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Identification of mutations associated with acquired resistance to sunitinib in renal cell cancer

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Sunitinib is one of the most widely used targeted therapeutics for renal cell carcinoma (RCC), but acquired resistance against targeted therapies remains a major clinical challenge. To dissect mechanisms of acquired resistance and unravel reliable predictive biomarkers for sunitinib in RCC, we sequenced the exons of 409 tumor-suppressor genes and oncogenes in paired tumor samples from an RCC patient, obtained at baseline and after development of acquired resistance to sunitinib. From newly arising mutations, we selected, using *in silico* prediction models, six predicted to be deleterious, located in *G6PD, LRP1B, SETD2, TET2, SYNE1*, and *DCC*. Consistently, immunoblotting analysis of lysates derived from sunitinib-desensitized RCC cells and their parental counterparts showed marked differences in the levels and expression pattern of the proteins encoded by these genes. Our further analysis demonstrates essential roles for these proteins in mediating sunitinib cytotoxicity and shows that their loss of function renders tumor cells resistant to sunitinib *in vitro* and *in vivo*. Finally, sunitinib resistance induced by continuous exposure or by inhibition of the six proteins was overcome by treatment with cabozantinib or a low-dose combination of lenvatinib and everolimus. Collectively, our results unravel novel markers of acquired resistance to sunitinib and clinically relevant approaches for overcoming this resistance in RCC.

Tumor Markers and Signatures

Key words: sunitinib, renal cell carcinoma, biomarkers, resistance, MCL-1, mTORC1, *G6PD*, *LRP1B*, *SETD2*, *TET2*, *SYNE1*, *DCC*
Additional Supporting Information may be found in the online version of this article.

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What's new?

Sunitinib, a tyrosine kinase inhibitor, is one of the most commonly used targeted therapeutics for treatment of metastatic renal cell carcinoma (mRCC). However, the majority of patients who initially respond eventually develop acquired resistance. In the present study, by analyzing sequential tumor biopsies from an mRCC patient who developed acquired resistance to sunitinib, the authors identified mutations in genes whose loss of function conferred sunitinib resistance to tumor cell lines and xenografted mice and went on to identify critical downstream action mechanisms. The findings may be relevant for the development of predictive biomarkers and new therapeutic strategies in mRCC.

Introduction

Development of acquired resistance is a common phenomenon in most cancer patients that initially respond to targeted therapies. The study of sequential biopsies, obtained at baseline and at the moment of progression, from these patients has allowed the identification of newly acquired mutations in the driver genes, which explain the development of acquired resistance. This strategy has been pursued to identify mutations that conferred resistance to imatinib in chronic myeloid leukemia[1] and in gastrointestinal stromal tumors[2] and was subsequently implemented for epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI)[3] and *EML4-ALK* translocations[4] in nonsmall cell lung cancer, EGFR inhibitors in colorectal cancer,[5] and BRAF inhibitors in melanoma,[6] among others, as reviewed elsewhere.[7]

Sunitinib constitutes a mainstay treatment for metastatic renal cell carcinoma (mRCC), but unfortunately, most patients who respond eventually develop acquired resistance.[8] We hypothesized that next-generation sequencing (NGS) of tumors obtained at baseline and at progression from mRCC patients developing acquired resistance after initial responses could identify mutations that confer acquired resistance to therapy and that such mutations could affect tumor driver genes or related pathways, helping to identify predictive biomarkers for mRCC (i.e., reverse identification).[7]

Methods

Patient selection

We identified mRCC patients treated with sunitinib that presented a clinical response followed by development of acquired resistance and that had tumor samples available at baseline and at progression. Obtaining samples at progression was justified by the routine care of the patients (i.e., confirmation of progression or resection of progressing lesions that was clinically indicated for the patient's benefit). Only patients with baseline tumor samples collected <6 months before treatment initiation and after tumor progression were eligible for the study, in order to improve the chronological correlation between the molecular profile of the tumor and the clinical events analyzed. The study was designed following the DESIGN guidelines.[7] Patients signed informed consent and the protocol was approved by our institution's ethical review board.

Tumor DNA sequencing

DNA was extracted from fresh-frozen tumoral tissue after pathological selection using a Maxwell® 16 MDx Instrument (Promega, Promega Biotech Ibérica S.L., Madrid, Spain) and quantified by Qubit (Qiagen, Madrid, Spain).

We sequenced sequential paired samples from the same patient in an Ion S5 NGS system using the Ion AmpliSeq™ Comprehensive Cancer Panel (ThermoFisher Scientific, Madrid, Spain), which targets the exons of 409 tumor-suppressor genes and oncogenes. In brief, 25 ng tumor DNA per primer pool for a total of 100 ng input DNA was used for sequencing. The Ion AmpliSeq™ Comprehensive Cancer Panel is optimized for library construction with the Ion AmpliSeq™ Library Kit 2.0. Libraries are then ready for template preparation on the Ion OneTouch™ System and sequencing on the Ion S5™ Sequencer.

Criteria for selection of candidate mutations associated with acquired resistance to sunitinib

The novel mutations arising at progression were filtered according to minor allele frequency (MAF; those with MAF > 0.1% in ExAC or 1,000 genomes databases were excluded) and prioritized according to their effect on protein function and location and on prediction of pathogenicity using Alamut® Visual v2.7.2. (Interactive Biosoftware, Rouen, France). Genomic variants were analyzed and interpreted with Alamut® Visual, a software application that uses information from different public databases such as NCBI, EBI, and UCSC, as well as other sources including gnomAD, ESP, Cosmic, ClinVar, or HGMD and CentoMD. This software uses several criteria to identify pathogenic variants, including predicted changes in aminoacid sequences that may interfere with the protein function. The tool also provides nucleotide conservation data through many vertebrate species, with the phastCons and phyloP scores, amino acid conservation data through orthologue alignments, and information on protein domains. Finally, Alamut® Visual integrates several missense variant pathogenicity prediction tools and algorithms and offers a window dedicated to the *in silico* study of variants' effect on RNA splicing.

Reagents

Antibodies were purchased from the indicated sources and used at a dilution of 1:1,000–1:2,000: anti-MCL-1 (Santa Cruz Biotechnology, Heidelberg, Germany); anti-pS6K1, anti-S6K1, anti-pS6, anti-S6, anti-pGSK3β, anti-GSK3β, anti-pERK, anti-ERK and SETD2 (Cell Signaling Technology, Frankfurt am Main, Germany); lipoprotein receptor-related protein 1B (Sigma Aldrich, Vienna, Austria); SYNE1 and TET2 (Bethyl Laboratories, Montgomery, USA); DCC (BD, Schwechat, Austria) and G6PD (Abcam, Cambridge, UK). Sunitinib was purchased from Sigma Aldrich (Sigma Aldrich, Vienna, Austria) and cabozantinib, lenvatinib, everolimus, and axitinib from Selleck Chemicals (Munich, Germany).

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Tissue culture

RCC (A-498 and ACHN) cell lines were kind gifts from Dr. Axel Ullrich (Max-Planck Institute, Martinsried, Germany). The authenticity of those cell lines has been proven by DNA profiling using GenePrint 10 Promega kit. Sunitinib-desensitized paired cell lines were generated by continuous culturing in the presence of increasing doses of sunitinib as described as described elsewhere.[9] All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine unless otherwise indicated. All cells were maintained in a humidified tissue culture incubator at 37°C in 5% CO_2. Cell death was assessed by trypan-blue exclusion assay.

Immunoblotting

Whole cell lysates were prepared by directly lysing cells growing in culturing dishes or collected cell pellets in lysis buffer (40 mM Hepes pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and EDTA-free protease inhibitors [Roche] containing 0.3% CHAPS). Lysates were cleared by centrifugation at 13000*g* for 15 min at 4°C, quantified using BioRad DC protein assay reagent followed by mixing 1:1 with 4% sodium dodecyl sulfate (SDS), 100 mM Tris-Cl pH 6.8, 20% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol added immediately before use and heating at 94°C for 7 min. Equal amounts of proteins were then electrophoresed on 8–15% SDS-polyacrylamide gel electrophoresis gels. Gels were run at 50 V (stacking gel)/90 V (separation gel) on a Protean III apparatus (BioRad). Gels were transferred onto nitrocellulose and probed with the appropriate primary antibody for a variable incubation time depending on the experimental design, followed by the corresponding secondary antibodies diluted 1:1,000–2,000. The proteins were visualized by enhanced chemiluminescence using a ChemiDoc apparatus (BioRad) according to the manufacturer's instructions.

RNA interference

shRNA pLKO.1 lentiviral constructs were purchased from Open Biosystems. Target sequences are as follows:

Scrambled: GTGGACTCTTGAAAGTACTAT.
SETD2#1: CCTGAAGAATGATGAGATAAT.
SETD2#2: GCCCTATGACTCTCTTGGTTA.
LRP1B#1: CGGCATTTACAGTCCCTGATA.
LRP1B#2: GCTGTAAAGATCAAGATGAAT.
SYNE1#1: GCAGTTTAACTCAGACTTGAA.
SYNE1#2: GCGTAGTGATAAGACTGATTT.
TET2#1: GCCAAGTCATTATTTGACCAT.
TET2#2: CAGTCTAATGTACGAACTTTA.
DCC#1: CCATCCAATGTAGTAGCCATT.
DCC#2: GCGTCTCTACTGATGATATAA.
G6PD#1: GTCGTCCTCTATGTGGAGAAT.
G6PD#2: CAACAGATACAAGAACGTGAA.

Lentiviral transduction

The pLKO.1 vectors and package plasmids were cotransfected into packaging HEK293T cells and the viral supernatants were collected, supplemented with polybrene (8 μg/mL) and used to infect target cells in four cycles of transduction, 2 hr each over two consecutive days.

Xenografts

Six-week-old female nude CD1 mice (n = 5) purchased from Charles River Laboratories received subcutaneous flank injections of 1 × 10^6 A-498 cells transduced with the indicated shRNAs and suspended in 200 μL saline. After the tumors were established, mice received either dextrose–water vehicle or sunitinib (30 mg/kg) dissolved in 5% dextrose–water and administered by daily gavage for the indicated time points. Tumor growth was monitored by bidimensional measurements obtained with a caliper. Experiment on animals was approved by Ethics Committee in accordance with EU 86/609 Directive (Council Directive 86/609/EEC of November 24, 1986 on the approximation of laws, regulations, and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes). Mice were housed according to the guidelines set out in Commission Recommendation 2007/526/EC—June 18, 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes.

Statistics

Results are representative of three independent experiments and are expressed as the mean, and error bars indicate SEM. A Student's t test was applied to assess significance.

Results

Identification of mutations associated with sunitinib resistance in clinical samples

From a cohort of 108 mRCC patients treated with sunitinib, only one patient met the requirements of presenting acquired resistance and having tumor biopsies at baseline and after development of resistance with sufficient tissue to perform NGS in both samples. This patient underwent a partial nephrectomy in December 2007 (baseline sample) to excise a relapsing clear-cell RCC. In March 2008, he presented an abdominal relapse and started sunitinib 50 mg/24 hr in four weekly cycles, followed by 2 weeks of rest. He experienced a partial response that lasted until October 2010, when he developed adrenal and vertebral progression that compressed the spinal cord, thus requiring surgical resection in December 2010 (sample at progression; Figs. 1*a*–1*d*).

Sequencing of paired samples from this patient with the Ion AmpliSeq™ Comprehensive Cancer Panel revealed 5,210 differential genetic variants between both samples. Six mutations that were predicted to be deleterious according to the Alamut® Visual v2.7.2 were located in *G6PD, LRP1B, SETD2, TET2, SYNE1*, and *DCC* (Table 1).

Sensitivity to sunitinib in cancer cell lines is associated with differential expression of the resistance-associated proteins

We compared the expression of the proteins encoded by the genes that harbored the identified mutations in lysates derived

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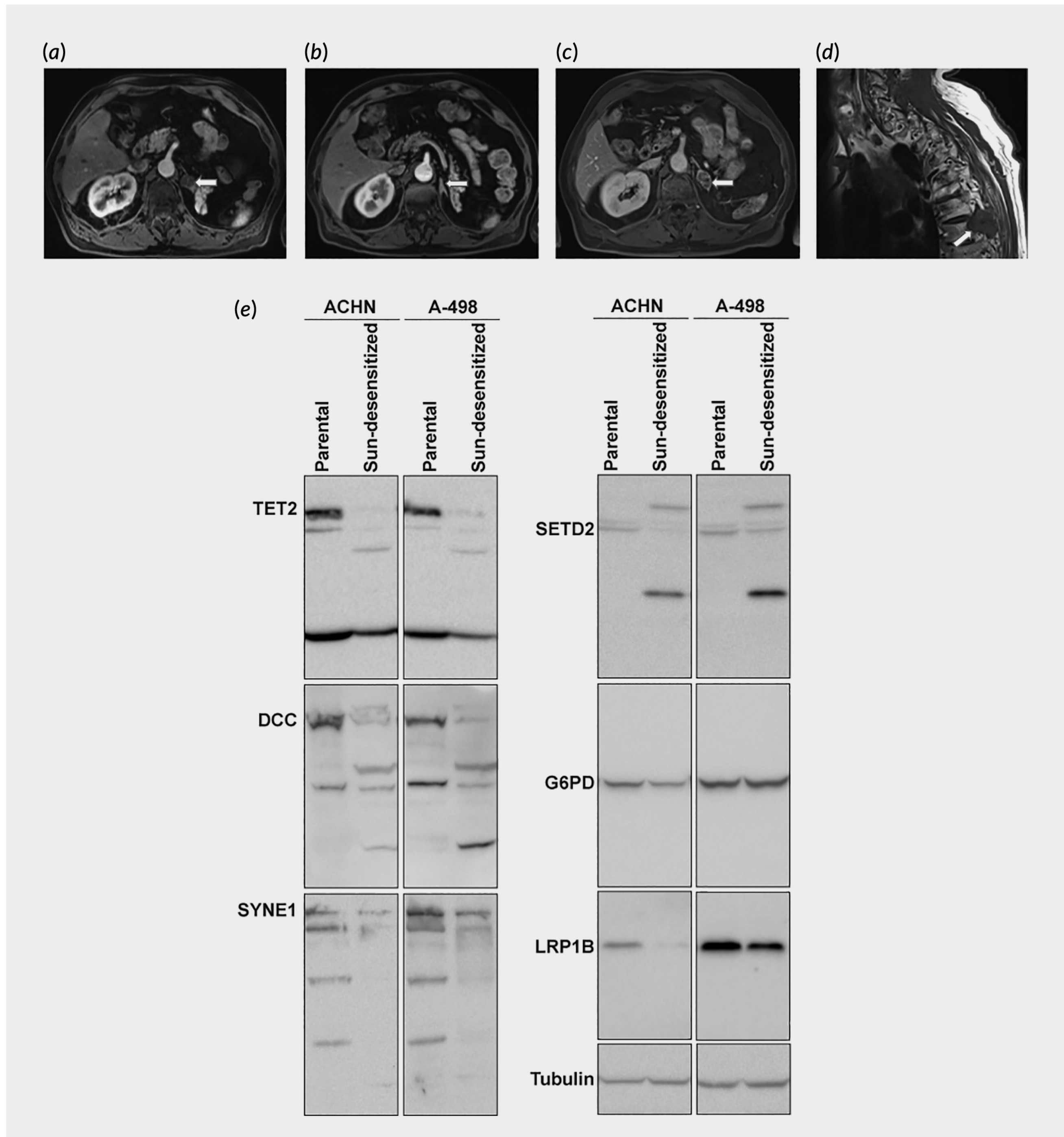

Figure 1. Identification of mutations associated with sunitinib resistance in clinical samples and cell lines. (*a–d*) Clinical evolution and radiological images of the patient studied. (*a*) March 2008: Patient starts sunitinib after abdominal progression. Arrow shows an adrenal metastasis. (*b*) March 2009: MRI confirms a major response of the adrenal metastasis (arrow). (*c* and *d*) October 2010: patient presents adrenal (*c*) and spine progression (*d*) while receiving sunitinib (arrows). The spinal lesion is resected in December 2010 to avoid medullary compression. (*e*) Immunoblotting analysis of lysates prepared from either parental or sunitinib-desensitized cells with antibodies against G6PD, LRP1B, SETD2, TET2, SYNE1, and DCC showed differential expression for all the proteins studied.

from either parental or sunitinib-desensitized cancer cells lines by immunoblotting analysis (Fig. 1*e*). All resistance-associated proteins showed differences between parental and sunitinib-desensitized cancer cells in terms of the levels of the full-length proteins as well as in the patterns of other bands detected on the blots. Generally, the levels of the full-length

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Table 1. Mutations developing in the tumor upon acquired resistance predicted to be deleterious

Genomic coordinate	Gene	Reference sequence	Nucleotide change	Aminoacid change	Adverse predictors[1]	ExAC frequency	ID[2]
chr2:141660694	*LRP1B*	NM_018557.2	c.3561T›G	p.Cys1187Trp	8/9	Not reported	Not reported
chr3:47165678	*SETD2*	NM_014159.6	c.448C›T	p.His150Tyr	3/9	Not reported	Not reported
chr4:106155236	*TET2*	NM_001127208.2	c.137C›T	p.Pro46Leu	1/9	1/245,542	COSM3598848
chr6:152748848	*SYNE1*	NM_033071.3	c.5101G›A	p.Gly1701Ser	0/9	Not reported	Not reported
chr18:50592494	*DCC*	NM_005215.3	c.1219G›A	p.Gly407Arg	8/9	1/121,256	rs142822433; COSM106302
chrX:153760967	*G6PD*	NM_000402.4	c.1192G›A	p.Glu398Lys	9/9	Not reported	rs868970288; COSM1599109

[1]Adverse predictors are considered when the variant is classified as a real mutation by any of the following nine: MutationTaster, MutationAssessor, FATHMM, FATHMM-MKL, MetaSVM, MetalR, Provean, LRT, and SIFT.
[2]ID refers to the identification of the specific mutation in dbSNP and/or COSMIC.

proteins were reduced in sunitinib-desensitized cells as compared to the parental counterparts, with varying degrees among the six proteins. These differences were more evident for SETD2 or TET2, whereas they were modest or absent for G6PD. Additionally, immunoblotting analysis using antibodies against four of the six proteins (TET2, DCC, SETD2, and SYNE1) showed differential band patterns between sunitinib-desensitized and parental cells.

Sunitinib enhances the expression of the resistance-associated proteins

As the identified proteins exhibited mutations in the sunitinib-resistant tumor and differential expression in sunitinib-desensitized cell lines, we aimed to explore their potential contribution to sunitinib cytotoxicity. To this end, we examined the levels of the six proteins in a sensitive RCC cell line treated with cytotoxic doses of sunitinib. Immunoblotting analysis showed that sunitinib treatment increased the expression of the resistance-associated proteins, again with varying magnitudes across the six proteins (Fig. 2*a*).

Knockdown of the identified proteins desensitizes cancer cells to sunitinib *in vitro*

Next, we aimed to examine whether the identified proteins contribute to sunitinib cytotoxicity. We silenced each of the six genes in sunitinib-sensitive RCC cells using two specific shRNAs (Supporting Information Fig. S1*a*) and explored the effects on sunitinib cytotoxicity. Our data show that knockdown of each of the six identified proteins impeded sunitinib-induced cell death and imparted tumor cells with varying degrees of resistance against cytotoxic doses of sunitinib (Fig. 2*b*).

Knockdown of the resistance-associated proteins impedes antitumor effects of sunitinib *in vivo*

Consistent with our observations in cell cultures, knockdown of the identified proteins in tumor xenografts derived from the A-498 RCC cell line impeded the tumor-restraining effects of sunitinib (Fig. 2*c* and Supporting Information Fig. S1*b*), further confirming the relevance of those proteins in mediating the antitumor effects of sunitinib and indicating that their loss of function renders tumors refractory to sunitinib.

Knockdown of resistance-associated proteins impedes sunitinib-evoked modulation of oncogenic pathways

Next, we aimed to gain deeper insight into the mechanisms by which the identified proteins mediate the antitumor effects of sunitinib. We recently reported that modulation of the prosurvival MCL-1 protein and mTORC1 signaling downstream of the ERK and GSK3β pathways plays a crucial role in determining the response to sunitinib.[9–11] Consistently, we observed that treatment of sunitinib-sensitive RCC A-498 cells with cytotoxic doses of sunitinib was associated with time-dependent reduction in MCL-1 levels and inhibition of mTORC1 activity as assessed by the phosphorylation of downstream targets (Fig. 3*a*). However, sunitinib failed to trigger similar effects in sunitinib-desensitized A-498 cells, further confirming the relevance of those signaling events to sunitinib response and resistance (Fig. 3*a*). As our results indicate that knockdown of each of the six resistance-associated proteins renders tumor cells resistant to sunitinib, we assessed whether this was also associated with modulation of those pathways.

Immunoblotting analysis showed that in control A-498 cells either not infected (Mock) or infected with scrambled shRNA, treatment with cytotoxic doses of sunitinib triggered a decline in MCL-1 levels and inhibition of mTORC1 activity which correlated with dephosphorylating (and thus activation) of GSK3β and to moderate dephosphorylating (and thus inhibition) of ERK. By contrast, sunitinib generally failed to trigger similar effects in A-498 cells depleted of each of the resistance-associated proteins (Fig. 3*b*). Of note, knockdown of each of the resistance-associated genes had differential effects on those signaling pathways: for instance, inhibition of TET2, DCC, and SETD2 had the most pronounced effect on MCL-1 levels, whereas knockdown of LRPB1, SYNE1, and TET2 had the most pronounced effect on mTORC1 signaling (Fig. 3*b*).

Taken together, these results indicate that the six resistance-associated proteins identified are critically required for sunitinib-triggered modulation of MCL-1, mTORC1, ERK, and GSK3β, all events ultimately involved in sunitinib cytotoxicity. In tumor cells exhibiting loss-of-function of those proteins (either due to mutations

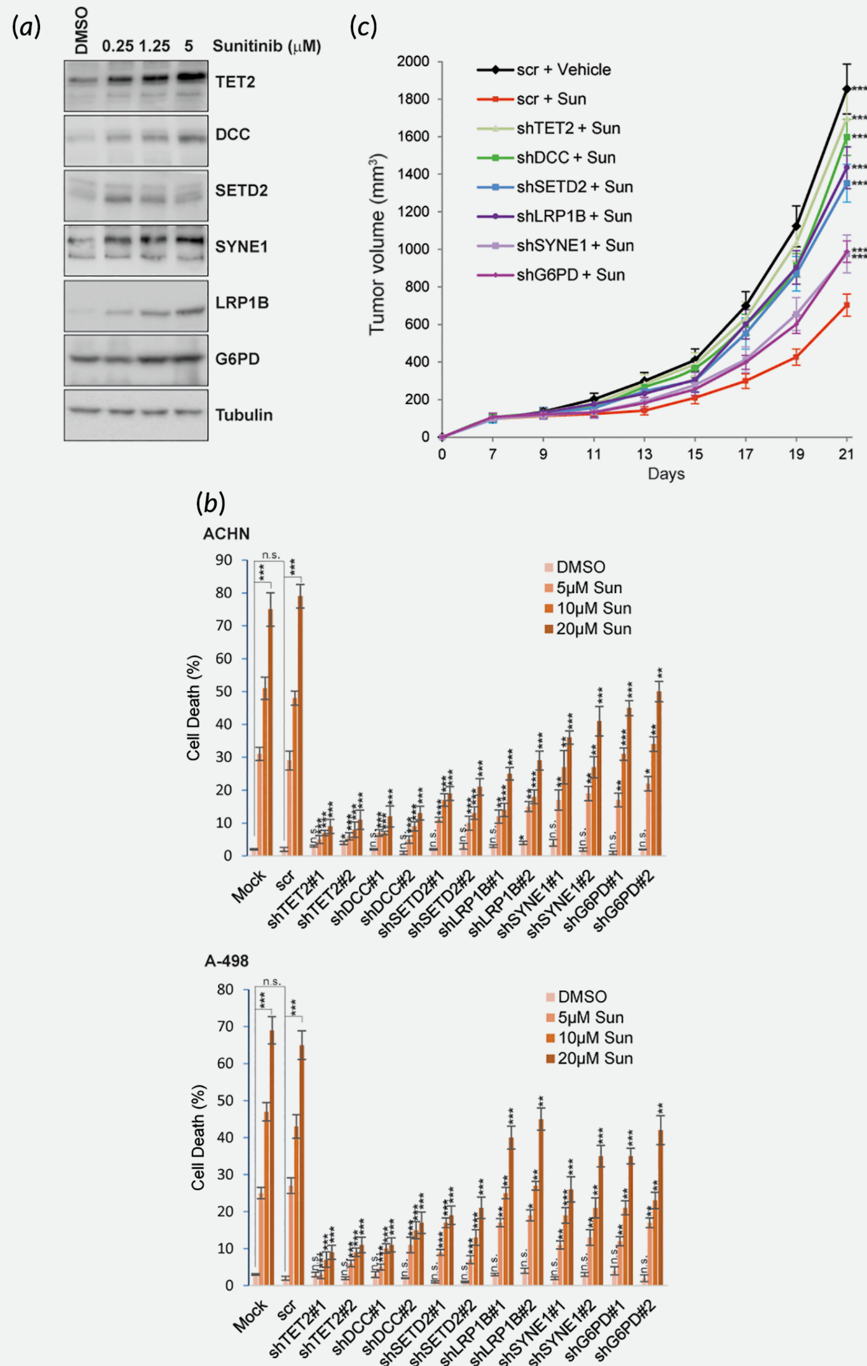

Figure 2. Candidate proteins are enhanced by sunitinib and mediate its cytotoxicity. (*a*) Immunoblotting analysis, using antibodies against the six resistance-associated proteins, of cell lysates derived from A-498 cells treated with either dymethyl sulfoxide (DMSO) or the indicated concentrations of sunitinib for 24 hr showed that sunitinib increased their expression. (*b*) Percentage of cell death of parental ACHN and A-498 cells infected with the indicated shRNAs and treated with DMSO or the indicated concentrations of sunitinib for 48 hr to assess the effects of depleting the six resistance-associated proteins on the response of sunitinib-sensitive renal cancer cell lines to sunitinib. (*c*) *In vivo* growth of tumor xenografts derived from A-498 cells transduced with the indicated shRNAs. After establishment of xenografts, mice were treated daily with either 30 mg/kg sunitinib or vehicle administrated by oral gavage. Tumor growth was followed at the indicated time points and showed that depletion of the resistance-associated proteins render tumors less sensitive to treatment with sunitinib. Student *t* test: ***$p < 0.0005$, **$p < 0.005$, *$p < 0.05$, n.s. = nonsignificant *versus* control tumors derived from cells transduced with scrambled shRNA and treated with sunitinib. [Color figure can be viewed at wileyonlinelibrary.com]

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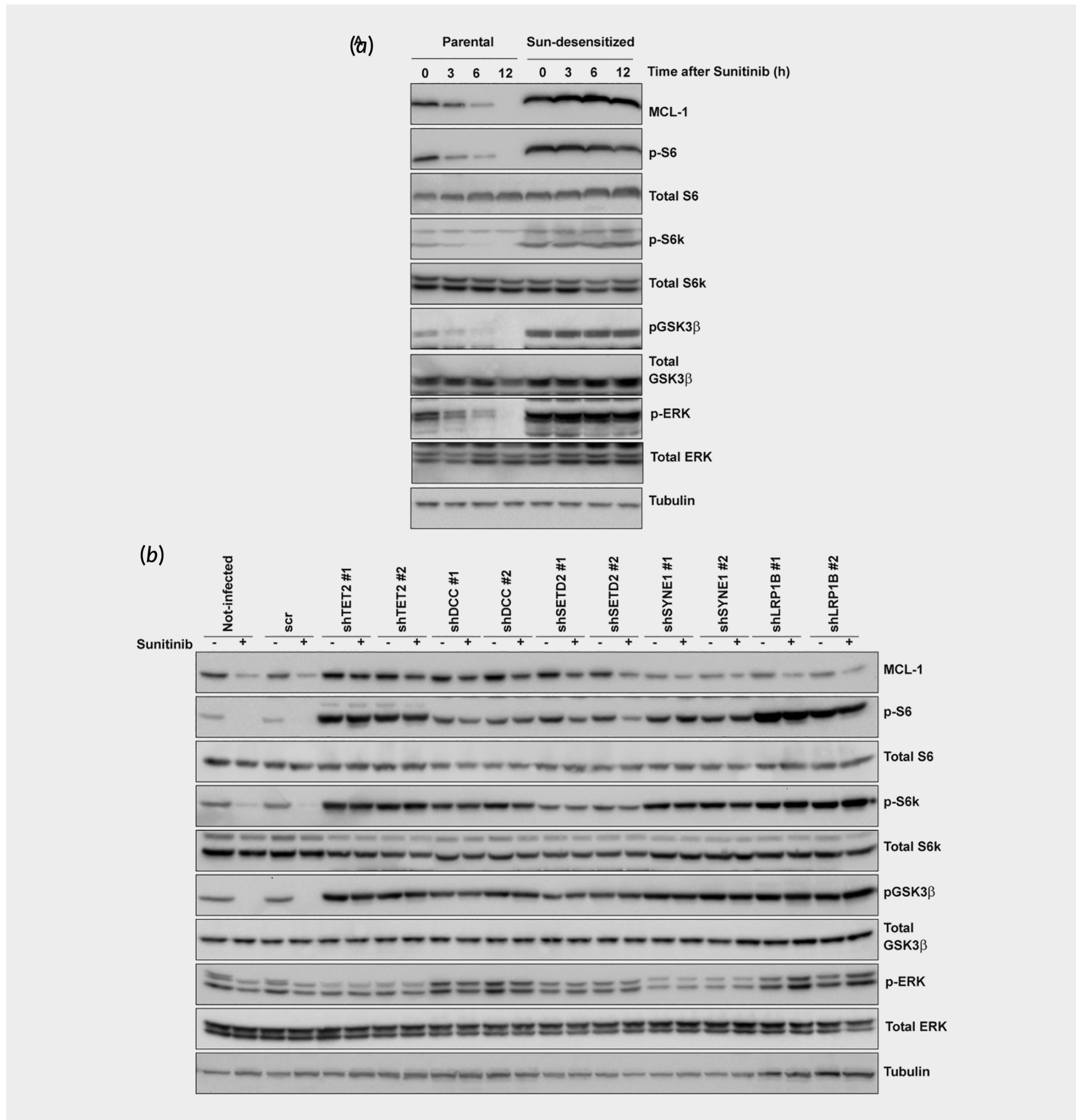

Figure 3. Sunitinib-induced modulation of oncogenic pathways is mediated by resistance-associated proteins. (*a*) Immunoblotting analysis using the indicated antibodies of cell lysates derived from parental or sunitinib-desensitized A-498 cells treated with 5 μM sunitinib for the indicated time points showing that Sunitinib modulates oncogenic pathways in parental—but not in desensitized—cells. (*b*) Immunoblotting analysis using the indicated antibodies of cell lysates derived from A-498 cells stably infected with the indicated shRNAs and treated with either DMSO or 5 μM sunitinib for 24 hr showing that depletion of resistance-associated proteins impedes sunitinib-evoked modulation of oncogenic pathways.

or shRNA-mediated depletion), sunitinib fails to trigger these events, which impedes its cytotoxicity and ultimately leading to emergence of resistance. These results also strongly suggest that the mutations detected in these genes in patient sample are loss-of-function mutations.

Activity of cabozantinib and lenvatinib plus everolimus in paired sunitinib sensitive and resistant renal cancer cell lines

Primary or acquired resistance to sunitinib eventually develops in most mRCC patients. Having gained insight into mechanisms of

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acquired resistance, we next aimed to attempt clinically relevant approaches to tackle these resistance mechanisms. Recently, cabozantinib[12] and the combination of lenvatinib plus everolimus[13] have shown clinical activity in mRCC patients previously treated with TKI, including sunitinib. Moreover, cabozantinib has shown improved progression-free survival over sunitinib in a randomized Phase II study performed in the first-line setting.[14] Our results show that sunitinib-desensitized cells were more sensitive to cabozantinib and to the combination of lenvatinib and everolimus (Figs. 4*a* and 4*b*) than their parental counterparts. Notably, sunitinib-desensitized cells exhibited comparable sensitivity to their parental counterparts when treated with either axitinib or single agent lenvatinib or everolimus (Fig. 4*c* and Supporting Information Fig. S2). These results indicate specifically increased sensitivity to cabozantinib or to the combination of lenvatinib and everolimus rather than general sensitization to cytotoxic stimuli *per se*. Further immunoblotting analysis showed that cabozantinib or the combination of lenvatinib and everolimus greatly diminished the elevated MCL-1 levels and mTORC1 signaling in sunitinib-desensitized cells (Fig. 4*d*).

Moreover, parental ACHN and A-498 cells infected with the indicated shRNAs and treated with cabozantinib or lenvatinib plus everolimus for 48 hr showed that depletion of the resistance-associated proteins rendered cells more sensitive (Fig. 4*e*), as opposed to the results seen with sunitinib in Figure 2*b*. These results support the potential relevance of these pathways in the activity of cabozantinib and lenvatinib plus everolimus in patients resistant to sunitinib that is observed in the clinical setting.

Discussion

Sunitinib, a TKI, is one of the most commonly used targeted therapeutics for treatment of mRCC. However, the majority of patients who initially respond eventually develop acquired resistance. Identification of the mechanisms by which tumors acquire resistance after treatment with sunitinib and other targeted therapeutics and approaches for resensitization are thus of outmost importance.

We have recently identified intrinsic mechanisms of resistance to sunitinib.[9] However, it remained elusive to determine whether these mechanisms contribute to acquired resistance to sunitinib and if so, what are the arising upstream mutations associated with the activation of these mechanisms in tumors that acquire resistance.

In the present study, by analyzing sequential tumor biopsies from an mRCC patient who developed acquired resistance to sunitinib, we identified mutations in genes whose loss of function conferred sunitinib resistance to tumor cell lines in culture and xenografted in mice and went on to identify critical downstream mechanisms.

From all the newly arising mutations identified in the sample obtained after development of acquired resistance, we selected those predicted to be pathogenic according to Alamut® Visual v2.7.2. Our analysis identified six mutations located in *G6PD, LRP1B, SETD2, TET2, SYNE1*, and *DCC*. We showed that the six proteins encoded by these genes were differentially expressed in paired sunitinib-sensitive and resistant cancer cell lines. The levels of the full-length proteins were lower and additional lower molecular weight bands detected in sunitinib-desensitized cells as compared to their parental counterparts.

Furthermore, treatment with cytotoxic doses of sunitinib was associated with an increase in the levels of most of the six proteins. This increase mediated cytotoxic effects of sunitinib as knockdown of each of the proteins with specific shRNAs in sunitinib-sensitive cancer cell lines decreased sunitinib cytotoxicity *in vitro* and allowed *in vivo* escape of tumor xenografts from sunitinib treatment.

Taken together, our data thus suggest that loss-of-function mutations in the six genes mediate acquired resistance to sunitinib.

The presence of single nucleotide variants that result in a loss of function of the encoded protein is frequent in cancer when they affect tumor-suppressor genes and/or DNA repair genes, at least affecting the allele that carries the mutation. There are several examples of this type of mutations affecting TP53 (the classical p. Arg175His; p.Arg273His and many others) and DNA repair genes. In many instances, for example, the somatic mutations in ATM, a gene belonging to the repair machinery, it is frequent to find a single mutation at the somatic level, being a proprietary loss of function mutation in the tumor, whereas the presence of two inactivating mutations at the germline constitutes the inherited and autosomal recessive ataxia telangiectasia. For example, the DCC mutation that we identified (p.Gly407Arg; c.1219G>A; COSM106302) is an exon 7 alteration predicted as likely pathogenic due to the previous description in tumors (COSMIC), absence in the general population databases (ExAC, gnomAD), and the hallmarks considered by the *in silico* predictors: it affects a highly conserved nucleotide (phyloP: 9.29 [−20.0;10.0]), a highly conserved amino acid (up to *C. elegans* [considering 12 species]), and the protein domains: immunoglobulin-like domain, immunoglobulin I-set immunoglobulin subtype 2, and immunoglobulin subtype. Due to these characteristics, several clinical resources yield an interpretation of this variant as a full loss of function mutation (cancer genome interpreter, e.g.).

Interestingly, the six genes that harbored mutations at the time of acquired resistance have been previously implicated in tumorigenesis, and some specifically with RCC. *SETD2* is a well-known tumor-suppressor gene encoding a histone methyltransferase that frequently harbors inactivating mutations in clear cell[15–17] and type 2 papillary RCC[18] and displays significant tumor heterogeneity across branched evolution of RCC.[19] Loss of heterozygosity at the *DCC* locus, 18q21.3, has been reported in 13.5% of RCC patients,[20] and DCC protein underexpression is frequently observed in clear-cell RCC and correlates with worse prognosis.[21] *TET2* is a tumor-suppressor gene that is frequently methylated in several tumors, including RCC.[22] The tumor-suppressor low-density *LRP1B* is underexpressed in human RCC tissue and cell lines, and its knockdown promotes anchorage-independent cell growth, migration, and invasion of RCC cells.[23] The synaptic

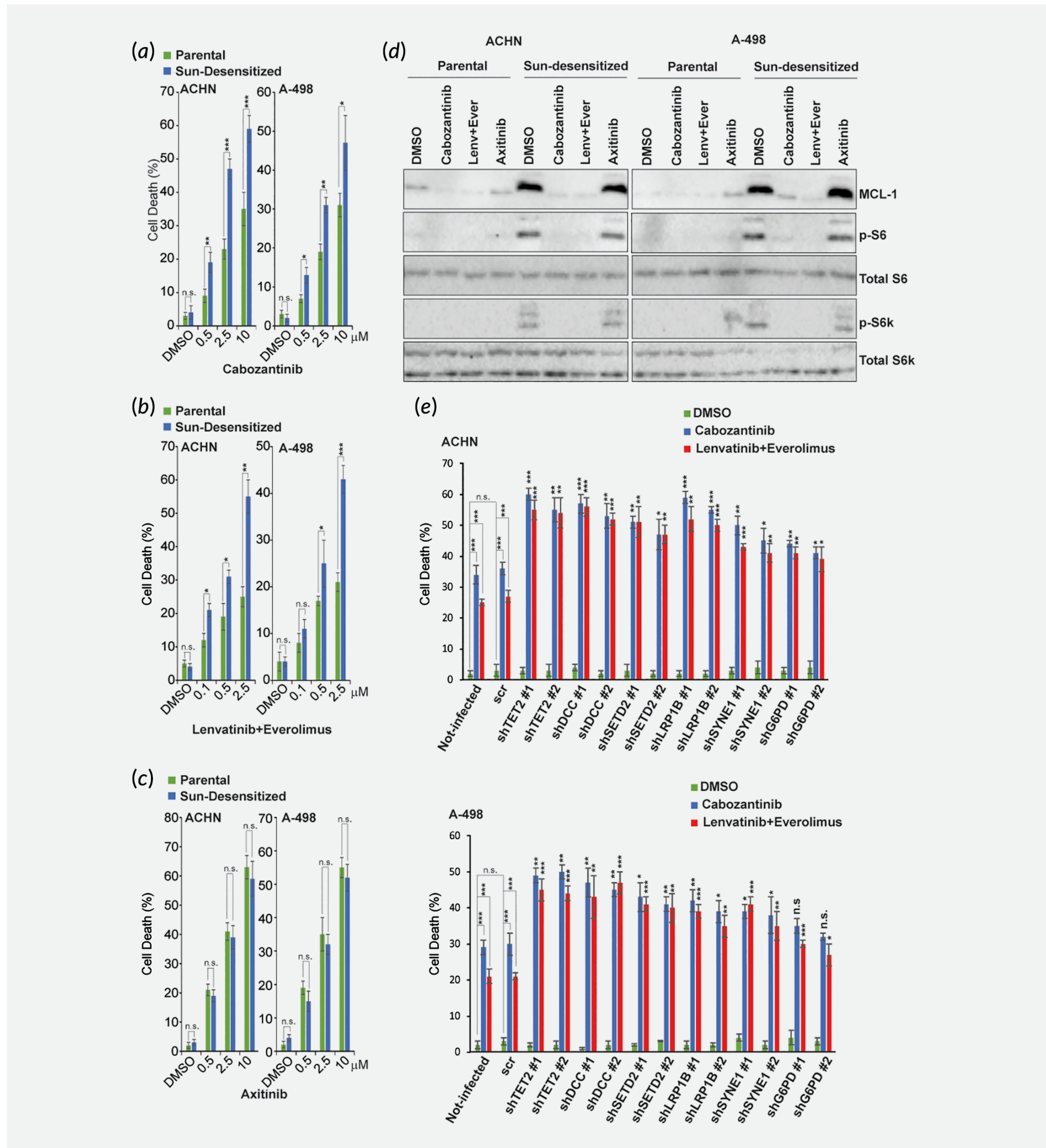

Figure 4. Sunitinib-resistant cells are sensitive to cabozantinib and to the combination of lenvatinib plus everolimus. (*a*–*c*) Percentage of cell death of sunitinib-desensitized *versus* parental ACHN and A-498 cells treated with DMSO or the indicated concentrations of cabozantinib (*a*), lenvatinib plus 100 nM everolimus (*b*) or axitinib (*c*) for 48 hr, showing higher sensitivity of sunitinib-desensitized cells compared to their parental counterpart cells. Student *t* test: ****p* < 0.0005, ***p* < 0.005, **p* < 0.05, n.s. = non-significant *versus* parental cells treated with the corresponding treatment. (*d*) Immunoblotting analysis of sunitinib-desensitized *versus* parental ACHN and A-498 cells treated with DMSO, cabozantinib (10 µM), lenvatinib (2.5 µM) plus everolimus (100 nM), or axitinib (10 µM) for 48 hr showing the inhibition of elevated MCL-1 levels and mTORC1 signaling in sunitinib-desensitized cells by cabozantinib or the lenvatinib-everolimus combination. (*e*) Percentage of cell death of parental ACHN and A-498 cells infected with the indicated shRNAs and treated with DMSO, cabozantinib (10 µM), lenvatinib (2.5 µM) plus everolimus (100 nM) for 48 hr showing that depletion of the resistance-associated proteins render cells more sensitive. Student *t* test: ****p* < 0.0005, ***p* < 0.005, **p* < 0.05, n.s. = nonsignificant *versus* control cells transduced with scrambled shRNA and treated with the corresponding treatment unless indicated otherwise on the graph. [Color figure can be viewed at wileyonlinelibrary.com]

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nuclear envelope protein 1 (*SYNE1*, Nesprin-1) is a human protein encoded by the *SYNE1* gene, which is frequently methylated in lung adenocarcinoma and lung cancer cell lines.[24] Although the latter five genes exert established tumor-suppressor functions, G6PD plays tumor-promoting roles and has been reported to be frequently overexpressed in RCC human samples and higher levels significantly correlated with Fuhrman grade, TNM stage, and overall survival.[25] Incubation of 786-O, 769-p, and A-498 RCC cell lines with sunitinib downregulated 86 phosphopeptides including G6PD, while upregulating Axl signaling.[26] Our analysis showed only modest or no difference in G6PD levels between parental and sunitinib-desensitized RCC cells. Consistently, treatment of parental RCC cells was not associated with a significant change in G6PD levels, in contrast to the five other proteins, and G6PD depletion had the least effect on sensitizing RCC cells to sunitinib. Interestingly, G6PD is the rate-limiting enzyme of the pentose phosphate pathway, an anabolic metabolic pathway parallel to glycolysis, which generates NADPH and pentoses, as well as the precursor for the synthesis of nucleotides ribose 5-phosphate, suggesting a link between metabolic alterations in cancer cells and resistance to sunitinib.

Furthermore, we investigated whether there is a link between the identified resistance-associated proteins and the mechanisms that we recently showed to determine the response and intrinsic resistance to sunitinib, namely modulation of MCL-1 level and of mTORC1 signaling through modulating upstream GSK3β and ERK signaling.[9,10] Induction of MCL-1 and mTORC1 acts as prosurvival stress responses geared toward impeding sunitinib cytotoxicity and imparting cancer cells and tumors with resistance against sunitinib. However, higher levels of stress exerted by cytotoxic doses of sunitinib beyond the capacity of tumors cells to adapt conversely elicit opposite effects and negatively modulates MCL-1 level and mTORC1 activity, which mediate cell death. MCL-1 is an antiapoptotic member of the BCL-2 family of proteins that is often upregulated in tumors and has been shown to contribute to drug resistance and relapse. The tumor-promoting properties of MCL-1 have been largely attributed to its anti-apoptotic functions. However, emerging reports suggest that MCL-1 is also implicated in other cellular processes that contribute to its tumorigenic potential.[27,28] mTORC1 signaling is another crucial factor in determining the response to anticancer agents and is implicated in diverse cellular processes including regulation of cellular energetics, autophagy, survival and proliferation.[11,29]

Our results showed that loss-of-function of the identified resistance-associated proteins in cells desensitized to sunitinib either by continuous exposure to increasing doses or by depletion of the identified six proteins impeded sunitinib-evoked modulation of these oncogenic pathways. These findings link mechanisms of intrinsic and acquired resistance to sunitinib and indicate that reactivation of mechanisms of intrinsic resistance upon acquiring the identified mutations contributes to acquired resistance after a period of treatment with sunitinib. It remains to be explored how modulation of each of the six proteins identified here as determinants of response/resistance to sunitinib feeds into MCL-1/mTORC1 axis. Given the diverse cellular effects, the six proteins exert as well as the wide spectrum of mechanisms of regulation of both MCL-1 levels and mTORC1 activity, it is likely that the six proteins differentially modulate distinct signaling events and/or cellular processes that ultimately lead to modulation of MCL-1/mTORC1. Indeed, our analysis shows differential modulation of ERK and GSK3β signaling by those proteins upstream of MCl-1/mTORC1.

We devised and tested clinically relevant approaches to overcome acquired resistance to sunitinib. Cancer cells which acquire resistance to certain anticancer agents through up-regulation of particular molecular pathways become addicted to these pathways for survival and hence highly sensitive to their inhibition by other agents, a phenomenon widely known as "collateral sensitivity."[30] We found that cells which acquire resistance to sunitinib either after continuous exposure in the culture or as a result of depletion of the identified six proteins were particularly sensitive to treatment with cabozantinib or with a combination of lenvatinib and everolimus. Both treatments effectively target mTORC1/MCL-1, on which sunitinib-resistant cells are essentially dependent for survival. Therefore, the inhibition of such targets would render cell lines more sensitive to cabozantinib and lenvatinib plus everolimus. The increased sensitivity of the cell lines resistant to sunitinib to both treatments could also represent the identification of some of the specific targets that induce their clinical efficacy in patients that have progressed to sunitinib and other TKIs[12,13] and the increased efficacy reported for cabozantinib over sunitinib in the first line setting.[14] Although these results further support our observations, additional studies are required to validate them. Additional therapeutic strategies to sensitize tumors to sunitinib have also been suggested.[31]

It will be interesting to examine in a larger patient cohort if our findings represent general mechanisms of acquired resistance to sunitinib. Nevertheless, the validation work we have carried out in *in vitro* and *in vivo* models supports the general relevance of the identified proteins to the phenotypes of sensitivity and resistance to sunitinib.

In conclusion, the identification of mutations associated with acquired resistance to sunitinib contributes to reveal mechanisms of action and resistance to this drug and might help to develop reliable biomarkers for sunitinib in mRCC patients.

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