Cancers evolve by a reiterative process of clonal expansion, genetic diversification, and clonal selection in which the tissue provides the context for cancer cell evolution. Cancer can be considered as an ecosystem continuously adapting to changing environments, where the driving force is represented by intrinsic mutability of cancer cells, following the rules of Darwinian evolution (1, 2). The competition for space and resources allows colonization of other organs, giving rise to distant metastases. Little is known about how multiple alterations coexist in the same cancer lesion. In some cases, an early genetic alteration is taken over by a new one impinging on the same molecular pathway, thereby bypassing the early mutation. The late mutation confers resistance to therapy targeting the early mutation, but renders cancer cells sensitive to specific inhibitors of the latter (3).

The role of aberrant activation of the MET oncogene in driving the malignant progression and metastasization has been widely documented (4, 5). MET is genetically altered at uncommonly high frequency in cancers of unknown primary site (CUP) (6), a mysterious, lethal clinical entity, featuring multiple metastases in the absence of a detectable primary tumor. Constitutive MET activation occurs through several mechanisms including gene amplification, activating genetic mutations, transcriptional up-regulation, or ligand-dependent autocrine or paracrine mechanisms (7). Activated MET receptors propagate a intricate system of signaling cascades leading to the acquisition of cell motility, proliferation, and escape from apoptosis. Taken together, these biological events recapitulate a biological program defined as invasive growth (8). The versatility of MET-mediated biological responses is sustained by qualitative (i.e., engagement of dedicated signal transducers) and quantitative (i.e., either recruitment of adaptor amplifiers or desensitization through internalization, degradation (9) or negative feedback by phosphorylation of regulatory residues (10, 11)) signal modulation. A number of MET kinase inhibitors have been developed over the last 10 y and are currently in clinical trials (5). In cancer cell lines or patient-derived tumor xenografts, it has been shown that only tumors displaying MET genetic lesions (mostly amplification) respond to MET blockade with apoptosis and/or cell cycle arrest in vitro (12) and tumor growth inhibition in vivo (13). Similar to the case of oncogenic kinase receptor, resistance to targeting drugs occurs and is mediated by aberrant activation of other downstream signal transducers (14, 15).

MET-dependent signals are organized in pathways that are shared among different tyrosine kinase receptors, including the MAPK cascade (RAS-RAF-MEK-ERK). The BRAF Ser/Thr kinase (16) is mutated in ∼8% of all cancers (17), with almost all mutations falling in the kinase domain (18, 19). The most common mutation encodes the constitutively active BRAF-V600E oncoprotein, specifically targeted by vemurafenib (20). Despite the initial success of this and other BRAF inhibitors, drug resistance is invariably observed (21). Resistance is mostly a result of reactivation of the MAPK pathway that occurs through stromal extracellular or cell-autonomous survival signals mediated by tyrosine kinase
receptors such as AXL (22) or MET (23, 24). MEK inhibitors display clinical efficacy to overcome resistance to BRAF blockade (25–27).

Here we identified and characterized a molecular mechanism of resistance in a CUP tumor harboring two concomitant mutations affecting MET and BRAF. In this tumor, inhibition of either oncogene was ineffective because of reactivation of MET quenched by a previously unknown mechanism of negative feedback by BRAF. These findings unravel the existence of a mechanism of resistance to target monotherapy.
Results and Discussion

We here report a tumor that concomitantly harbors an amplification of the MET oncogene and an activating mutation in BRAF. This tumor was a liver metastasis of a CUP. Although we cannot chart the evolutionary trajectory of this case (CUP1.13), it is likely that the mutation in BRAF, a downstream MET signal transducer (28), occurred as a stochastic secondary event after the acquisition of MET gene amplification. This is reminiscent of the sequence of mutations emerging when tumor genetic evolution is driven by the selective pressure of targeted inhibitors such as HER-2 inhibitory antibodies in breast cancer (29, 30) or BRAF inhibitors in melanoma (31), which cause the emergence of resistance to specific direct inhibitors manageable for in vivo treatment. Nevertheless, inhibition of BRAF pathway was ineffective as well (Fig. 2A). However, concomitant treatment with the two drugs inhibiting MET and MEK significantly impaired tumor growth (Fig. 2C). These data were strengthened by measuring the percentage of cells entering S-phase by incorporation of the thymidine analog G′-deoxyuridine (11). As result, BRAF inhibition unifies and accelerates phosphorylation of amplified MET (4, 5). Ligand-independent activation of the MET kinase by amplification and overexpression has been established in a wide spectrum of tumors (for a review, see ref. 7).

In CUP1.13, MET was eightfold amplified (Fig. 1 A and B), overexpressed and exposed at the cell surface (Fig. 1C), but unexpectedly silent (Fig. 1D). MET amplification was combined to a second uncommon downstream oncogenic mutation, G469A, affecting BRAF (Fig. 1E) (17). Next-generation analysis of a panel of 241 cancer-related genes detected only one additional mutation, SMO R726K (17). Next-generation analysis of a panel of 241 cancer-related genes detected only one additional mutation, SMO R726K (17). Next-generation analysis of a panel of 241 cancer-related genes detected only one additional mutation, SMO R726K (17). Next-generation analysis of a panel of 241 cancer-related genes detected only one additional mutation, SMO R726K (17). Next-generation analysis of a panel of 241 cancer-related genes detected only one additional mutation, SMO R726K (17). Next-generation analysis of a panel of 241 cancer-related genes detected only one additional mutation, SMO R726K (17). Next-generation analysis of a panel of 241 cancer-related genes detected only one additional mutation, SMO R726K (17).
CUP1.13 PDX (L1.13), TAK-632 (33), a specific BRAF inhibitor suitable for in vitro experiments, or AZD-6244, inhibiting MEK in the downstream pathway, restored MET Tyr-phosphorylation (Fig. 3 A and B). These observations unveil a previously unrecognized negative feedback loop between MEK and the BRAF pathway. This loop, fixed in cancer cells harboring an activating mutation of BRAF, may operate in physiological conditions as well, providing a mechanism to restrict MET signaling in time. A similar regulatory feedback loop operates in the case of epithelial growth factor receptor (EGFR) (34). To check the specificity of the loop in L1.13 overexpressing MET, we screened 49 tyrosine kinase receptors by antibody-based phosphoproteomics. In these cells, other than a slight effect on EGFR, MEK inhibition unbridles kinase activity of MET specifically (SI Appendix, Fig. S1). To get insight in the mechanism underlying this phenomenon, we considered the relatively fast kinetics (peak of MET phosphorylation at 30 min, plateau at 2 h, and drop from 6 h on; Fig. 3B) and focused on posttranslational mechanisms. The kinetics suggest the involvement of activation/inactivation of Ser/Thr phosphatases/kinases that regulate MET kinase activity. Treatment with sodium fluoride, a selective Ser/Thr phosphatase inhibitor (35), completely inhibited rephosphorylation of MET in the presence of MEK inhibitor (Fig. 3C). We thus focused on the Ser/Thr phosphatase PP2A, known to dephosphorylate MET at Ser985 (36). We previously showed that MET phosphorylation on the regulatory Ser985 represents the major regulatory site responsible for MET kinase inhibition (11). Treatment of L1.13 cells with AZD-6244 decreased PP2A phosphorylation on Tyr307 (Fig. 3D). This is known to trigger phosphatase activity of PP2A (37) and was found to reduce MET Ser985 phosphorylation (Fig. 3E), and to activate MET kinase (Fig. 3F). Moreover, in a separate experiment, two specific PP2A siRNAs completely inhibited the feedback loop and fully prevented MET rephosphorylation in the catalytic domain by impairing dephosphorylation of the Ser985 (Fig. 3 F and G and SI Appendix, Fig. S2). This finding is in agreement with phosphoproteomics studies showing that AZD-6244 downmodulates MET Ser985 phosphorylation (38).

In L1.13 cells, ERK phosphorylation was strongly decreased by treatment with AZD-6244, but not completely lost (Fig. 3 A–C). Full inhibition was, however, observed after concomitant treatment with the MET inhibitor JNJ-38877605 (Fig. 4A). This observation suggests that, given the branching nature of the signaling network emanating from MET (39), the residual ERK activity after MEK inhibition is driven through an alternative pathway, such as the PI3K pathway (40). Accordingly, blockade of PI3K in L1.13 cells, where MET was reactivated by the MEK inhibitor, completely abrogated ERK activity (Fig. 4B), supporting the involvement of the MET/PI3K/AKT axis.

Taken together, these data provide the mechanistic explanation for the negative feedback loop between BRAF and MET in this cancer cell line, via inactivation of the phosphatase PP2A (Fig. 5). Even if other mechanisms may be envisaged, the circuit involving BRAF/MEK and PP2A is necessary and sufficient for the feedback of MET inhibition.

It is an accepted notion that tumors are composed by genetically heterogeneous subclones, some of which rise while others shivel to deal with stromal selective pressure (41, 42). A subclone harboring an early oncogenic mutation may develop a later mutation more effective in promoting adaptation and the emergence of a progeny endowed with increased malignancy. If the late mutation impinges on the same signaling pathway, its targeting should be, in principle, effective. The data reported here unveil and explain a paradox of targeted therapy: quenching the effects of a late mutation restores sensitivity to drugs targeting what it is likely to be an earlier mutation. We show that, in the case of MET and BRAF oncogenes, the paradox is generated by an inhibitory feedback loop, mediated by a protein serine phosphatase. The key role of phosphatase PP2A as negative regulators of signal transduction has been already observed in the case of other tyrosine kinase receptors (43). At first sight, it is counterintuitive, considering treating a patient featuring a BRAF

![Image](https://i.imgur.com/5.jpg)
mutated tumor with a MET inhibitor, as BRAF is downstream MET in the signal transduction cascade. On the contrary, these data provide a strong rationale for combinatorial therapy of MET-amplified/BRAF-mutated patients, who have poor clinical response to each inhibitor in monotherapy.

**Materials and Methods**

**Tumor Sample.** Informed consent for research use was obtained from the patient (ClinicalTrials.gov identifier: NCT03347318) at the enrolling institution FPO-IRCCS (Candiolo) before tissue banking, and study approval was obtained from the ethics committee of FPO-IRCCS (Candiolo).

**Cell Cultures and Inhibitors.** Primary cell line L.1.13 was derived from PDX1.13. The tumor was explanted, digested with Collagenase I (Sigma-Aldrich), and cultured in RPMI medium 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich). Cell genetic tumor identity has been identified by short tandem repeat profiling (Cell ID; Promega).

A549 were purchased from American Type Culture Collection, A2780 from European Collection of Authenticated Cell Cultures, and maintained in their original culturing conditions according with supplier guidelines.

AZD-6244 was purchased from Sequoia Research Products, TAK-632 from Selleck chemicals, NaF from Sigma Aldrich, and BEZ-625 from Selleck Chemicals, and JNJ-38877605 was kindly provided by Janssen Pharmaceutica NV.

**siRNA.** siRNA (Ambion) were transfected by Lipofectamine RNAiMAX (Invitrogen), according to manufacturer’s instructions, and harvested 72 h after transfection.

**PPP2CA Assay.** Clone: s10958 (#58); s10959 (#59). Scrambled siRNA: Silencer Select Negative Control No. 1 siRNA.

**Nucleic Acid Extraction.** gDNA was extracted with Maxwell RSC Blood DNA Maxwell RSC Whole Blood DNA Kit (Promega), according to the manufacturer’s protocol.

**qRT-PCR.** Gene copy number was performed by real-time PCR in triplicate on ABI PRISM 7900HT thermal cycler (Life Technologies), using Human TaqMan probes from Thermo Fisher Scientific: MET (assay ID Hs04993403_cn), EGFR (assay ID: Hs00942325_cn). RNase P was used as endogenous reference gene (TaqMan Copy Number Reference Assay, human, RNase P).

**FISH Analysis.** FISH analysis for the detection of MET gene copy number was performed on 5-μm paraffin-embedded tissue sections according to standard techniques. The tissue sections were incubated with the MET (7q11.31) 5' dual-color probe (Kreatech; Leica Biosystems) and counterstained with DAPI I (Vysis/Ambion). The FISH analysis was performed with the fluorescence microscope BX61 (Olympus) and the automated FISH imaging platform Bioview (Abbott Molecular). An average of 100 nonoverlapping interphase nuclei with intact morphology was analyzed using H&E-stained sections as a hysto-topographic reference. MET gene was considered amplified when the MET/CEP 7 Ratio was ≥ 2 (44).

**Mutational Analysis.** gDNA was examined with OncoCarta Panel v1.0 (www.readcube.com/articles/10.1038/nmeth.f.254). Next-generation sequencing on 241 genes was performed using Illumina MiSeq. A custom pipeline was used for next-generation sequencing, to call somatic variations when supported by at least 1% allelic frequency and 5% Fisher’s test significance level.

**Flow-Cytometric Analysis.** Cells (2 x 10^7) were incubated with the APC conjugated mouse anti-MET (FAB3852A, clone 95106; R&D Systems Inc.) and conjugated mouse anti-Actin (sc-1616), and P-PP2A (sc-271903) were from Santa Cruz Bio Technology; anti-MET (Tyr1234/1235) (Clone D26), anti-Erk (Thr202/ Tyr204) (Clone D13.14.4E), anti-ERK, anti PP2A (catalog number #2038), anti-P-AKT (Ser473; Clone D9E), and anti-AKT (Clone 40D4) were from Cell Signaling; anti-Vinculin (catalog number V9131) was from Sigma; and P-MET (Tyr1234/1235) (assay ID: s10958) was from Thermo Fisher Scientific.

**Immunohistochemistry.** Twenty-four hours before sacrifice, mice were intraperitoneally injected with 75 μg/mouse EdU. Immunofluorescence was carried out on formalin-fixed paraffin-embedded (FFPE) tissues. Slides were stained using the Click-IT Edu AlexaFluor 555 Imaging Kit (Life Technologies), following the manufacturer’s instructions, and with DAPI. At least 15 images have been acquired for each treatment group. Quantitative analyses of colocalization were carried out with ImageJ software (https://imagej.nih.gov/ij/).

In Vivo Experiments. PDX was obtained by s.c. implantation in the flank of a female NOD (nonobese diabetic) SCID mouse, 5 wk old, purchased from Charles River Laboratories. Tumors were then passed until production of a cohort of 36 mice. Once tumors were established (average volume, 250/300 mm3), mice were randomized and divided into four cohorts (6 mice cohort) and treated daily by gavage with vehicle, AZD-6244 (25 mg/kg), JNJ-38877605 (50 mg/kg), and a combination of the two drugs. Tumor size was evaluated weekly. All animal procedures were approved by the Italian Ministry of Health and the internal Ethical Committee for Animal Experimentation of Candolfo Cancer Institute, FPO-IRCCS.

**Immunofluorescence and Immunohistochemistry.** Twenty-four hours before sacrifice, mice were intraperitoneally injected with 75 μg/mouse EdU. Immunofluorescence was carried out on formalin-fixed paraffin-embedded (FFPE) tissues. Slides were stained using the Click-IT Edu AlexaFluor 555 Imaging Kit (Life Technologies), following the manufacturer’s instructions, and with DAPI. At least 15 images have been acquired for each treatment group. Quantitative analyses of colocalization were carried out with ImageJ software (https://imagej.nih.gov/ij/).

**Immunohistochemistry.** Immunohistochemistry was carried out on FFPE tissues. Slides were incubated with the primary antibodies: P-ERK1/2 rabbit mAb (Th202/Thr204, clone D13.14.4E; Cell Signaling Technology) and P-MET (Tyr1234/1235) (5AF2480 and R&D Systems. Anti-rabbit secondary antibody (Dako Envision + System-horseradish peroxidase–labeled polymer, Dako) has been used. Immunoreactivities were revealed by incubation in DAB solution (DakoCytomation Liquid DAB Substrate Chromogen System; Dako). Slides were counterstained in Mayer’s haematoxylin. A negative control slide was processed with secondary antibody without primary antibody incubation. Images were captured with 40x objective, and representative images were acquired.

**Statistical Analysis.** Statistical analyses were performed by two-tailed Student’s t test and two-way ANOVA, using the GraphPad Prism software. All experiments, except the in vivo trials and phosphokinase array analysis, were repeated at least three times. Figures show one representative experiment, reporting the average of the technical replicates. Statistical significance: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

**ACKNOWLEDGMENTS.** We acknowledge the constructive criticisms of C. Boccaccio, L. Trusolino, and T. Crepaldi, and M. Milan for helpful discussion. We thank L. Casorzo for performing FISH analysis, and the NGS facility, and in particular G. Cisafiulli. A particular thanks to M. Pirra Piscatelli for passionate bench work and C. Fanizza for her contribution to the revision. We also thank B. Martinoglio, M. Buscarnio, R. Albano, and S. Glove for skilled technical assistance. We thank E. Casanova for performing FACS experiments. Finally, we thank A. Cignetto for secretarial assistance. This work was supported by AIRC IG (Associazione Italiana per la Ricerca sul Cancro Investigator Grant), Project 15372; AIRC Special Project for the MCO 2010 MCO (Molecular Clinical Oncology), Project 9970; Grant FPRC (Fondazione Piemonte per la Ricerca sul Cancro - onlus) SxMille Ministero della Salute 2013; and Grant FPRC SxMille Ministero della Salute 2014 (to P.M.C.). Ministero della Salute, Ricerca Corrente 2018.