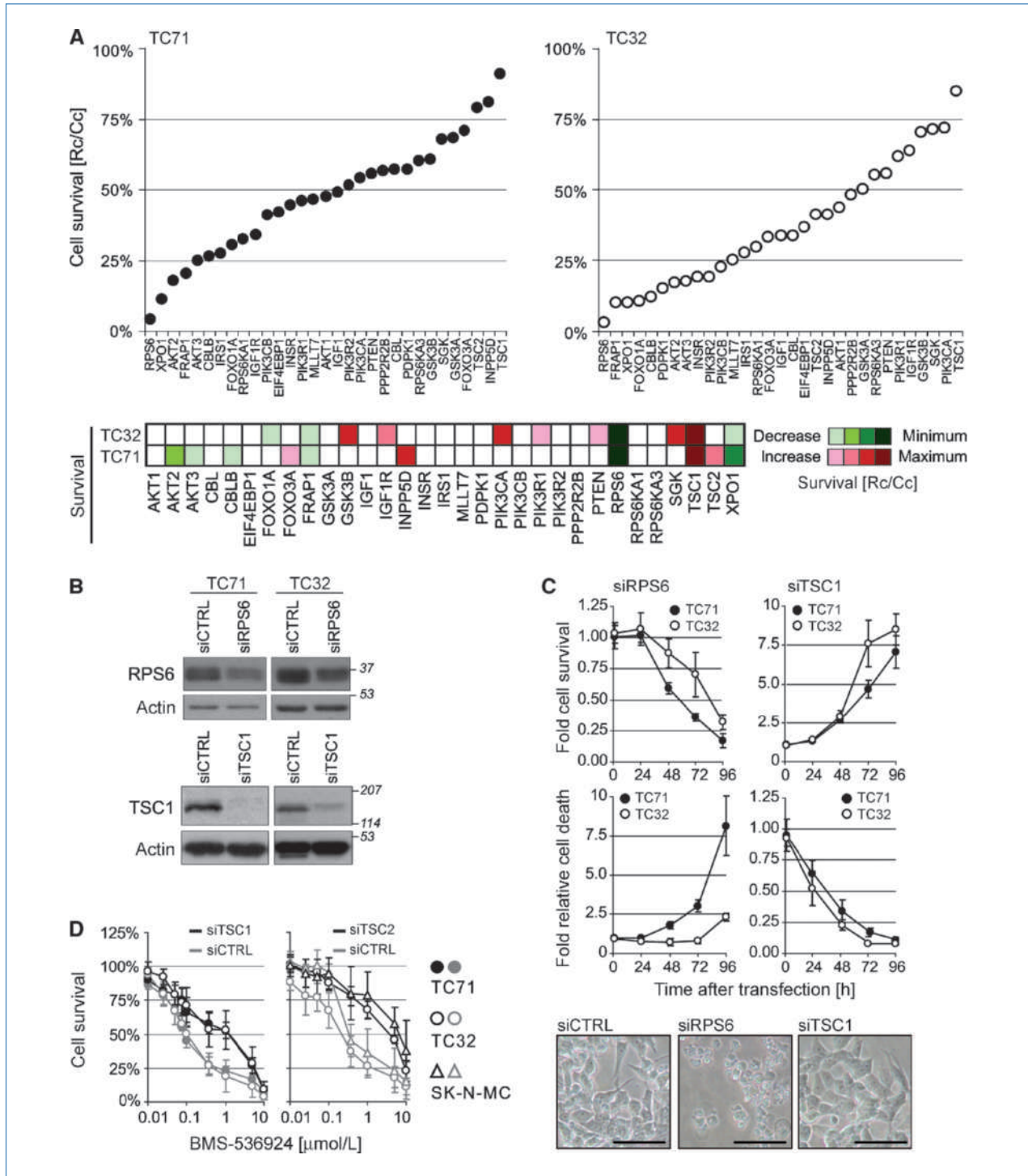
Figure 2. siRNA screening of the IGF1R signaling pathway. A, top, siRNAs rank ordered by effect on cell survival relative to transfection reagent alone (Rc/Cc); bottom, screening hits presented as heat map (for definitions, see Supplementary Materials and Methods). B, confirmation of protein knockdown. Cells were transfected with siRNAs targeting *RPS6* (siRPS6) and *TSC1* (siTSC1) or nontargeting control siRNAs (siCTRL) and analyzed after 20 h (*RPS6*) or 96 h (*TSC1*). C, time course analysis. In cells transfected as in B, survival was analyzed by luminescence and cell death by EthD-1 uptake. Top, survival was plotted relative to siCTRL; middle, cell death was graphed relative to siCTRL and surviving cell fractions to take into account that EthD-1 fluorescence intensity is directly linked to cell numbers remaining at each time point (for comparison, see absolute values in Supplementary Fig. S2D). Points, mean (*n* = 3); bars, SD. Bottom, microscopic images of TC71 cells at 96-h time point. Scale bars, 100 μm. D, TSC1/TSC2 knockdown induces BMS-536924 resistance. Cells were transfected with siTSC1, siRNAs targeting *TSC2* (siTSC2), or siCTRL and, 48 h later, exposed to BMS-536924 for another 48 h. Survival analyzed by luminescence (TSC1) or WST-1 (TSC2) is graphed relative to non–BMS-536924–treated control. Points, mean (*n* = 3); bars, SD.
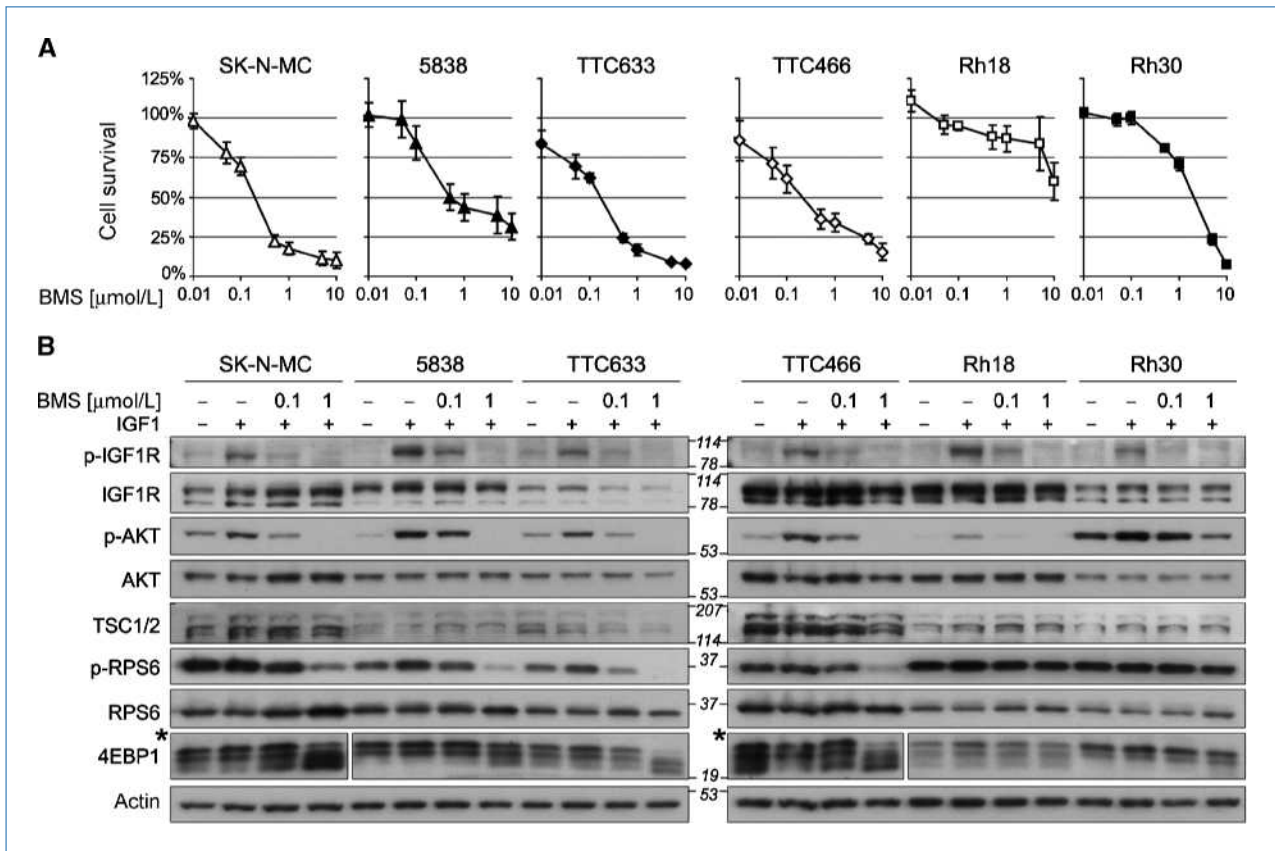
Figure 3. Distal IGF1R pathway signaling responses correlate with BMS-536924 sensitivity. A, dose-response series of EFT and RMS cell lines treated with BMS-536924 for 72 h. Survival was measured by WST-1 assay and graphed relative to nontreated controls. Points, mean ($n \geq 3$); bars, SD. B, IGF1R signaling in cells treated with BMS-536924 or DMSO vehicle for 1 h followed by IGF1 (100 ng/mL) for 15 min. Cell lysates were probed as indicated; actin was loading control. All panels are equal exposures. Asterisk indicates shorter exposure.

Knockdown of MST1R sensitizes sarcoma cells to BMS-536924

Based on the above, we reasoned that alternative pathways might regulate RPS6 activation in BMS-536924–sensitive and BMS-536924–resistant cell lines. Because numerous PTKs converge on mTORC1 signaling, activation of another PTK could contribute to BMS-536924 resistance. To explore this systematically, we performed screens using an siRNA library targeting all 88 known human PTKs to identify PTKs whose inactivation enhances BMS-536924 activity. Briefly, parallel sets of TC71 and TC32 cells were transfected with a library of siRNA pools (four siRNA duplexes/target gene), followed after 48 hours by BMS-536924 treatment of one set and vehicle treatment of the other. A sublethal dose of BMS-536924 ($\sim$ED$_{10}$; Supplementary Fig. S4A) was selected to ensure detection of both sensitizing and antagonizing siRNAs. After another 48 hours, fractional survival was determined for siRNAs alone (Fig. 4A; Supplementary Fig. S4B) and siRNAs plus BMS-536924 (Supplementary Fig. S4B). A sensitivity index was calculated for each cell line (see Supplementary Materials and Methods; ref. 36) to define those siRNA pools with either sensitizing or antagonistic effects on BMS-536924 activity (Fig. 4B). Protein knockdown and cell survival effects of individual siRNAs were validated for selected PTKs (Fig. 5A; Supplementary Fig. S2B; data not shown). In the nondrug-treated screen, knockdown of several PTKs significantly impaired fractional survival in both cell lines, including IGF1R, EPHB2, FES, Src family kinases HCK and LCK, LMTK2, NTRK2, MUSK, PDGFRβ, and PTK9L (Fig. 4A), highlighting these PTKs as potential therapeutic targets in EFTs to be further studied elsewhere. Here, we focused on BMS-536924–sensitizing siRNAs because corresponding proteins may contribute to drug resistance.

Those siRNAs sensitizing both lines to BMS-536924 included MST1R (macrophage-stimulating 1 receptor), LTK, and FLT3 (Fig. 4B). The most pronounced effects were observed for MST1R, a MET-related PTK also known as RON (37). Overexpression and activation of MST1R is associated with poor clinical outcome in several cancers (38), but this PTK has not been studied in sarcomas. MST1R knockdown was confirmed by Western blotting (Fig. 5A), and alone actually promoted survival of TC71 and TC32 cells slightly, whereas slightly decreasing Rh30 survival (Fig. 5B). Four individual siRNAs to *MST1R* had similar effects on cell survival (data not shown). With BMS-536924, however, MST1R knockdown markedly sensitized all three cell lines to BMS-536924, with dramatically increased cell death and a left shift of

dose-response curves (Fig. 5C; Supplementary Fig. S5A and B). This occurred over a broad dose range with maximum sensitization observed near the ED_{50}s for each line (Supplementary Fig. S5C). Maximal sensitization occurred in BMS-536924–resistant Rh30 cells, where the ED_{50} was shifted by 10-fold from ~2 μmol/L to 200 nmol/L. Thus, MST1R knockdown was sufficient to restore BMS-536924 sensitivity to resistant cells. Interestingly, whereas INSR knockdown strongly sensitized TC71 cells to BMS-536924, the opposite was seen in TC32 cells (Fig. 4B; Supplementary Fig. S4B). The basis for this disparity is unknown and warrants further investigation.

MST1R knockdown blocks distal IGF1R signaling in BMS-536924–resistant cells

Next, we analyzed how BMS-536924 sensitization by MST1R knockdown correlates with IGF1R signaling in either
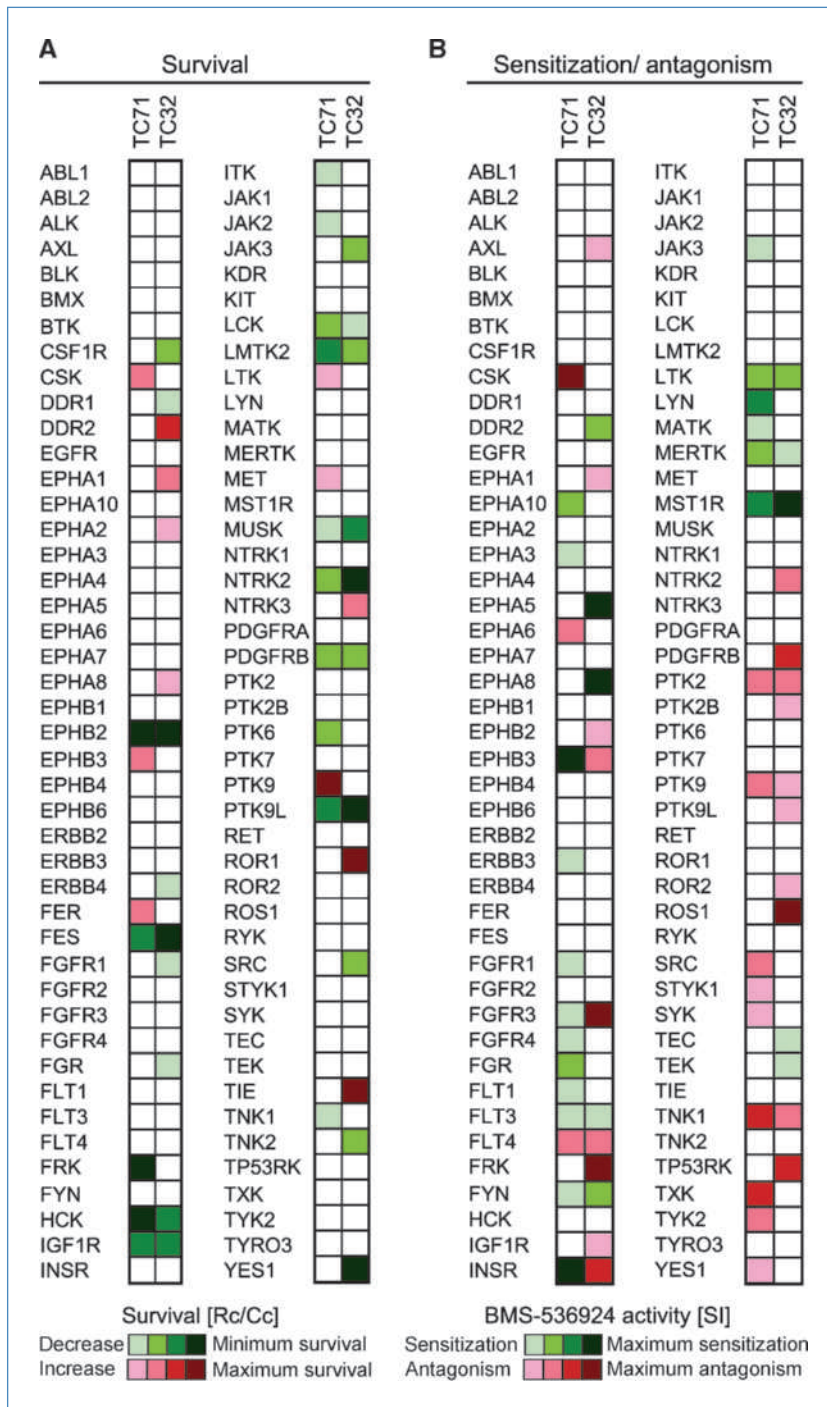

Figure 4. Synthetic lethal siRNA screens of all human PTKs. Screening hits are represented as heat maps (for definitions, see Supplementary Materials and Methods). A, cell survival effects of specific siRNAs alone compared with transfection reagent only (Rc/Cc). B, sensitizing and antagonizing siRNA effects on BMS-536924 activity as determined by sensitivity index (SI).
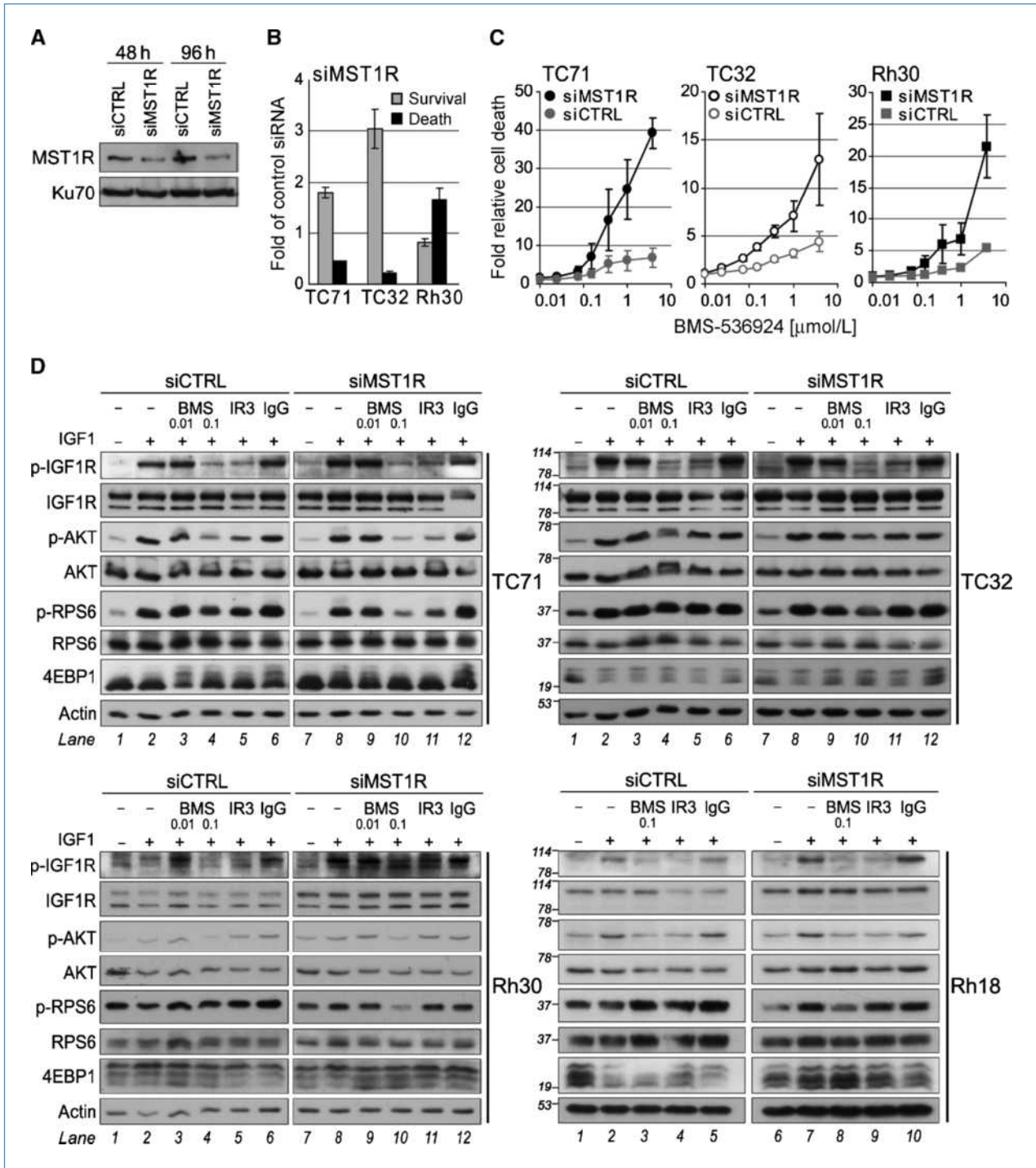
Figure 5. MST1R knockdown sensitizes sarcoma cells to BMS-536924. A, MST1R protein knockdown in TC32 cells transfected with siRNAs targeting *MST1R* (siMST1R) or nontargeting siCTRL. Ku70 was loading control. B, effects of MST1R knockdown on cell viability. Luminescence and EthD-1 uptake were measured 96 h after transfection. Survival was graphed relative to siCTRL, and cell death relative to siCTRL and surviving cell fraction. Columns, mean (*n* = 3); bars, SD. C, dose response in cells transfected 48 h previously with siMST1R or siCTRL and treated with BMS-536924 for 48 h. Cell death was analyzed by EthD-1 uptake and graphed relative to non-BMS-536924–treated control and surviving cell fractions (Supplementary Fig. S5A) to take into account effects of siRNAs alone (see B) and that EthD-1 fluorescence is directly linked to cell numbers remaining at each dose (for absolute values, see Supplementary Fig. S5B). Points, mean (*n* = 3); bars, SD. D, MST1R knockdown enhances BMS-536924 activity on distal IGF1R signaling components. Seventy-two hours after siRNA transfection, cells were treated for 1 h with BMS-536924 (0.01 and 0.1 μmol/L; 0.01 μmol/L not tested in highly resistant Rh18 cells), αIR3 antibody (100 ng/mL), or IgG control (100 ng/mL), followed by IGF1 (100 ng/mL, 15 min). Lysates were immunoblotted as indicated with actin as loading control.

BMS-536924–sensitive TC71 and TC32 versus BMS-536924–resistant Rh18 and Rh30 cells. We chose a low 10 to 100 nmol/L BMS-536924 dose range to critically assess drug sensitization. Several key findings emerged (Fig. 5D). First, MST1R knockdown alone did not alter expression or baseline activity of IGF1R or AKT in the cell lines (Fig. 5D; lanes 1 versus 7 for TC71, TC32, and Rh30, and lanes 1 versus 6 for Rh18). Second, MST1R knockdown alone had little effect on phospho-RPS6 levels, other than slightly decreasing baseline levels in the two BMS-536924–resistant cells, Rh30 (lanes 1 versus 7) and Rh18 (lanes 1 versus 6). Third, 100 nmol/L BMS-536924 inhibited IGF1R and AKT activation in all cell lines, but effects on RPS6 activation were not apparent in control siRNA–transfected cells, consistent with Fig. 3B. Fourth and most notably, MST1R knockdown markedly enhanced inhibitory effects of BMS-536924 on RPS6 phosphorylation in all cell lines (lanes 4 versus 10 for TC71, TC32, and Rh30, and lanes 3 versus 8 for Rh18). Therefore, MST1R contributes to activation of the essential distal IGF1R effector, RPS6, in BMS-536924–resistant cell lines. By comparison, αIR3 anti-IGF1R antibodies alone blocked IGF1R activation in all cell lines, but only minimally decreased AKT activation (Fig. 5D). Alone, αIR3 did not appreciably affect RPS6 phosphorylation in TC32, Rh30, or Rh18 cell lines, nor did it enhance effects of MST1R knockdown. In the αIR3-sensitive cell line TC71 (Supplementary Fig. S1B), RPS6 activation was minimally decreased by αIR3 alone and enhanced in combination with MST1R knockdown (lanes 2, 5, and 11). The basis for this discordance with BMS-536924 is unknown, but is consistent with its superior activity on survival of TC71 and TC32 cell lines compared with αIR3 (Supplementary Fig. S1B). Our PTK siRNA screening studies therefore reveal an unexpected role for MST1R in modulating distal IGF1R effectors, and highlight this PTK as a candidate therapeutic target for combination with IGF1R inhibitors.

MST1R is expressed in childhood sarcomas

We next analyzed mRNA expression of MST1R and its ligand, MST1, in 109 childhood sarcoma samples by gene expression profiling. All sarcoma subtypes showed variable but significantly higher MST1R expression than normal control bone marrow–derived MSCs (Fig. 6A). Similarly, MST1 expression was significantly higher in all sarcoma types other than alveolar RMS (Fig. 6B). Although MSCs were used as normal tissue controls for MST1R and MST1 mRNA expression in sarcomas, this does not infer overexpression in the latter, as the relationship of EFT and RMS to normal bone marrow–derived MSCs remains unclear. Western blotting confirmed variable MST1R protein expression in all five EFT cell lines tested, and in three of four RMS cell lines (see Fig. 6C). A phospho-specific MST1R antibody showed activation in all cell lines except TC71 and RD (Fig. 6C). Finally, MST1R expression was examined in primary sarcoma samples by immunohistochemistry on tissue microarrays. Eleven of 22 (50%) EFT, 15 of 21 (71%) alveolar RMS, and 12 of 25 (48%) embryonal RMS (ERMS) cases stained for MST1R, defined as membranous/cytoplasmic positivity in >10% of cells (Fig. 6D; Supplementary Table S2). Therefore,

although additional studies are required, our findings indicate that MST1R is expressed in some childhood sarcomas, consistent with a role for this PTK in activation of distal IGF1R effectors.

Discussion

There is an urgent need to develop new therapeutics for high-risk childhood sarcomas such as EFT and RMS. Mounting preclinical data and early promise in clinical trials have led to intense evaluation of IGF1R inhibitors in these diseases. Although there is clear efficacy of IGF1R-blocking antibodies in some patients, others remain resistant or become so during therapy. However, the biological basis for resistance remains unknown, and biomarkers to select patients most likely to benefit from IGF1R blockade and to monitor treatment response are poorly demarcated. Here, we used an siRNA screening approach to determine which elements of the IGF1R signaling axis are crucial for its prosurvival function in childhood sarcoma cells, and to investigate PTK networks that influence activity of the BMS-536924 IGF1R inhibitor. Our findings suggest that activation of the distal signaling molecule RPS6 plays a critical role in pro-oncogenic IGF1R signaling in both EFT and RMS cells, and that RPS6 activation is sustained in BMS-536924–resistant cells even when drug treatment blocks proximal IGF1R and AKT activation. Moreover, knockdown of MST1R restores BMS-536924 efficacy in highly drug-resistant cell lines, and this correlates with inhibition of RPS6 phosphorylation. We propose that MST1R is a potential modifier of IGF1R inhibitor sensitivity in childhood sarcoma cells through its ability to function as an alternative activator of distal IGF1R signaling molecules including RPS6.

Current biomarkers for IGF1R inhibitor efficacy focus on expression and/or activity of intrinsic proximal components of IGF1R signaling, including IGF1, IGFBPs, IGF1R itself, and AKT (1, 3, 18, 26). In contrast, our findings highlight an important role for distal IGF1R signaling elements in predicting response to IGF1R blockade. When we analyzed BMS-536924 efficacy in sensitive versus resistant cell lines, expression levels and activity of IGF1R pathway elements did not correlate with response. In fact, BMS-536924 blocked IGF1R and AKT phosphorylation even in resistant cells, indicating that proximal pathway elements do not necessarily accurately represent responses. These findings are supported by those of Kurmasheva and colleagues (39), which show a close correlation between RPS6 activity and in vitro and in vivo response to the anti-IGF1R antibody CP-751,871 in childhood sarcoma cell lines. Taken together, our studies highlight RPS6 as a key survival factor and its blockade as a critical indicator of the cytotoxic activity of IGF1R inhibitors.

As a component of the 40S ribosome subunit, RPS6 is critical for protein translation (40). RPS6 activation may represent a point of "oncogene addiction" in transformed cells that are undergoing high proliferative rates and require rapid protein translation, especially of key stress-adaptive mRNAs. As a downstream effector of PI3K-AKT–mediated mTORC1-dependent translational control, one approach to target RPS6
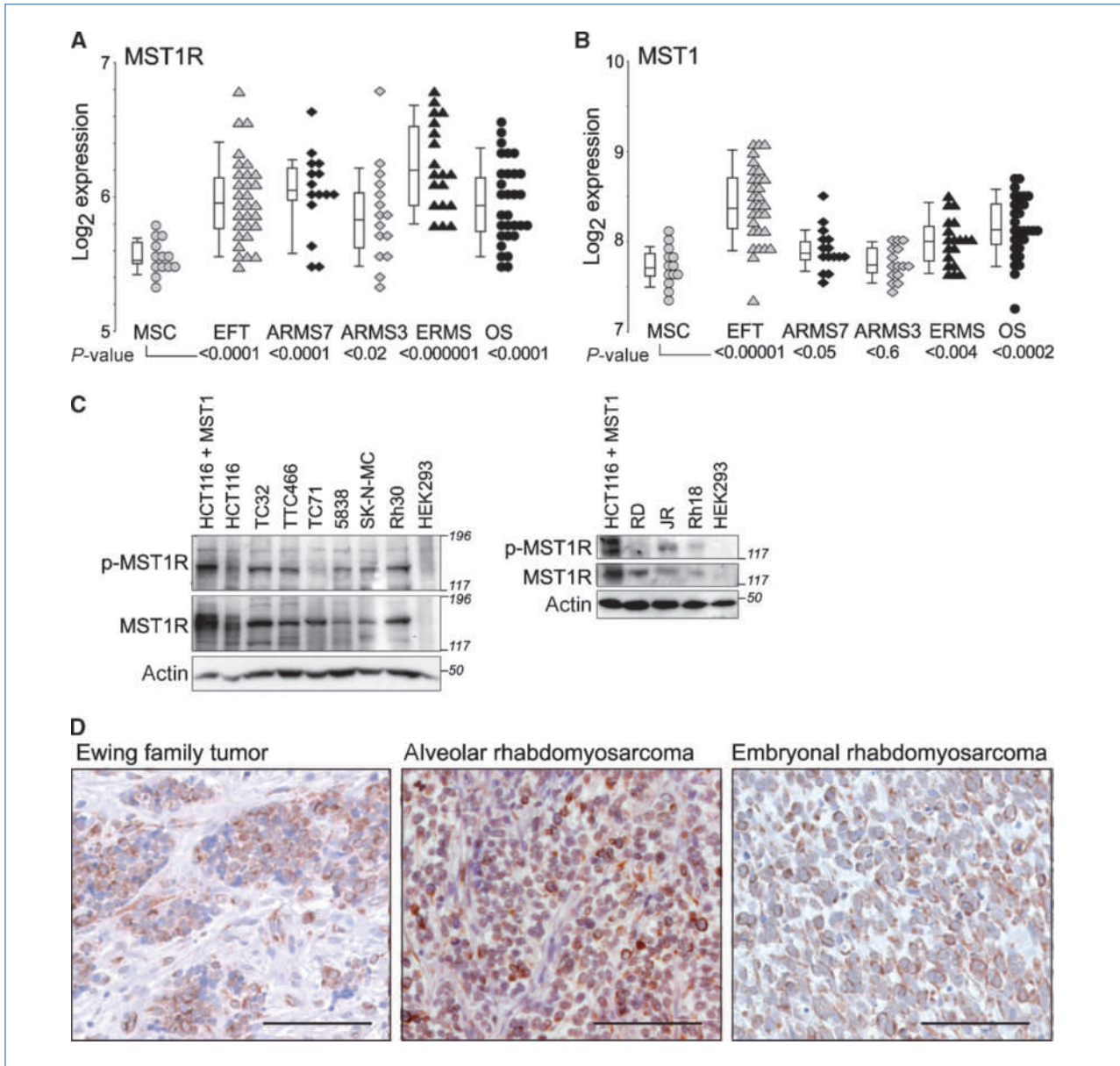
Figure 6. MST1R is expressed in childhood sarcomas. *MST1R* (A) and *MST1* (B) mRNA expression in 30 EFTs, alveolar RMS with PAX7-FKHR fusion (ARMS7) and PAX3-FKHR fusion (ARMS3; 15 each), 19 ERMS, 30 osteosarcomas (OS), and MSC controls as analyzed by U133A arrays. Symbols beside each box plot represent individual samples. Three individual *MST1* probes tested, probe 216320_x_at shown. C, left and right, MST1R protein expression and activation in sarcoma cell lines. Cell lines grown in serum-containing medium were analyzed for phosphorylated and total MST1R; HCT116 cells stimulated with MST1 (5 nmol/L, 20 min; lane 1) served as positive and HEK293 cells as negative controls. D, immunohistochemical analysis of MST1R protein expression in tissue microarrays of childhood sarcomas using antibodies to total MST1R. Shown are representative examples of highly positive MST1R-expressing EFT, ARMS, and ERMS primary tumors (i.e., >50% positive cells). Scale bars, 100 μm.

is via mTORC1 inhibitors such as rapamycin. Because IGF1R seems to be the major activator of the latter cascade in childhood sarcoma cells, combining IGF1R inhibitors with rapamycin is an attractive strategy to avoid stimulating this pro-oncogenic feedback loop, and preclinical and clinical studies are under way to test the long-term efficacy of this approach (http://www.clinicaltrials.gov; refs. 18, 39). However, previous preclinical studies report an uncoupling be-

tween IGF1R and AKT activation in RMS xenografts once cells became resistant to IGF1R-blocking antibodies (18), suggesting the presence of alternative mechanisms for activating the PI3K-AKT cascade and downstream pathways in childhood sarcoma cells.

Our combinatorial siRNA approach to screen for modifiers of BMS-536924 activity in EFT cells identified MST1R knockdown as dramatically sensitizing cell lines to BMS-536924

over a broad dose range, potently restoring BMS-536924 cytotoxic activity, and overcoming primary drug resistance. Activation of receptor PTKs as a mechanism to confer resistance to targeted inhibitors in human cancers is well described (41). It is widely known that PTKs do not act independently, but form redundant cellular networks to activate common pathway elements, and as such can confer resistance to specific kinase inhibitors (41). For example, EGFR and other HER family receptors are known to mediate resistance to IGF1R inhibitors and vice versa, and cotargeting both PTKs results in enhanced antitumor activity (26–28). It is therefore conceivable that MST1R similarly modulates IGF1R inhibitor activity through activation of distal molecules such as RPS6. MST1R, also called RON (recepteur d'origine nantais) or PTK8, is a MET family receptor PTK (37). Activated by its specific ligand MST1 (hepatocyte growth factor–like), MST1R activates many similar pathways as MET, including PI3K-AKT and Ras-ERK, to regulate cell proliferation, adhesion, and motility (38, 42). MST1R overexpression and activation is associated with poor clinical outcome in various epithelial cancers, but this PTK has not been studied in childhood tumors. By gene expression profiling, MST1R mRNA expression was detected not only in EFT and RMS but also in osteosarcoma primary tumors. MST1R mRNA levels alone did not affect event-free or overall survival in the EFT or RMS cohorts tested (data not shown). However, analysis in an IGF1R inhibitor–treated cohort may be necessary to rigorously assess influences of MST1R expression on survival. In addition, we confirmed MST1R protein expression by Western blotting in EFT and RMS cell lines as well as in primary tumors by immunohistochemistry. Interestingly, MST1R was activated in almost all EFT and RMS cell lines tested based on MST1R phosphotyrosine antibody reactivity. Whether this represents ligand-dependent versus constitutive activation remains to be determined, as does MST1R activation in primary sarcoma samples. If MST1R indeed modifies IGF1R inhibitor responses, then it may represent a tractable biomarker for IGF1R inhibitor sensitivity. Moreover, combination therapy targeting both IGF1R and MST1R may provide a viable treatment option in childhood sarcomas, particularly with therapeutic MST1R and MET inhibitors being in preclinical and clinical development, and we are currently exploring this possibility. Additional synthetic lethal siRNA screens in cell lines with known primary or secondary resistance to IGF1R inhibitors may reveal further PTKs whose cotargeting improves activity of IGF1R inhibitors in childhood sarcomas.

Small-molecule tyrosine kinase inhibitors with activity against both IGF1R and INSR are emerging as a second, potentially more potent class of targeting compounds compared with blocking antibodies that target IGF1R alone (1–3). In the current study, we used the dual specificity IGF1R/INSR kinase inhibitor BMS-536924 and confirmed *in vitro* activity in EFT cell lines and reports of EFT cell lines being more frequently sensitive to IGF1R inhibition (six of six) than RMS cell lines (zero of two; refs. 26, 29, 43). BMS-536924 activity was superior to the αIR3 monoclonal IGF1R antibody, which showed only modest inhibition of cell survival. How BMS-536924 compares with humanized antibodies currently in clinical development remains to be determined. Our data point to a potential role for small-molecule IGF1R kinase inhibitors in the therapy of childhood sarcomas, particularly EFTs.

In summary, our findings indicate that other PTKs may converge on common distal signaling elements to mediate resistance to IGF1R inhibitors, suggesting that new combinatorial targeting strategies are required to effectively block IGF1R signaling in drug-resistant tumors. In the screening approach presented here, we identified MST1R as one potential combinatorial target. More generally, our results underscore the need to fully understand how PTK networks may function together in a particular tumor type to successfully implement PTK inhibitor strategies.

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