**MET and KRAS gene amplification mediates acquired resistance to MET tyrosine kinase inhibitors.**

# *MET* and *KRAS* Gene Amplification Mediates Acquired Resistance to MET Tyrosine Kinase Inhibitors

Virna Cepero1, J. Rafael Sierra[1], Simona Corso[1], Elena Ghiso[1], Laura Casorzo[1],Tim Perera[2], Paolo Maria Comoglio[1], and Silvia Giordano[1]

**Author Affiliations**

[1]Institute for Cancer Research and Treatment, University of Torino Medical School, Candiolo, Turin, Italy and [2]Ortho Biotech Oncology Research and Development, Beerse, Belgium

**Author Notes**

Current address for V. Cepero and J.R. Sierra: University Health Network, Ontario Cancer Institute and Princess Margaret Hospital, Toronto, Ontario, Canada M5G 2M9.

**Corresponding Author**:
Silvia Giordano, Institute for Cancer Research and Treatment, University of Torino Medical School, S.P.142 Km 3.9, 10060 Candiolo, Turin, Italy. Phone: 39-011-9933833; Fax: 39-011-9933225; E-mail: silvia.giordano@unito.it.

## ABSTRACT

The establishment of the role of MET in human cancer has led to the development of small-molecule inhibitors, many of which are currently in clinical trials. Thus far, nothing is known about their therapeutic efficacy and the possible emergence of resistance to treatment, a problem that has been often observed with other receptor tyrosine kinase (RTK) inhibitors. To predict mechanisms of acquired resistance, we generated resistant cells by treating MET-addicted cells with increasing concentrations of the MET small-molecule inhibitors PHA-665752 or JNJ38877605. Resistant cells displayed *MET* gene amplification, leading to increased expression and constitutive phosphorylation of MET, followed by subsequent amplification and overexpression of wild-type (wt) *KRAS*. Cells harboring *KRAS*amplification progressively lost their MET dependence and acquired KRAS dependence. Our results suggest that *MET* and *KRAS* amplification is a general mechanism of resistance to specific MET inhibitors given that similar results were observed with two small inhibitors and in different cell lines of different histotypes. To our knowledge, this is the first report showing that overexpression of wt KRAS can overcome the inhibitory effect of a RTK inhibitor. In view of the fact that cellular models of resistance to inhibitors targeting other tyrosine kinases have predicted and corroborated clinical findings, our results provide insights into strategies for preventing and/or overcoming drug resistance.

## INTRODUCTION

The frequent alteration of tyrosine kinases in human malignancies led them to be considered as targets for antineoplastic therapies; this resulted in the development of several inhibitors that have shown a strong clinical activity (1–5). The concept of “oncogene addiction” has added further rationale to the use of targeted therapies (6, 7). In general, targeted therapies induce tumor regression in a good percentage of patients. However, almost invariably, responsive patients develop resistance to the treatment and undergo tumor relapse (8). A challenge associated to targeted therapies is, therefore, to predict mechanisms that could cause resistance to the treatment and to find ways to circumvent these hurdles.

The recent introduction in cancer therapy of several selective tyrosine kinase inhibitors (TKI) has had a dramatic effect in oncology. However, after the first excitement following the initial results, the problem of acquired drug resistance has become more and more important and still represents a crucial limitation. The use of *in vitro* and preclinical models, as well as the evaluation of clinical samples, allowed the identification of several molecular mechanisms responsible for acquired resistance to TKIs (8).

The receptor for hepatocyte growth factor (HGF), encoded by the *MET* gene, which is deregulated in many tumors, is becoming an important candidate for targeted therapies (9,10). Recent studies by our and other groups have shown that tumor cells displaying *MET*amplification, which results in receptor overexpression and ligand-independent activation, feature oncogenic addiction. In these tumors, inhibition of MET results in impairment of cell growth and in cell death, both *in vitro* and *in vivo* (11–16). In recent times, agents targeting MET—the majority being small-molecule kinase inhibitors—have entered in clinical trials (17–19).

In our work, we have established cell lines resistant to long-term treatment with MET inhibitors and studied the molecular mechanisms involved in the onset and the development of resistance. We show that prolonged treatment with specific MET inhibitors drove amplification, overexpression, and constitutive activation of the receptor. Moreover, we observed that cells progressively amplified *KRAS*, resulting in increased expression and activation of wild-type (wt) KRAS and in activation of the mitogen-activated protein kinase (MAPK) pathway. We show that amplification of wt *MET* and *KRAS* is found in cells of diverse histotypes, resistant to different inhibitors. Moreover, unexpectedly, we observed that resistance to treatment was reversible and that the alterations leading to resistance were lost after drug withdrawal.

## MATERIALS AND METHODS

### Cell lines

GTL16 and EBC-1 cell lines, which harbor *MET* amplification, were cultured in either DMEM or RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. GTL16 cell line is a clonal cell line established in our lab (as described in refs. 20, 21, derived from the poorly differentiated gastric carcinoma cell line MKN45). After cloning, multiple vials of GTL16 cells were frozen; on thawing, cells were kept in culture for not more than 20 passages. No further characterization of this cell line has been performed, except for the analysis of the karyotype. EBC-1 cell line, derived from a metastatic skin tumor of a patient with lung squamous cell carcinoma, was purchased in 2008 from the Japan Cancer Research Resources Bank. To characterize the cells, the cell bank performed isoenzyme analysis. On arrival, cells were not kept in line for more than 20 passages. Further authentication of the cell line has not been performed by the authors. Resistant cells were generated by exposure to increasing doses of the inhibitor. See Supplementary Materials and Methods for detailed information.

### mRNA and genomic DNA analysis

Total mRNA extracts were obtained using Trizol extraction kit (Ambion–Applied Biosystems). RNA (1 μg) was retrotranscribed using M-MLV retrotranscriptase minus H (Promega) in a final volume of 40 μL, according to the manufacturer's protocol. Genomic DNA was extracted using Wizard SV Genomic DNA Purification System (Promega). cDNA (1 μL) or genomic DNA (50 ng) was amplified and analyzed using Power SYBR Green PCR Master Mix (Applied Biosystems) by real-time quantitative PCR using ABI Prism 7900HT (Applied Biosystems). Specific primers were designed using Primer3 web tool (22). Sequences are available on request. Gene expression and gene copy number fold increase were determined by using the following formula: fold increase = $2^{\wedge} - [(C_t$ of

target gene − $C_t$ of control gene)$_{resistant}$ − ($C_t$of target gene − $C_t$ of control gene)$_{parental}$], where $C_t$ means cycle threshold.

**Genomic sequence analysis**

Exon sequencing of *MET* (13–20), *EGFR* (18, 19, and 21), *PI3KCA* (9 and 20), *B-RAF* (15),*KRAS* (2–3), and *HRAS* (2–3) was performed by automated sequencing by ABI Prism 3730 (Applied Biosystems).

**Western blot analysis**

Whole-protein extracts were prepared, quantified using the BCA Protein Assay kit (Pierce), and analyzed using the chemiluminescence ECL kit (Amersham). Primary antibodies anti–phospho-AKT (Ser$^{473}$), anti-AKT, anti–phospho-p44/42 MAPK, and anti-p44/42 MAPK were purchased from Cell Signaling. Antibodies anti–phospho-MET1234-1235 and anti-phosphotyrosine were from Upstate. Antibodies against KRAS (F234) and anti–β-actin (I-9) were from Santa Cruz Biotechnology. Monoclonal antibodies anti-MET DL21 was obtained as described (23). Secondary antibodies were purchased from Amersham.

**Pull-down assay for RAS**

Pull-down assays were performed using glutathione *S*-transferase (GST)–RAF–CRIB, which specifically binds to the GTP-bound form of RAS, and Western blots were developed using anti-KRAS antibody (F234).

**Fluorescence in situ hybridization analysis**

Fluorescence *in situ* hybridization (FISH) probes for KRAS (RP11-295I5) were purchased from Blue Gnone. Probes for the MET locus (LSID7S522, spectrum orange), CEP7, and CEP12 (spectrum green) were purchased from Vysis Abbott.

**Comparative genomic hybridization analysis**

Comparative genomic hybridization (CGH) analyses were performed by NimGenetics. Analyses were performed by comparing genomic DNA from GR150 versus GTL16 wt and GR300 versus GR150 using a 244K CGH array developed by Agilent, Inc.

**Biological assays**

For growth curve and cell viability assays, cells were seeded in 96-well plastic culture plates (3000 per well) in the presence of the indicated drugs or vehicle (DMSO). Resistant cells were kept in the presence of PHA-665752 (Tocris Bioscience). Cell viability was assessed using a luminescence assay (CellTiter-Glo Luminescent Cell viability Assay, Promega). Anchorage-independent growth in soft agar was performed as described (24), and colony formation was quantified with the Alamar Blue indicator dye (Trek Diagnostic Systems). All the measurements were recorded using a DTX 880 Multimode plate reader (Beckman-Coulter). Small interfering RNA (siRNA) against MET [previously described (11)] was electroporated using Amaxa Technology (Instrumentation Laboratory), and short hairpin RNA (shRNA) against KRAS sequence (5′-GUGCAAUGAGGGACCAGUA) was cloned into an inducible lentiviral vector as previously described (11). wt KRAS cDNA was cloned into the pRRL2 lentiviral vector by following a directional cloning strategy using a*Bam*HI-*Xho*I digestion. MAPK inhibitors were purchased from Sequoia Research Product (sorafenib), Promega (U0126), and Cayman Chemicals (PD0325901).

### Xenograft transplantation experiments

All animal procedures were approved by the Ethical Commission of the University of Turin and the Italian Ministry of Health. GTL16 wt or GR300 cells ($3 \times 10^5$) were injected s.c. into the right posterior flanks of 6-week-old female CD-1 *nu/nu* mice (Charles River Laboratories). Treatment with JNJ38877605 at half of the maximum tolerated dose or vehicle was initiated after early tumor appearance (approximate volume, 10 $mm^3$). Tumor volume was monitored every 3 or 4 days for 24 days, calculated as described (25).

### Statistical analyses

For experimental data, two-tailed Student's *t* tests were calculated using GraphPad Prism web tool software. A *P* value of <0.05 was considered significant.

## RESULTS

### Establishment of cell lines resistant to MET inhibitors

GTL16 gastric cancer cells are the prototype of "MET-addicted cells," displaying *MET*amplification (26), overexpression, and constitutive activation (27, 28). Targeting MET in GTL16 cells leads to a strong impairment of their viability and growth ability (11, 13–16).

To study possible mechanisms of acquired resistance to MET kinase inhibitors, we exposed GTL16 cells to increasing concentrations of the MET inhibitor PHA-665752 (29,30), generating several cell lines resistant to different increasing doses of this inhibitor. Resistant cells were analyzed for their biological and biochemical properties. The growth rate of the resistant cells, which were always kept in the presence of PHA-665752, was only slightly decreased in comparison with wt cells (a representative example is shown inFig. 1A). Moreover, resistant cells displayed a significant increase in the $IC_{50}$ (half-maximal inhibitory concentration) when compared with wt cells (Fig. 1B), and were able to grow in anchorage-independent conditions in a similar manner as parental cells (Fig. 1C).

To evaluate cross-resistance between PHA-665752 and other MET inhibitors, we treated PHA-665752–resistant GTL16 cells with comparable doses of JNJ38877605, a specific MET inhibitor of the same class, currently undergoing clinical trials (31), and found that cells showed a similar behavior in terms of cell viability and MET status when treated with either compound (Supplementary Fig. S1A and B).

To evaluate if the resistance to PHA-665752 generated *in vitro* was also maintained *in vivo*, we performed xenografts in nude mice. Because PHA-665752 has poor pharmacokinetic properties and low oral bioavailability (17), we daily treated the grafted animals with the orally available JNJ38877605, to which the cells are cross-resistant (Supplementary Fig. S1A and B). As shown in Fig. 1D, mice injected with GTL16 wt cells did not develop tumors in the presence of JNJ38877605 ($P = 0.0015$), whereas resistant cells were able to originate tumors ($P = 0.0222$), which were only slightly smaller than those generated by wt cells in the absence of JNJ38877605 ($P = 0.1659$) or by the same resistant cell growing in the absence of the inhibitor ($P = 0.3347$). These results indicate that resistant cells generated *in vitro* are able to originate tumors *in vivo* in the presence of therapeutic doses of the MET inhibitor.

**Resistance to MET inhibitors correlates with MET gene amplification**

The biochemical analysis of resistant cells showed an increased amount of MET protein, which initially paralleled the increase in the concentration of the drug (Fig. 2A, top), thus allowing the maintenance of the basal phosphorylation levels (Fig. 2A, middle). This was concomitant with an increment in MET mRNA, evaluated by quantitative reverse transcription-PCR (qRT-PCR; Supplementary Fig. S2A).

To evaluate if the observed MET overexpression was due to gene amplification, a known mechanism of resistance to small inhibitors (8), we performed qRT-PCR on genomic DNA and found an increase in gene copy number in the resistant cells when compared with the parental ones (Fig. 2B). The number of gene copies progressively increased to reach a maximum in cells resistant to 150 nmol/L PHA-665752 (GR150) and started to decrease in cells resistant to higher doses of the drug.

*MET* overamplification was confirmed by FISH and CGH analysis on PHA-665752–resistant cells. As shown in Supplementary Fig. S2C, CGH analysis of GR150 cells displayed a gain in the genomic area comprising the *MET* locus when compared with wt cells. On the contrary, cells resistant to a higher dose of PHA-665752 (300 nmol/L, GR300) displayed partial loss of the same genomic area. These observations agree with the results obtained by qRT-PCR (see Supplementary Fig. S3 for complete CGH analyses).

In GTL16 wt cells, *MET* is already amplified (11 copies) and the amplicon is localized outside the chromosome 7 (33) on a marker chromosome (M1; ref. 26). Performing FISH analysis, we found that, also in resistant cells, the increased *MET* copies were not located on chromosome 7 (Fig. 2C).

**Acquired resistance to MET inhibitors is an adaptive process**

To discriminate whether the observed amplification was due to selection of a preexisting subpopulation or if it was acquired during the treatment, we isolated different clones of GTL16 wt cells by limiting dilution and treated two of them (clones 16.1 and 16.2) with increasing doses of the inhibitor to induce resistance (as described in Supplementary Materials and Methods). We found that, in resistant cells obtained from both clones, the number of *MET* copies rose with the increase of the inhibitor concentration, similarly to what was observed in the bulk population of GTL16 wt cells (Supplementary Fig. S4A). This finding reveals that the observed genomic alterations were acquired during the selection.

We also induced resistance in GTL16 wt cells with JNJ38877605, another MET-specific kinase inhibitor, using the same procedure described for PHA-665752. We thus obtained cell lines resistant to different concentrations of the drug. Analysis of JNJ38877605-resistant cells revealed an increase in *MET* copy number (Fig. 2D), suggesting that *MET*amplification is not a specific response to PHA-665752 treatment but a more general mechanism of resistance to selective MET TKIs.

**A second hit is responsible for resistance to high doses of MET inhibitors**

As previously mentioned, MET phosphorylation was maintained up to 150 nmol/L PHA-665752. In cells resistant to higher doses, the level of MET and its phosphorylation started to decrease (Fig. 2A). Moreover, after silencing MET in GR300 cells using siRNAs, we observed that cell viability was almost unaffected compared with what observed in wt and GR150 cells, which are still dependent on MET signaling (Fig. 3A; Supplementary Fig. S2D; $P < 0.0001$). The cells resistant to higher doses of the drug showed a sustained activation of MAPK [extracellular signal-regulated kinase (ERK) 1/2] even if the levels of MET phosphorylation were low (Figs. 2A and 3B).

All these data suggest that in cells resistant to high concentrations of inhibitor, a new molecular event has taken place and MET activation is no longer the event mediating resistance. To further investigate this possibility, we first discarded the presence of mutations in the receptor by sequencing all the transmembrane and cytoplasmic portions of*MET* in the parental and derived cell lines. In addition, no mutations were found in the signal transducers that are more frequently aberrantly activated in human tumors and are responsible for increased activation of ERK1/2, such as *EGFR, KRAS, HRAS, B-RAF*, and*PI3KCA* (Supplementary Fig. S5).

To evaluate if other tyrosine kinases were differentially activated in resistant cells, we performed anti-phosphotyrosine blots on proteins immunoprecipitated with anti-phosphotyrosine antibodies, finding no qualitative differences in tyrosine phosphorylation patterns (Supplementary Fig. S4C); this result suggests that it is unlikely that other tyrosine kinases (receptor or cytoplasmic) were significantly activated in resistant cells. In particular, we also looked at the activation of epidermal growth factor receptor (EGFR) family members, known to cross talk with MET, and insulin-like growth factor-I receptor, but we did not find increased phosphorylation in GR300 cells (data not shown).

The analysis of the qRT-PCR and CGH data showed the amplification of the genomic region containing *KRAS* in GR300 cells (Fig. 3C; Supplementary Figs. S3 and S6A). FISH analysis showed that the extra copies of *KRAS* were progressively acquired as double minutes outside chromosome 12 (where *KRAS* is positioned; Fig. 3D).

The levels of expression of KRAS in GR300 cells were strongly increased, whereas the levels of HRAS were unchanged (Fig. 4A, i; $P < 0.0001$). As shown in Fig. 4A (i and ii), KRAS protein progressively increased with increasing doses of the drug. Moreover, pull-down experiments conducted with GST-RAF showed that the augmented expression of KRAS was paralleled by its enhanced activation, without substantial changes in the ratio of the active molecule (Fig. 4A, iii). Likewise, we found a progressive increase in the number of *KRAS* copies in resistant GTL16 clones (16.1 and 16.2; Supplementary Fig. S4B). This finding reveals that amplification of both *MET* and *KRAS* was acquired during the treatment. Moreover, also cells resistant to high doses of JNJ38877605 displayed an increase in *KRAS* copy number (Fig. 4B).

To prove that the increase of KRAS-mediated signaling was involved in resistance, we silenced KRAS expression by transducing the cells with an inducible (Tet-ON) system containing a short-hairpin RNA against KRAS (Supplementary Fig. S6B). Although KRAS silencing was almost ineffective in wt cells (Supplementary Fig. S6C), it resulted in decreased ability of resistant cells to grow in anchorage-independent manner in soft agar (Fig. 4C; $P < 0.0001$). As a mirror experiment, GTL16 cells overexpressing exogenous wt KRAS developed resistance to PHA-665752 (Fig. 4D).

To further validate our observations, we induced resistance to PHA-665752 in the non–small cell lung carcinoma cell line EBC-1, which displays *MET* amplification and high sensitivity to MET inhibitors. Resistant cells displayed an increased expression and phosphorylation of MET (Fig. 5A, top; Supplementary Fig. S7A), as a result of a further*MET* amplification (Fig. 5B). Moreover, resistance to increasing doses of inhibitor was coupled to high ERK1/2 activation (Fig. 5A, bottom) sustained by increased levels of KRAS (Fig. 5A; Supplementary Fig. S7B) due to *KRAS* amplification (Fig. 5B). *MET* and *KRAS*amplification was confirmed by FISH analyses (Fig. 5C and D).

All these results suggest that amplification of both *MET* and *KRAS* can be a general mechanism of resistance to selective MET TKIs.

**Acquired resistance to MET inhibitors retains dependence on the MAPK pathway**

Because resistant cells display a strong activation of ERK1/2, we wondered if they were still responsive to MAPK inhibitors. Therefore, wt and resistant cells were treated with the specific MAPK inhibitors U0126 and PD0325901, or sorafenib (initially identified as a RAF inhibitor and now used in clinics as a multitarget inhibitor), and their ability to grow in either anchorage-dependent or anchorage-independent conditions was evaluated. MAPK inhibitors effectively decreased the anchorage-independent growth ability of resistant cells (Fig. 6A) as well as their viability in anchorage-dependent (adherent) conditions (Supplementary Fig. S8A–C). Western blots to confirm the effects of MAPK inhibitors on MET downstream pathways are shown in Supplementary Fig. S8D. These results suggest that blocking pathways downstream to KRAS could be an option to overcome the KRAS-mediated resistance to MET inhibitors.

**Acquired resistance to MET inhibitors is reversible**

To evaluate if resistant cells have acquired a permanent resistant phenotype, we cultured the GR300 cells in the absence of PHA-665752 (GR300-0PHA). Approximately 1 month later, we analyzed these cells and found that they had lost the increased expression of both MET and KRAS (Fig. 6B). The decreased level of protein expression was accompanied by loss of gene copies for both *MET* and *KRAS* (Fig. 6C; $P < 0.0001$), reaching a number of copies similar to that of the wt cells. Likewise, clones of GR300 obtained by single-cell dilution that underwent withdrawal of the inhibitor displayed a significant decrease in *KRAS*copies (Supplementary Fig. S9; $P < 0.0001$). To show that cells kept in the absence of PHA-665752 had lost their resistance to the inhibitor, we treated them again with the drug. As shown in Fig. 6D, the cells were no longer able to grow in the presence of the same doses of drug to which they had been previously resistant, thus behaving as wt cells. A similar behavior was also observed in cells explanted from tumors resistant to JNJ38877605 *in vivo*. When tumors were excised and cells were put in culture, in the absence of the inhibitor, they lost resistance to the drug.

## DISCUSSION

The tyrosine kinase receptor for HGF, encoded by the *MET* gene, has very recently become a target for molecular therapies. Because little is known thus far on the possible mechanisms of resistance to treatment with MET inhibitors, we generated *in vitro* resistant cells by using a protocol similar to those used to establish cells resistant to other TKIs. We thus treated MET-addicted cells with increasing concentrations of the inhibitor in a stepwise manner, generating cell lines resistant to increasing concentrations of the drug. To investigate the mechanisms responsible for resistance, at first, we ruled out the presence of newly occurring mutations that could potentially interfere with the interaction between the receptor and the drug. Instead, we found that resistant cells show a progressive increase in the amount of MET protein that, even in the presence of sustained concentrations of drug, allows the maintenance of a level of tyrosine phosphorylation (and hence pathway activation) comparable with that observed in parental untreated cells. By silencing MET expression in these resistant cells, we observed a strong impairment of their growth, indicating that these cells were still addicted to MET. We found that this increased protein expression was due to *MET* gene amplification, leading to a total copy number of ~60. A similar mechanism of resistance has been observed in the case of other tyrosine kinases, such as *Bcr-Abl* in patients affected by chronic myelogenous leukemia, where the amplification of the *Bcr-Abl* gene is responsible for loss of response to imatinib, and the increase in the dose of the drug can confer again responsiveness to treatment (34).

In our models, FISH analysis showed that the acquired copies of *MET* are not located on chromosome 7 (where the *MET* gene is positioned; ref. 33) but on a marker chromosome (26). This suggests a mechanism of progressive acquisition of additional *MET* copies as a consequence of asymmetric partitioning of the marker chromosome at mitosis. It is likely that cells gaining *MET* extra copies have a selective advantage under the selective pressure of the drug. The observed increase of gene copies is not due to selection of a preexisting population of cells, but rather to an adaptive process, because the *MET* gene underwent amplification also in a clonal population (generated by limiting dilution cloning of wt GTL16 cells) exposed to the drug.

It is puzzling to note that the most frequent genetic alteration of *MET* in human tumors is gene amplification, rather than mutation (35). This behavior is quite different from that of other tyrosine kinases, such as EGFR or c-Kit, which are more frequently mutated in cancer. Interestingly, *MET* amplification takes place also in tumors treated with inhibitors of other receptor tyrosine kinases (RTK) and mediates resistance to treatment (36–38). An example is the case of some lung cancers treated with gefitinib, where *MET* amplification and overexpression leads to ERBB3 transphosphorylation, thus inducing resistance to inhibitors of the EGFR family (39). One possible explanation for *MET* being frequently amplified relies on the fact that the *MET* gene is located within a known chromosomal common fragile site, FRA7G (33). Hellman and colleagues have shown that conditions that interfere with DNA replication (called “replication stress”) induce a perturbation of chromatin organization of this region, predisposing it to breakage (40). In their work, they hypothesize that this replication stress exerts a selective pressure for gene amplification that disappears once the oncogene has attained sufficient amplification. The increased number of *MET*copies, counteracting the activity of the anti-MET drug, rescues cell proliferation.

Interestingly, the number of *MET* copies increased with the dose of the inhibitor up to a threshold, after which it started to decrease. This decrease was accompanied by a reduction of the amount of the MET protein and of its total phosphorylation. At this point, a second unrelated event took place in the cells, which had become independent from MET expression (shown by the fact that MET silencing did not severely impair their viability). We have identified this second event in the progressive amplification of *KRAS*, driving receptor-independent MAPK activation. Amplification of *KRAS* led to an increased expression and activation of the KRAS protein. Moreover, parental cells overexpressing exogenous wt KRAS were capable to overcome the effects of MET inhibitors. In the same line, previous works published by our group showed that overexpression of constitutively active KRAS, bRAF, or AKT could induce the onset of biological resistance to MET inhibitors (41).

The impaired viability of resistant cells on KRAS silencing indicates their dependence on KRAS. On the other hand, parental cells were unaffected by KRAS silencing, likely due to redundant roles of HRAS and KRAS in these cells. This phenomenon, known as “oncogene switch,” has already been reported as a consequence of inhibition of different tyrosine kinases, but it usually involves the activation of other kinases, driving parallel signaling pathways (42). In human tumors, KRAS is almost invariably activated as a consequence of point mutations, with only a few cases where *KRAS* gene amplification has been described (43, 44). It is interesting to note that, as for *MET*, also the *KRAS* gene is located inside a chromosomal common fragile site, on chromosome 12 (45), and thus, it is likely that the same mechanisms responsible for *MET* amplification can account also for this second event.

Because specific anti-RAS drugs are not available, we tested the ability of compounds acting downstream RAS (such as U0126, PD325901, and sorafenib) to impair cell viability. We observed that cells resistant to MET inhibitors that underwent *KRAS* amplification are indeed sensitive to these drugs.

All the molecular events that we have described were not restricted to a single cell line or to a single inhibitor, which indicates that they can represent a more general mechanism to generate cell resistance to small kinase inhibitors against MET.

Finally, we have observed that these cells lost their acquired resistance to MET inhibitors in the absence of the selective pressure. After culture in the absence of PHA-665752, resistant cells lost the acquired amplification of both *MET* and *KRAS* and once again became sensitive to MET inhibition. One possible explanation for this finding relies on the fact that the amplified oncogenes *MET* and *KRAS* are extrachromosomal in the cells examined. Removal of the inhibitor results in an excess of signal transduction that may induce cellular stress, known to lead to loss of extrachromosomal DNA (46, 47). Cells that underwent loss of *MET* and *KRAS* extra copies have thus an advantage and become the prevalent population in the absence of MET inhibitor. These observations suggest that the mechanism of resistance we observed may be less stable than others already described (such as the appearance of point mutations) and that, possibly, an intermittent therapy, favoring loss of amplified copies, could give better results. Reversibility of the resistant phenotype after drug withdrawal has been previously observed by other groups using other inhibitor for RTKs (48).

This is one of the first preclinical studies highlighting mechanisms of resistance to long-term exposure to selective MET kinase inhibitors (49, 50) and the first report, to our knowledge, showing that amplification of *MET* and *KRAS* genes mediates resistance to MET kinase inhibitors. The prediction of molecular mechanisms of resistance to MET inhibitors may provide insights to strategies aimed at preventing and/or overcoming drug resistance.

## Disclosure of Potential Conflicts of Interest

P.M. Comoglio received research support and/or consultation fees from Bayer-Shering, Boehringer-Ingelheim, Johnson & Johnson, and Servier; T. Perera is a full-time employee of Janssen Pharmaceutica and a shareholder of Johnson and Johnson.


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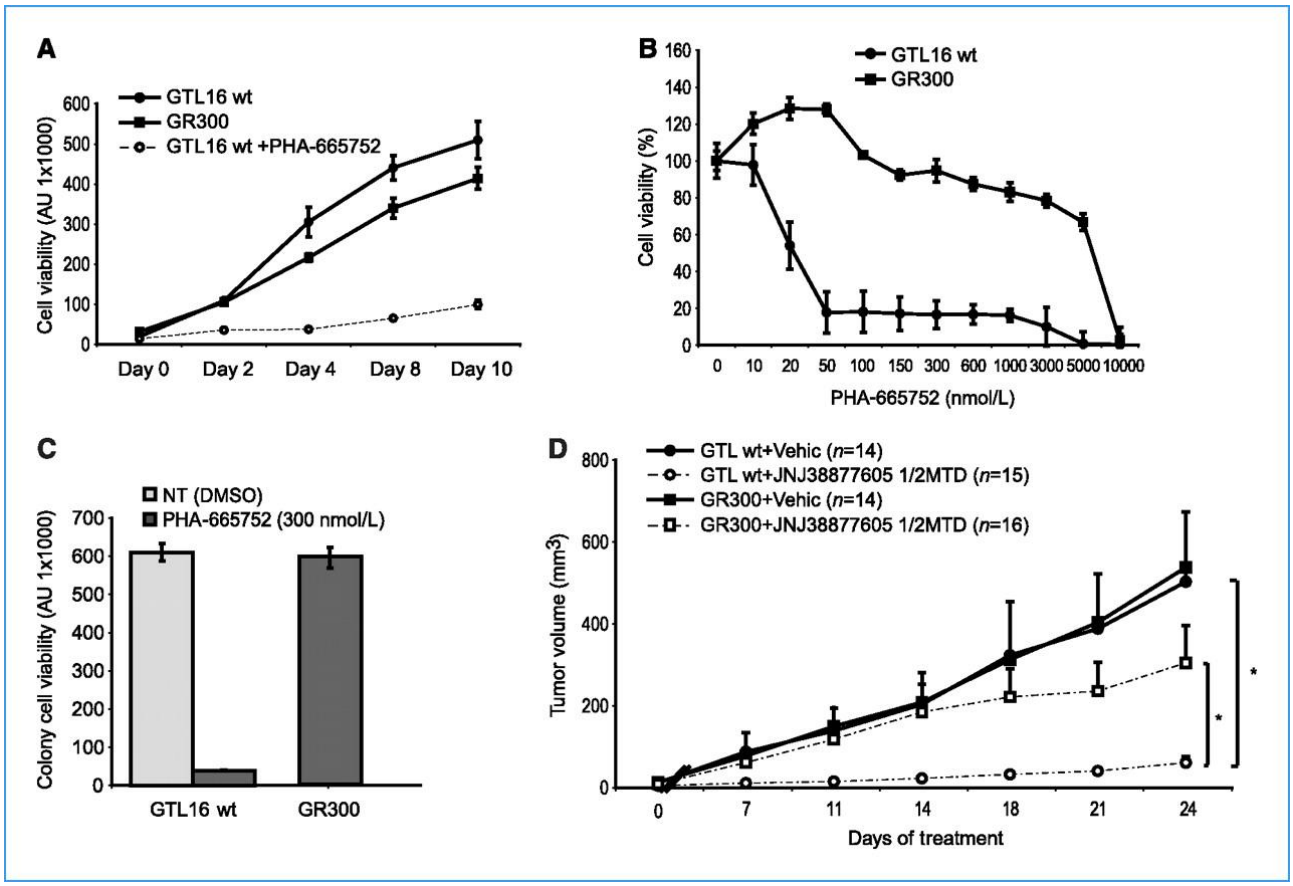


**Figure 1.**

Characterization of PHA-665752–resistant GTL16 cells. Growth ability (A), sensitivity to PHA-665752 (B), and anchorage-independent growth (C) were evaluated by cell viability assays in GTL16 wt cells and resistant to 300 nmol/L PHA-665752 (GR300). Resistant cells were always kept in the presence of the inhibitor. D, mice s.c. injected with either wt or PHA-665752–resistant GTL16 cells were subjected to the indicated treatments. Mean tumor volume ± SE was evaluated at the indicated times. Vehic, vehicle; 1/2 MTD, half maximum tolerated dose; *n*, number of tumors per experimental group. *, $P < 0.05$.

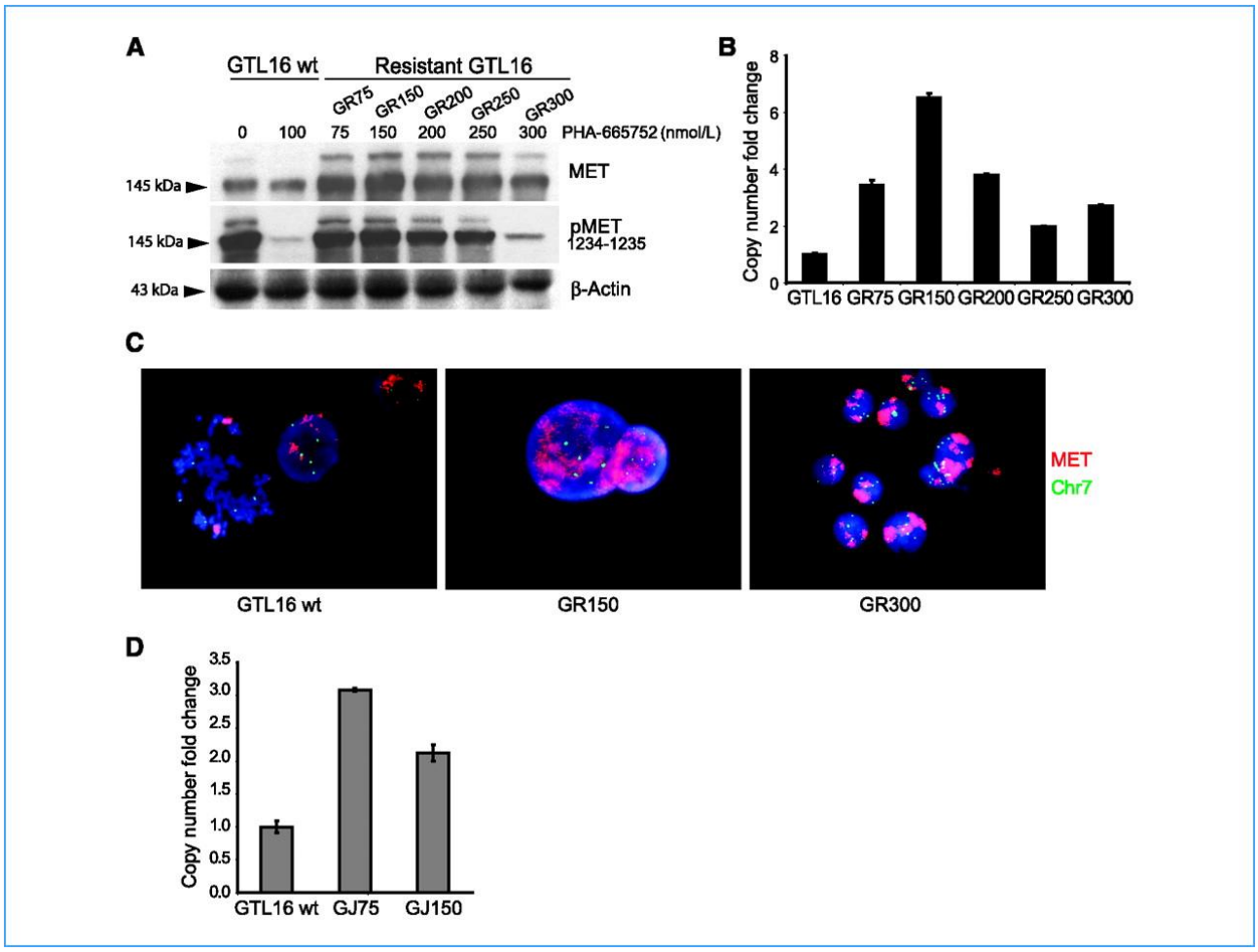


**Figure 2.**

Resistant GTL16 cells display *MET* amplification and overexpression. A, Western blot analysis of MET expression (top) and tyrosine phosphorylation (middle) in GTL16 cells resistant to different concentrations of PHA-665752. pMET, tyrosine phosphorylated MET. Probing with β-actin was used as loading control. B, evaluation of *MET*amplification by qRT-PCR performed on genomic DNA in the different PHA-665752–resistant cell lines. C, FISH analyses of GTL16 wt cells and resistant to increasing doses of PHA-665752. Red, MET locus; green, chromosome 7 centromere (CEP7). D, evaluation of *MET* amplification by qRT-PCR performed on genomic DNA in different JNJ38877605-resistant cell lines. GR and GJ: GTL16 cell resistant to PHA-665752 or JNJ38877605, respectively, followed by the dose to which they are resistant (in nmol/L).

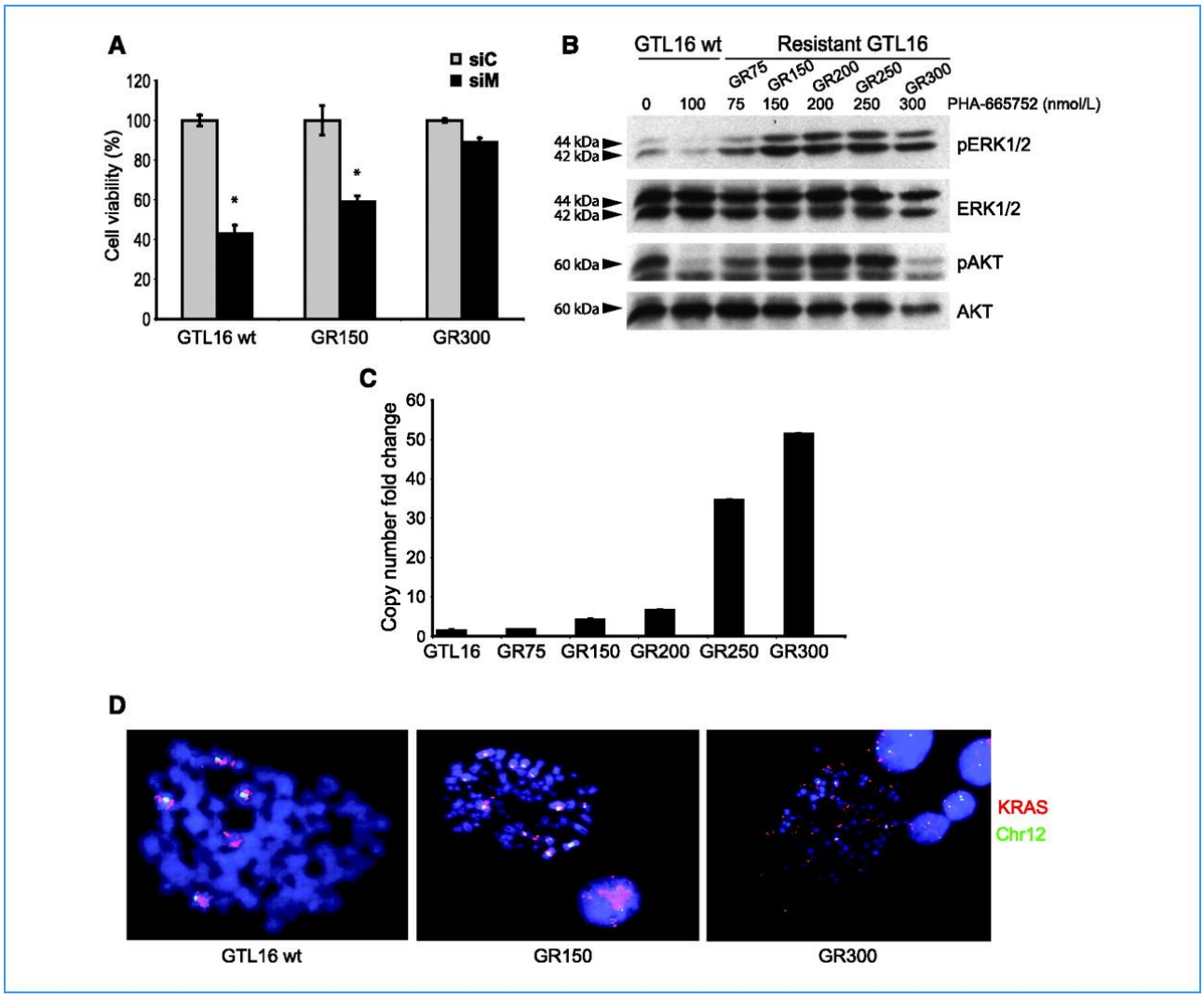


**Figure 3.**

*KRAS* amplification as a second event of resistance. A, to evaluate cell dependence on MET expression, GTL16 wt and resistant cells were transfected with MET-specific siRNAs (siM) or control siRNAs (siC). *, $P < 0.0001$. Cell viability was evaluated after 4 d. B, activation of ERK1/2 and AKT was evaluated in wt and resistant GTL16 cells. pERK1/2, phospho-ERK1/2; pAKT, phospho-AKT. C, evaluation of *KRAS* amplification by qRT-PCR performed on genomic DNA in different cell lines resistant to PHA-665752. D, FISH analysis displays *KRAS* (red) amplification in resistant cells. Green, chromosome 12 centromere (CEP12).

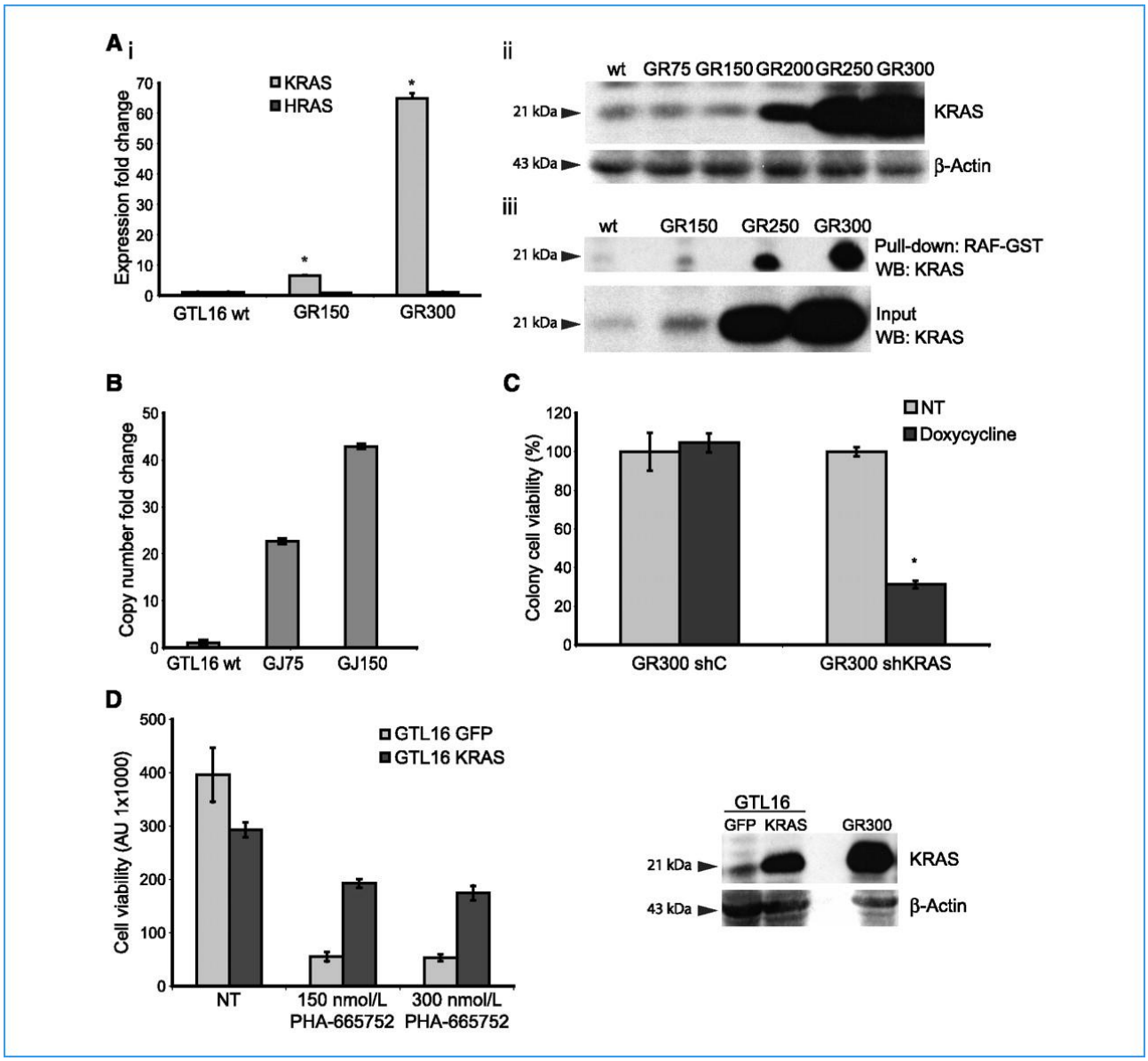


**Figure 4.**

KRAS is overexpressed and activated in resistant cells. A, i, expression of KRAS and HRAS, evaluated by qRT-PCR performed on RNA extracted from wt, GR150, and GR300 cell lines. *, $P < 0.0001$. ii, Western blot analysis of KRAS expression in resistant cell lines shows an increment of KRAS protein expression correlated with an increase of active KRAS, as shown by the RAF-GST pull-down assay (iii). B, evaluation of *KRAS* amplification by qRT-PCR performed on genomic DNA in different JNJ38877605-resistant cells. C, GR300 cells were transduced with inducible (Tet-ON) lentiviral vectors containing a short hairpin directed against KRAS (shKRAS) or a control shRNA (shC). Cell ability to survive in anchorage-independent manner was evaluated on induction (doxycycline) of shRNA expression. KRAS silencing severely impaired cell ability to grow in anchorage-independent manner. *, $P < 0.0001$. D, exogenous expression of wt KRAS induced the biological resistance to the MET inhibitor on GTL16 cells when transduced with a lentiviral vector containing wt KRAS exposed to different doses of PHA-665752; expression of KRAS was confirmed by Western blot.

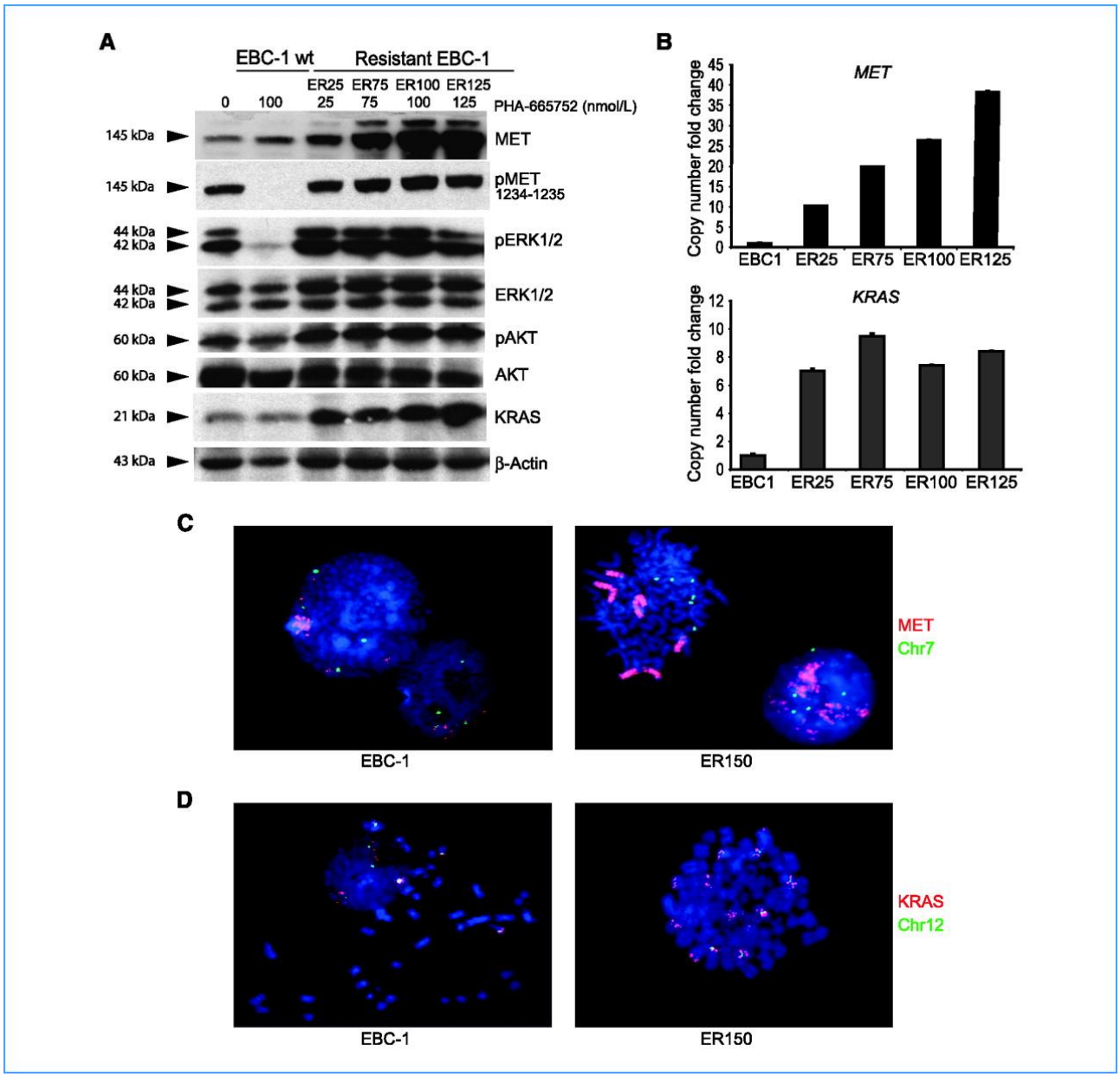


**Figure 5.**

PHA-665752–resistant EBC-1 cells display *MET* and *KRAS* amplification. A, Western blot analysis of MET and KRAS expression, and activation of MET, ERK1/2, and AKT in EBC-1–resistant cells. B, qRT-PCR analyses showed an increase of *MET* and*KRAS* gene copy number. FISH analysis showed increased copy number of (C) *MET*and (D) *KRAS* (MET locus, red; chromosome 7, green; KRAS, red; chromosome 12, green). ER: resistant EBC-1, followed by the dose to which they are resistant (in nmol/L).

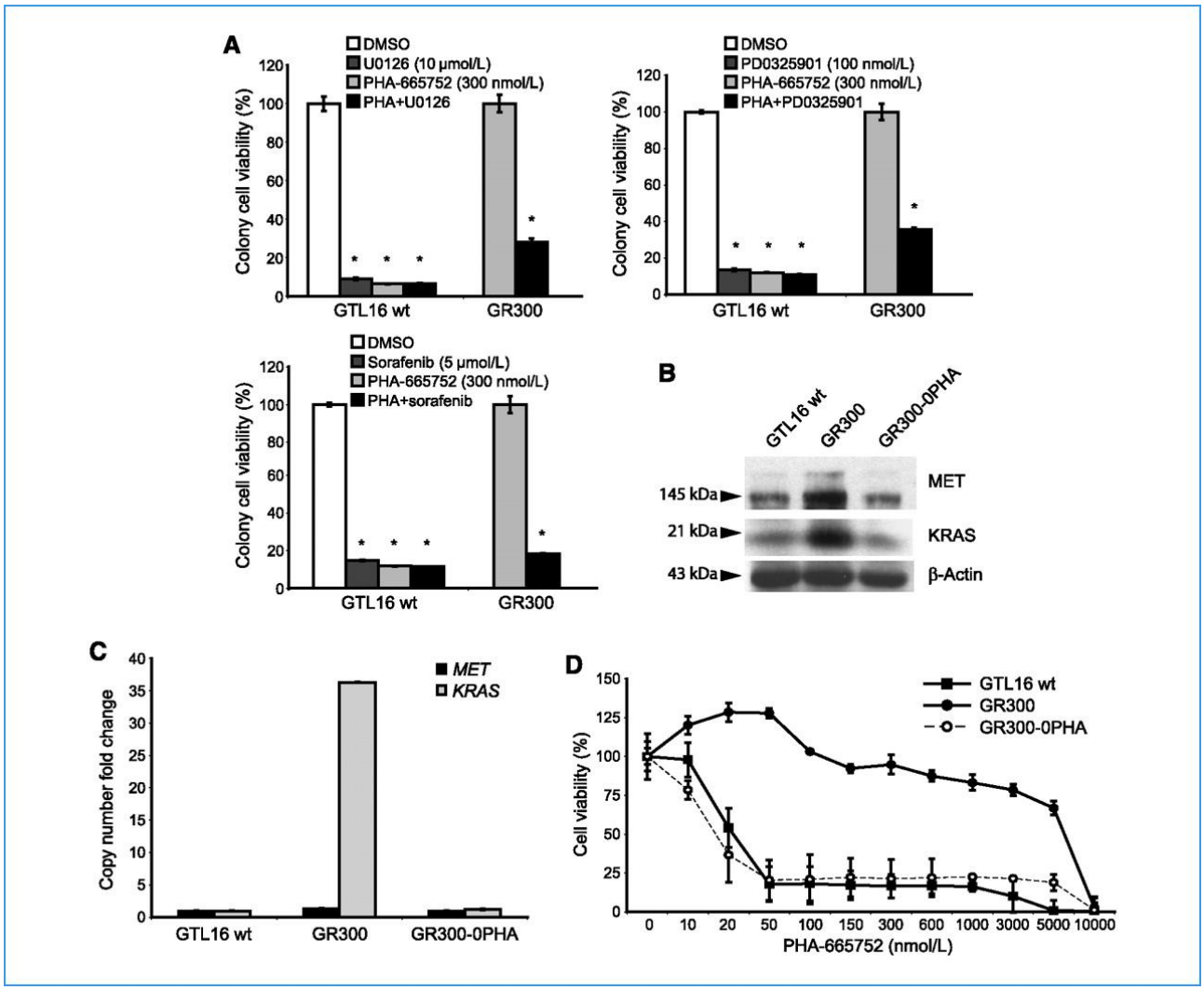


**Figure 6.**

PHA-665752–resistant cells are sensitive to multitarget and MAPK inhibitors, and acquired *MET* and *KRAS* amplification is reversible on removal of the inhibitor. A, anchorage-independent growth in soft agar of parental GTL16 and GR300 cells is severely impaired in the presence of U0126, PD0325901, or sorafenib. *, $P < 0.0001$. GR300 cells kept in the absence of PHA-665752 (GR300-0PHA) display *MET* and*KRAS* protein levels (B) and gene copy number (C) similar to those observed in the parental cell line. D, sensibility to PHA-665752 was evaluated by cell viability assays for GTL16 wt, GR300, and GR300-0PHA cells.