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Reviving 'oncogenic addiction' to MET bypassed by BRAF (G469A) mutation

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1   Abstract

Cancer clonal evolution is based on accrual of multiple driving genetic alterations that are expected to cooperate and progressively increase malignancy. Increasing evidence exists, which indicates that the interactions of multiple activated oncogenes can impair the efficacy if targeted therapies are based on single agents. However, the molecular mechanisms underlying such crosstalks are not completely understood.

Here, we report a Cancer of Unknown Primary site (CUP) tumor harboring 8-fold MET amplification combined with a downstream activating mutation of BRAF (G469A). Surprisingly, we found that, although highly expressed, the MET kinase was dephosphorylated and inactive in this tumor. Accordingly, in a patient-derived xenograft (PDX) originated from the same tumor, the specific MET inhibitor JNJ-38877605 failed to inhibit tumor growth. Similarly, treatment with a MEK inhibitor was also ineffective, despite the constitutive activation of BRAF. However, the concomitant inhibition of both kinases resulted in significant retardation of tumor growth. Mechanistically, we observed that inhibition of BRAF - or the downstream MEK signaling cascade - resulted in a strong and specific phosphorylation of MET, reviving the addiction to the amplified oncoprotein by inducing its sustained activation. The rapid activation of MET that follows BRAF (G469A) inhibition depends on the suppression of a negative feedback loop dependent on phosphorylation of Ser-985 in MET intracellular domain, which is a known negative regulator of MET kinase activity. Specifically, we found that MEK inhibition activates the protein phosphatase-2A (PP2A), which in turn dephosphorylates the MET Ser-985. These data identify a novel negative feedback loop between BRAF and MET that provide a new mechanism of resistance to targeted monotherapy. Furthermore, the effective tumor growth inhibition that we observed upon

combined inhibition of MET and BRAF, suggests that such combinatorial approach deserves further investigation as new therapeutic option for tumors bearing coexisting genetic alterations of MET and BRAF.

2 Introduction

2.1 Cancer pathogenesis

In the past decades, the extensive studies on cancer have shed light in the understanding of its pathogenesis. It is now an accepted notion that cancer arises through a multistep, mutagenic process by which cancer cells accumulate a common set of properties including unlimited proliferation potential, self-sufficiency in growth signals, and resistance to anti-proliferative and apoptotic signals. Moreover, tumors acquire support from surrounding stromal cells, attract new blood vessels to bring nutrients and oxygen, evade immune detection, and eventually metastasize to distal organs (Hanahan and Weinberg, 2000). The phenotypic traits of cancers are prompted by genetic alterations comprising the gain-of-function mutation, amplification, and/or overexpression of key oncogenes together with the loss-of-function mutation, deletion, and/or epigenetic silencing of key tumor suppressors (Hahn and Weinberg, 2002).

During cancer development, phenotypes displayed by cancer cells are the substantial aberrant activation of existing cellular programs normally present during embryogenesis and tissue homeostasis (*i.e.* cell proliferation, migration, polarity, apoptosis, and differentiation). Accordingly with Darwinian principles, cancer evolves through random mutations and clonal selection of cells able to adapt, survive and proliferate to selection dictated by the microenvironment and under conditions that would be lethal to normal cells.

2.2 Oncogene addiction

Comprehensive genotyping studies performed on tumor specimens are undoubtedly revealing the coexistence of overlapping mutations on different oncogenes owning to the same pathway. The identification of specific driver mutations responsible for the development and progression of cancer, along with a large number of passenger mutations that are irrelevant for the cancer phenotype, has revolutionized cancer treatments. A number of tumors are completely dependent on one single overactive driver oncogene for their survival and growth, and cancer cells undergo cell-cycle arrest and apoptosis if this oncogene is therapeutically inactivated (Sharma and Settleman, 2007). These tumors are addicted to the driver oncogene and responsive to therapy targeting constitutively hyperactive form of the molecule, which derives from point mutations, gene amplification or chromosomal translocation (Herbst et al., 2004; Sharma et al., 2007). The concept of 'oncogene addiction' was first introduced by Bernard Weinstein in 2000, by observing that some cyclin-D overexpressing cancers reverse their malignant phenotype upon cyclin-D depletion by RNA interference (Weinstein, 2002). After more than 15 years, the oncogene addiction concept maintains full validity and remains a model of how mechanistic studies can have immediate translational relevance. Three models seem to clarify the molecular mechanisms of oncogene addiction: genetic streamlining, oncogenic shock and synthetic lethality (Figure 1). The genetic streamlining hypothesis is based on the fact that cancer cells undergo constant genetic drift as a consequence of the selective pressure exerted by the tumorigenic process and by the tumor microenvironment. Cancer cells lose nonessential functions necessary for cell viability or that do not provide any increase in cellular fitness. Nevertheless, the silencing of these functions renders cancer cells much more

susceptible to inhibition of one or more of the pathways still active in cancer cells leading to rapid reduction in cellular fitness. In the 'oncogenic shock' model, addictive oncoproteins trigger at the same time pro-survival and pro-apoptotic signals. While under normal conditions, the pro-survival outputs dominate over the pro-apoptotic ones, the blockade of the addictive oncoprotein induces a rapid decline in the activity of survival pathways destabilizing this balance toward death-inducing signals, leading to death (Sharma and Settleman, 2007). The third model, the synthetic lethality model, is based on state that a gene A is in a synthetic lethal relationship with a gene B when loss of function of either gene A or gene B alone is fully compatible with cell viability, whereas, loss of activity of both A and B gene products is lethal for the cell. Because the gene that is synthetic lethal in combination with the cancerous mutation is usually wild type, the loss of function of the cancerous mutation induces lethality (Weinstein and Joe, 2008).

The concept of oncogene addiction was first supported by evidences in genetically engineered mouse models of human cancers together with mechanistic studies in human cancer cell lines and, later, by clinical trials involving specific molecular targeted agents. Accordingly, it has been observed that activation of the c-myc oncogene in the hematopoietic cells led to the development of T-cell and myeloid leukemias in a transgenic mouse model. Conversely, when this gene was switched off, the leukemia cells displayed differentiation and apoptosis (Felsher and Bishop, 1999). Studies on human cancer cell lines show that these cells, although carrying several genetic abnormalities, are highly dependent on the activity of a single oncogene for continued cell proliferation and survival. An example is the inactivation of ErbB2 in HER2 amplified human breast cancer cell lines by means of antisense oligonucleotides that led

to proliferation inhibition while there was no specific effect of antisense oligonucleotides on breast cancer cell lines that had no amplification of HER2 (Colomer et al., 1994). Similarly, inhibition of MET oncogene caused extensive apoptosis in MET-amplified gastric cancer cell lines but not in cell lines without MET amplification (Smolen et al., 2006). The dependency of some tumors on one single oncoprotein for their growth supposes that it is the target for therapeutic intervention thus providing a rational to develop new targeted compounds against that oncoprotein for the treatment of various cancers (de Bono and Ashworth, 2010; Stegmeier et al., 2010).
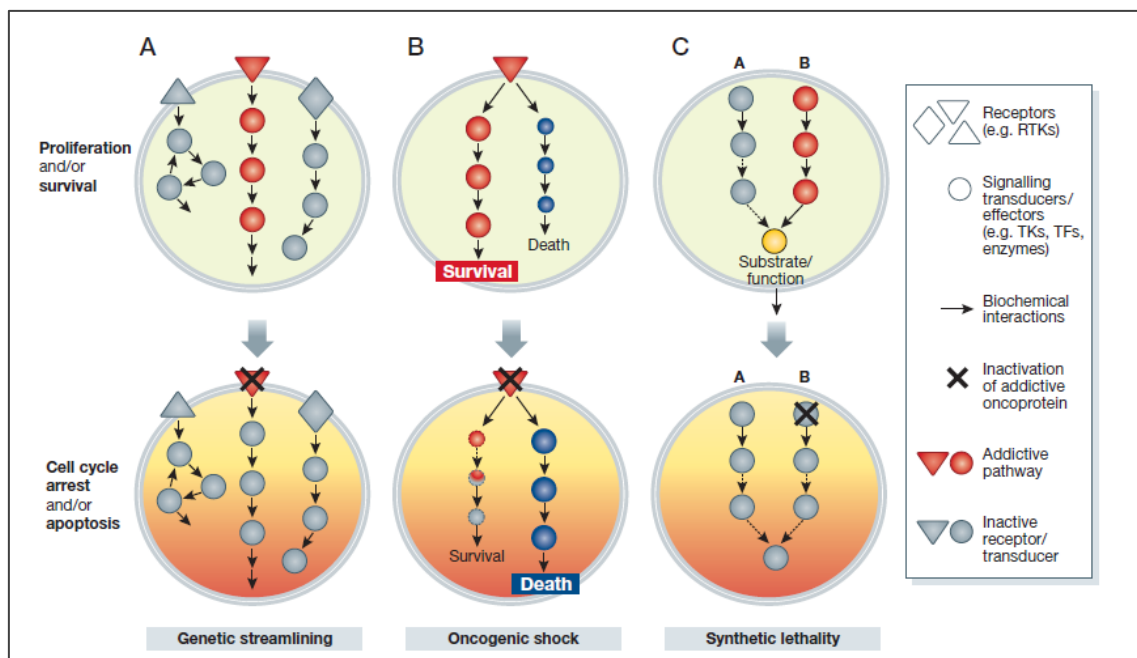
Figure 1: Models of oncogene addiction (Torti and Trusolino, 2011)

2.3   Targeted therapies

The study of oncogene addiction and the use of appropriate therapies gave rise to a series of successful clinical stories that prove the impact of targeted therapy in selected cohorts of patients. One of the most important translational application comes from the above mentioned HER2 amplified breast cancers; about 25−30% of breast cancers

overexpress HER2 protein due to gene amplification, a feature that correlates with adverse prognosis (Valabrega et al., 2007). Moreover, *in vitro* studies showed that HER2 amplification is a driver event in the onset and progression of breast cancers, thus rendering HER2 an attractive target for therapeutic inhibition. On these premises, agents to block HER2 activity, such as the humanized monoclonal antibody trastuzumab, were tested in clinical trials and are now approved for clinical use (Choudhury and Kiessling, 2004). Another example of oncoprotein that has been extensively studied for targeted therapy is EGFR in lung cancer. A number of prospective studies on patients affected by advanced NSCLC presenting activating EGFR mutations have demonstrated the benefit of EGFR inhibition. Indeed, in this setting 50–70% of such patients respond to therapy with EGFR inhibitors, and this correlates with improved progression free-survival and overall survival rates of treated patients (Pao and Chmielecki, 2010), underlying the importance of targeted therapy. In melanomas, as well as other types of cancers, BRAF is frequently hyperactivated due to single nucleotide substitutions (Gray-Schopfer et al., 2007). Based on this, several small-molecule inhibitory compounds against BRAF have been formulated. In particular, Vemurafenib, that is highly selective for the mutationally activated BRAF V600E allele, exhibited promising results in the preclinical setting. Such results were confirmed in a Phase 1 trial, which reported 81% overall response rate in mutated patients (Flaherty et al., 2010). The advent of targeted therapy raised new hopes for cancer treatment. However, successful translation of the oncogene addiction model into the rational and effective design of targeted therapeutics still faces major obstacles and only a small portion of patients effectively benefit from targeted therapies. Indeed, examples reported above are followed through a negative counterpart. In fact, only 30% of HER2-overexpressing metastatic breast cancer patients responded to anti-HER2 monotherapy (Valabrega et

al., 2007). Analogously, the small-molecule compounds gefitinib and erlotinib were built up to block the catalytic activity of EGFR but, when used in EGFR overexpressing lung cancers, the extent of clinical benefit from monotherapy with either drug induced a response rate of approximately 10% (Sequist and Lynch, 2008) as also in this case targeted inhibition of EGFR is effective in only a small subgroup of patients. Finally, tumor regrowth has been documented to occur in many patients affected by melanoma. This is in agreement with the notion that cancer cells are poorly sensitive to inhibition of the oncogenic driver whenever further alterations coexist, providing additional survival signals that relieve the consequences of target inactivation.

2.4   Primary and secondary resistances to targeted therapies

The failure of a small subset of patients to respond to therapy is defined primary or intrinsic resistance; conversely, most of patients, after an initial response, stop to benefit from therapy, a condition known as secondary or acquired resistance (Ellis and Hicklin, 2009). In particular, intrinsic resistance is clinically described as a lack of any positive, complete or partial, response to therapy; on the contrary, clinically acquired resistance is characterized by disease progression after an initial positive response to therapy or the stagnation of disease from therapy initiation over a duration of a minimum of six months (Jackman et al., 2010).

Mechanisms of resistance to targeted therapies develop through target reactivation by mutations/amplification in the target itself or other proteins interfering with drug activity; up- or down-regulation of the signaling components downstream or upstream of target thus activating targeted signaling pathway; reprogramming of a cell by activation of an alternative signaling pathway that can mimic or compensate for targeted

pathway (Asić, 2016) (Figure 2). While primary resistance evolves through selection during the tumorigenic process itself, in the case of secondary resistance treatment induces massive cell death and minor clones with improved fitness are selected during the course of therapy. Indeed, it is increasingly recognized that tumors contain a high degree of genetic and molecular heterogeneity within the same lesion (Gerlinger et al., 2012). Thus, secondary resistance can originate through acquisition of new genetic lesions under the pressure of therapy but it can also arise through selection, induced by treatment, of pre-existent subclones of cells already present in the original tumor that are intrinsically insensitive (Turner and Reis-Filho, 2012).

Numerous cases of primary and secondary resistance have been reported. As discussed before, primary and secondary resistances have been identified in HER2-amplified tumors that do not respond to trastuzumab. Actually, among HER2-amplified tumors, only a fraction of cases responds to therapy, due to the concomitant activation of alternative pathways (Berns et al., 2007; Freudenberg et al., 2009; Stemke-Hale et al., 2008). Similar to HER2, resistances also occurs in EGFR mutated lung cancers through the presence of additional alterations in the EGFR coding sequence co-existing with classical activating mutations or the presence of other genetic lesions that affect signaling downstream of EGFR (Pao and Chmielecki, 2010). Analogously, almost 20% of patients affected by melanoma initially do not respond to anti-BRAF monotherapy, due to intrinsic resistance while most of responsive patients eventually develop secondary resistance, through reactivation of the hit pathway, activation of tyrosine kinase receptors and activation of parallel signaling pathways (Manzano et al., 2016). The shared trait of all these examples is a cross-talk among oncogenes in which the inactivated target is bypassed by compensating activated oncogenes that may act within

the same targeted pathway, thus re-stimulating the pathway itself or in parallel axes that

are activated to substitute the blocked signaling.
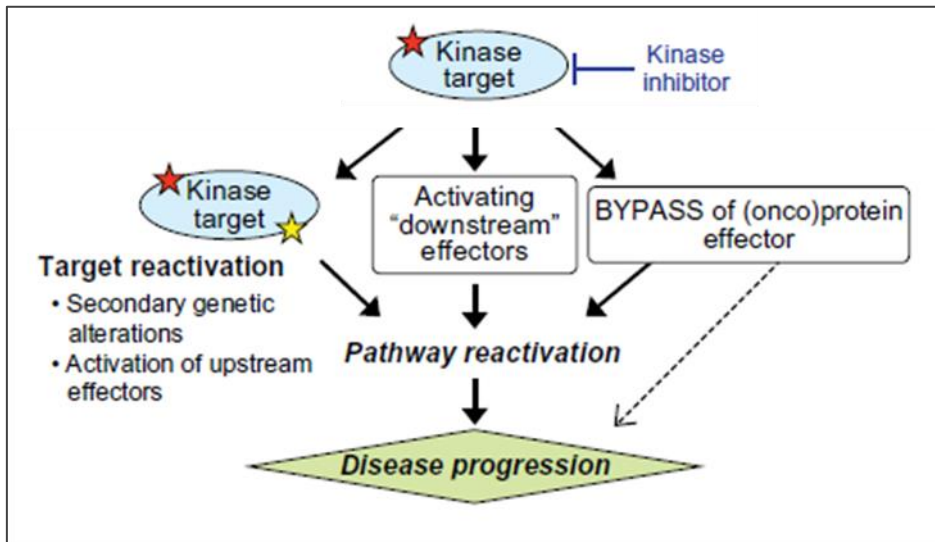
Figure 2: Mechanisms of resistance to targeted therapy (modified from (Garraway and Jänne, 2012))

2.5 Cross-talk among oncogenes: negative feedbacks and signaling pathway compensation

The limited efficacy of targeted therapies acting against genetically altered oncogenes is

usually caused by compensatory signals that substitute the inhibited target activity.

Negative feedback loops typically operate within oncogenic signaling pathways to avoid

signal overload and moderate signals in response to cellular requirements. Several

studies on signaling regulation and deactivation have highlighted the importance of

negative feedback control as a mechanism to ensure signaling thresholds compatible

with the induction of a physiological response (Casci et al., 1999; Fiorini et al., 2002;

Ghiglione et al., 1999; Golembo et al., 1996; Tsang and Dawid, 2004). The inhibition of

hyperactive feedbacks and consequently loss of the attenuating loops and overactivation

of oncogenic signaling has been implicated in the onset and progression of different

human cancers playing a key role in developing resistance to therapies (Chandarlapaty, 2012).

2.5.1 Negative feedbacks regulate signaling pathways in physiology

It is commonly accepted that signaling pathways are tightly regulated through negative feedbacks, confining in space and time signaling events, under physiologic conditions. Negative feedback regulation of signaling pathways occurs through a multitude of mechanisms. They include relatively fast-acting mechanisms that use the downstream effectors of the cascade to directly modify the activity of upstream components. Other slower mechanisms depend on the de novo expression of proteins, which in turn target, at multiple levels, the pathway to inhibit it (Ledda and Paratcha, 2007). All these mechanisms ensure that signaling dynamics can be controlled in a well-defined manner which can adapt promptly to changes in the homeostasis. Deregulation of this balance is a central mechanism by which cells escape external and internal self-limiting signals. In this scenario, protein phosphatases have a major role in negatively regulating such molecular networks. Nearly one-third of cellular processes are regulated via phosphorylation, where proteins transiently change from a phosphorylated to a dephosphorylated state and vice versa, through specific controls by protein kinases and protein phosphatases. Phosphatases are intensely involved in the regulation of tyrosine kinase receptors and their signaling pathways. Phosphatases are classified in two major classes, protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases (PSPs) (Alonso et al., 2004; Mumby and Walter, 1993). Among PTPs, PTP1B is the tyrosine phosphatase involved in EGFR inactivation (Lammers et al., 1993). It was shown that fibroblasts from PTP1B-deficient mice increased and sustained EGFR phosphorylation after growth factor treatment (Haj et al., 2003). Another example is the

tyrosine phosphatase BDP1, a regulator of HER2. The overexpression of BDP1 inhibited activation of HER2, while its suppression increased receptor phosphorylation. In addition, BDP1 was able to interfere with downstream signaling events, reducing MAPK activation (Gensler et al., 2004). However, autophosphorylation inhibitors of receptor tyrosine kinases downstream signaling have been described. PTEN is a phosphatidylinositol phosphatase implicated in negative signaling that specifically inhibits PI3K-Akt signaling pathway. The activity of Akt is regulated by PI3K via the synthesis of phosphatidyl inositol 3, 4, 5-triphosphate (PIP3). PTEN antagonizes PI3K by degrading PIP3 to phosphatidyl inositol 4, 5-biphosphate (PIP2). Deregulation of Akt through loss of functional PTEN has been implicated in the progression of different tumors (Simpson and Parsons, 2001). On the contrary, overexpression of PTEN in cancer cell lines inactivates Akt and induces cell cycle arrest (Lu et al., 1999).

Among PSPs, Protein Phosphatase-2A (PP2A) has an important role in regulating the activity of several critical cellular molecules including Akt, p53, c-Myc and β-catenin (Seshacharyulu et al., 2013). Because inhibition of its activity or loss of some of its functional subunits is a feature of neoplastic transformation, PP2A is now widely described as a tumor suppressor (Eichhorn et al., 2009). Consequently, it has been demonstrated that the PP2A inhibitor, okadaic acid, caused tumor development when injected into mice (Nagao et al., 1995).

2.5.2 Negative feedbacks and signaling pathways compensation mediate resistances in pathology

Incoming mechanistic studies are directing cancer therapy to the switch from an oncogene centered to a network-based point of view. The concomitant activation of alternative pathways as well as the modulation of feedback loops activity has been described as resistance mechanisms to targeted therapies. Examples include parallel

activation of IGF1 receptor signaling, overexpression of EGF family ligands and hyperactivation of PI3K-Akt signaling pathways in HER2-amplified breast cancer resistant to trastuzumab (Freudenberg et al., 2009). In these tumors, activation of the PI3K-Akt pathway is due to oncogenic mutations in the PIK3CA gene, encoding the catalytic subunit of PI3K, or loss of function of PTEN, encoding the phosphatase that attenuate PI3K activity (Berns et al., 2007). Also in NSCLC, sensitivity to EGFR monotherapy is impaired by the presence of other genetic lesions that affect signaling downstream of EGFR. For example, PTEN loss in NSCLC cells uncouples EGFR from negative feedback mechanisms, resulting in decreased proteasomal degradation (Sos et al., 2009; Vivanco et al., 2010). Similarly, feedbacks that mediate resistance to BRAF inhibitors have been studied in colon cancer. One of these characterizes multiple BRAF V600E mutant colon cancers in which the inhibition of EGFR by antibody drug or small molecule is strongly synergistic with BRAF V600E inhibition. In fact, authors demonstrated that the inhibition of BRAF causes a rapid feedback phosphorylation and activation of EGFR, through the reduction of EGFR-phosphatase (CDC25) activity (Prahallad et al., 2012). In 2014, Sun and co-workers showed that 6 out of 16 BRAF V600E melanoma acquired resistance to BRAF or MEK inhibitors via EGFR expression through the suppression of sex determining region Y-box 10 (SOX10) that leads to the activation of TGF-β signaling, thus causing upregulation of EGFR (Sun et al., 2014). In another work, it has been shown that BRAF- mutant thyroid cancer cells are refractory to RAF inhibitors. Here, the induction of HER3 transcription, through decreased promoter occupancy by the transcriptional repressors C-terminal binding protein 1 and 2 and by autocrine secretion of neuregulin-1, causes an increase in HER3 signaling and a consequent rebound in ERK signaling. In this case, inhibition of HER2, which prevented HER3 phosphorylation and subsequently MAPK activation, sensitizes

BRAF -mutant thyroid cancer cells to RAF or MEK inhibitors, providing a rationale for combining MAPK pathway antagonists with inhibitors of feedback reactivated HER signaling (Montero-Conde et al., 2013). The regulation of negative feedback pathways plays a key role in cancers, implicating them as an important mechanism of drug resistance. There is evidence that targeting an individual oncogene is only rarely effective likely because compensatory pathways are reactivated as a consequence of negative feedback suppression. This suggests that combinatorial therapies, targeting multiple interconnected pathways, are more likely to succeed.

2.6 MET oncogene networks

In the context of combinatorial therapy to overcome resistances, the MET oncogene plays a key role representing the prototype of interactions mediator. MET dynamically interacts with other cellular surface receptors and intracellular proteins, and the output signal triggered by activation of the receptor derives from the integration of this complex network. MET has been proved to interact with many different molecules influencing the response to therapy targeting MET itself and MET partners; HER2, EGFR and BRAF are some examples.

The involvement of MET in mediating resistance to anti-HER2 therapies was suggested in several models. For example, in HER2-positive metastatic breast cancer, MET amplification strongly correlated with poor outcome and resistance to trastuzumab (Minuti et al., 2012). Furthermore, studies on HER2-positive breast cancer cell lines and primary tumors revealed that MET blockade inhibited activation of Erk and Akt and sensitized cells to anti-HER2 treatment (Shattuck et al., 2008). Further evidences from gastric cancer models indicate MET and consequent Erk and Akt activation as a

mechanism of resistance to the HER2 inhibitors (Chen et al., 2012). Similarly to HER2, the cross-talk between MET and EGFR causes resistance to anti- EGFR inhibitors. In fact, 20% of patients with EGFR-mutated NSCLC develop resistance to EGFR inhibitors through the upregulation of MET pathway (D'Arcangelo and Cappuzzo, 2013). In this context MET activation is a consequence of MET amplification or upregulation of HGF expression which in turn leads to transactivation of HER3 and downstream PI3K/Akt activation, with consequent resistance to EGFR inhibitors (Bean et al., 2007; Engelman et al., 2007; Tang et al., 2008). These data provide a rationale for treatment of EGFR inhibitors resistant patients that display MET amplification or increased HGF expression, with MET inhibitors. Importantly, Turke and colleagues demonstrated that subpopulations of cells harboring MET amplification pre-existed to anti-EGFR therapy, suggesting the idea to co-treat patients with MET and EGFR therapies (Turke et al., 2010). Accordingly, co-treatment with anti-MET and EGFR inhibitors significantly enhanced tumor growth inhibition and caused regression in preclinical experiments (Wang et al., 2012). The involvement of MET in resistance to EGFR inhibition has also been extended to colorectal carcinoma where MET amplification was associated with resistance to EGFR inhibitors (Bardelli et al., 2013).

Besides mediating resistance to receptor tyrosine kinase inhibitors, MET has also been implicated in causing reduced sensitivity to downstream inhibitors. In patient-derived melanoma cell lines MET amplification and activation is a principal mechanism of primary resistance to BRAF inhibitors and genetic ablation of MET together with BRAF inhibition efficiently reduced cell growth and invasion (Vergani et al., 2011). More recently, Pietrantonio et al. reported another example of MET amplification as a new mechanism of resistance to anti-EGFR and BRAF combination therapies in a BRAF-mutated metastatic colorectal cancer. Here, a patient presented BRAF V600E

mutation and initially responded to combined EGFR and BRAF inhibitors. Then, resistance emerged due to the selection of pre-existing cells with increased MET gene copy number that underwent clonal expansion during treatment. Clinical benefit was achieved by the treatment with a MET inhibitor (Pietrantonio et al., 2016). However the scenario is more complex as MET overactivation can be mediated by other mechanisms, along with gene amplification, and it has been established a major role for MET in driving cancer progression, metastasis formation and dissemination.

2.6.1 MET functions and regulation

Considering the critical role of MET in interacting with other oncogenes and in mediating resistances, the study of its functions and regulation is extremely important. MET tyrosine kinase receptor is crucially involved in the control of the '*invasive growth*' genetic program. Such program is sustained by the biological process known as epithelial-mesenchymal transition (EMT). During EMT cells are able to release junctions that maintain the epithelial monolayer structure, rearrange cytoskeleton to change their polarity and move through the extracellular environment. Thus cells lose their epithelial phenotype to acquire a mesenchymal one. EMT is followed by cell migration and generation of new structures (Benvenuti and Comoglio, 2007).

Physiologically, MET is involved in embryonic development and organ regeneration. It contributes to establish the normal tissue patterning by orchestrating cellular proliferation, disruption of intercellular junctions, migration and invasion through the extracellular matrix (EMC) and protection from anoikis (programmed cell death induced upon cell detachment from EMC) (Birchmeier and Gherardi, 1998). In adult life this program is necessary during acute injury repair in normal tissues (Huh et al., 2004). Once bound by its ligand HGF (secreted by mesenchymal cells), MET

(expressed in epithelial cells and myoblast progenitors) kinase activity is switched on by receptor dimerization and auto-phosphorylation of the catalytic tyrosines (Y1234 and Y1235) located within the kinase activation loop. This results in subsequent phosphorylation of the docking tyrosines (Y1349 and Y1356) located within the docking site. The tyrosines of the docking site act as a degenerate motif for the recruitment of several intracellular signaling proteins and adaptors (Ponzetto et al., 1994). The different signaling proteins and adaptors are responsible for generating MET-specific biological activities by activating multiple signaling transduction pathways, including the Grb2-Ras-Mitogen-Activated Protein Kinase (MAPK) cascade, the Phosphatidylinositol-3 Kinase (PI3K) pathway and the Signal Transducer and Activator of Transcription (STAT) pathway (Figure 3).
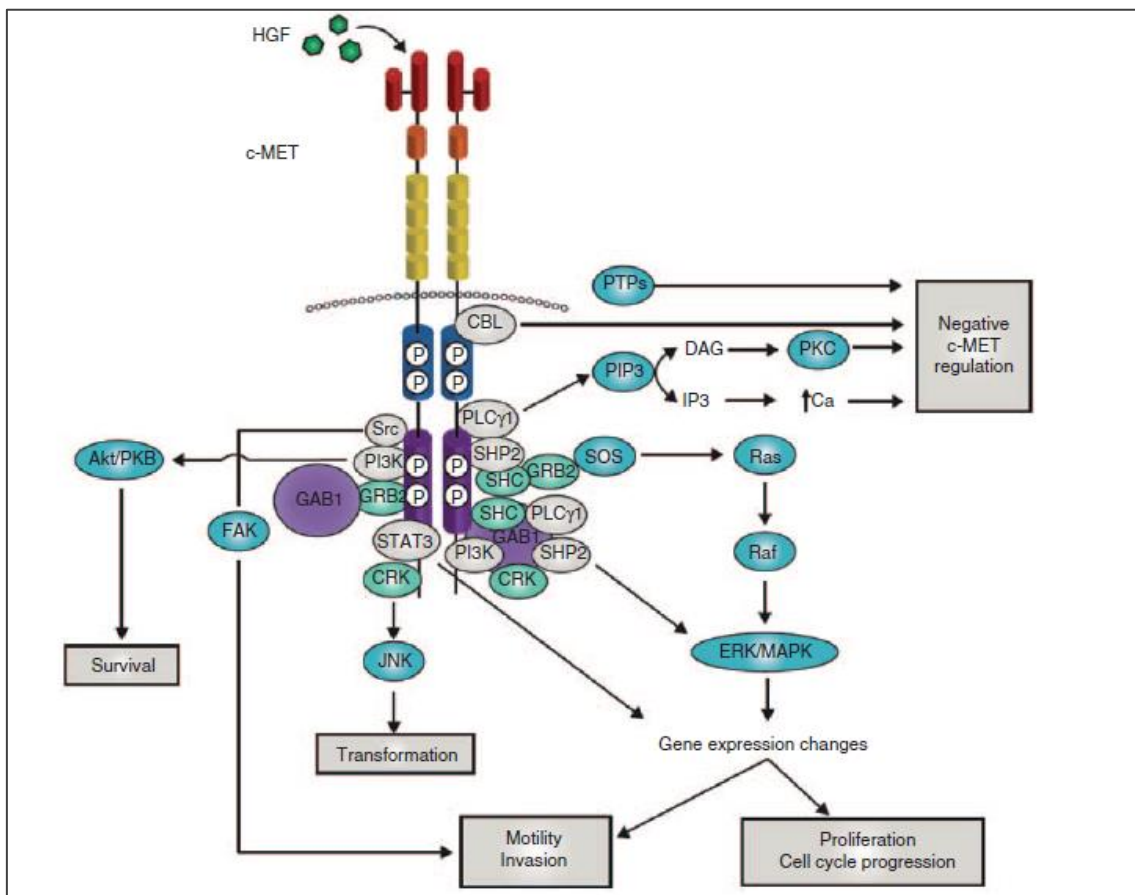

Figure 3: MET-mediated signaling (Organ and Tsao, 2011)

In physiological contexts, MET activation is tightly regulated through different mechanisms. MET is a substrate for several protein-tyrosine phosphatases, including the receptor PTPs density-enhanced phosphatase 1 (DEP1; also known as PTPRJ) and leukocyte common antigen related (LAR; also known as PTPRF) and the non-receptor PTPs PTP1B (also known as PTPN1) and T cell PTP (TCPTP; also known as PTPN2) (Machide et al., 2006; Palka et al., 2003; Sangwan et al., 2008). These phosphatases are involved in MET shutdown, triggering dephosphorylation of either the 'catalytic' (in the case of PTP1B and TCPTP) or the 'docking' tyrosines (DEP-1). In addition, recruitment of PLCγ results in activation of protein kinase C (PKC) that negatively regulates MET kinase phosphorylation and activity via Ser985 regulation (Gandino et al., 1990). Ser985, in the juxtamembrane domain, acts as a negative regulator of MET signaling. Under Ser985 phosphorylated conditions, kinase tyrosines are dephosphorylated, while its dephosphorylation, through recruitment of protein phosphatase-2A (PP2A), leads to MET kinase tyrosine phosphorylation and activation (Gandino et al., 1994; Hashigasako et al., 2004). MET signaling can be inhibited by receptor internalization. The endocytosis of ligand-activated receptor and its subsequent degradation in the lysosomal compartment is the most active form of receptor desensitization and signal inhibition (Sorkin and von Zastrow, 2009). Lysosomes trafficking involve Cbl activity. Cbl is an E3 ubiquitin-protein ligase that is recruited to Tyr1003 residue in the active receptor (Peschard et al., 2001). Cbl allows MET monoubiquitylation at multiple sites (Carter et al., 2004), and consequently its recognition by endocytic adaptors that contain ubiquitin-binding domains, its arrangement into clathrin-coated vesicles and delivery to the endosomal network. MET accumulation in multivesicular bodies sequesters the receptor from the cytosol thus switching off the signal. Lastly, MET undergoes proteolytic

demolition through fusion of the multivesicular bodies with lysosomes (Hammond et al., 2001). However, MET proteasomal degradation occurs in an ubiquitin-independent manner, via sequential proteolytic cleavages. Disintegrin and metalloprotease (ADAM)-mediated release of the extracellular domain (known as shedding), is a first step that lead to the production of a soluble N-terminal fragment and a membrane-anchored cytoplasmic tail. It is followed by proteolysis through γ-secretase, which produces a labile intracellular portion that is rapidly cleared by proteasome-mediated degradation (Foveau et al., 2009). The MET extracellular shedding reduces the number of receptor molecules on the cell surface, but also creates decoys that interact with both HGF (by sequestering the ligand) and full-length MET (by impairing dimerization and transactivation of the native receptor) to further inhibit MET signaling (Michieli et al., 2004). Conversely, MET deregulation is responsible for cancer progression, metastasis formation and dissemination (Trusolino and Comoglio, 2002).

2.6.2   Genetic-mechanisms of MET activation in cancer

Aberrant MET activation in cancers occurs through several mechanisms: HGF-dependent activation with establishment of autocrine or paracrine loops that release cells from the need of growth factors; receptor over-expression, which triggers receptor oligomerization and activation even in absence of ligands; somatic genetic lesions (including translocations, gene amplifications and activating mutations) which generate constitutively active receptors. However, MET activation in human cancers is mostly due to over-expression as a consequence of increased gene copy number or at transcriptional level. Enhanced MET expression has been described in a number of solid tumors including ovarian cancers (Di Renzo et al., 1994), osteosarcoma (Ferracini et al., 1995), renal (Natali et al., 1996), hepatocellular and non-small cell lung carcinomas

(Olivero et al., 1996); tumors of the upper gastrointestinal tract (Porte et al., 1998), gastric (Taniguchi et al., 1998) and oral squamous cell carcinomas (Morello et al., 2001); pancreatic (Di Renzo et al., 1995) and prostatic cancers (Humphrey et al., 1995). MET amplification was described in gastric cancers (Kijima et al., 2002; Kuniyasu et al., 1992), tumors of the upper digestive tract such as biliary tract (Nakazawa et al., 2005) and esophageal carcinomas (Miller et al., 2006) but also in lung cancers (Engelman et al., 2007; Lutterbach et al., 2007) and metastatic colorectal (Bardelli et al., 2013). Even though MET mutations are much less frequent, they have been described in several human tumors. Activating point mutations in MET kinase domain have been reported in hereditary and sporadic papillary renal-cell carcinomas (RCC) (Schmidt et al., 1997) and childhood hepatocellular carcinoma (HCC) (Park et al., 1999). Alterations in the juxtamembrane region were mainly found in gastric carcinoma (Lee et al., 2000), in lung cancers and pleural mesothelioma (Cipriani et al., 2009) and melanoma (Puri et al., 2007). MET mutations have been identified in clonal primary tumor cell outgrowths and lymph node metastases derived from head and neck squamous cell carcinoma (HNSCC) suggesting that mutations are selected during metastatic progression in these cancers (Di Renzo et al., 2000). In 2011, Stella et al. described an uncommonly high incidence of MET mutations in Cancers of Unknown Primary site of origin (Stella et al., 2011).

2.6.3 Non-genetic mechanisms of MET activation in cancer

It is now widely accepted that the development of human cancers is not only due to the sequential accumulation of somatic mutations but also to the interaction of oncogenes with tumor microenvironment. For example, hypoxia stimulates transcription of MET resulting in higher levels of protein expression. Thus hypoxic regions of human tumors

show increased expression of MET while MET expression becomes very low in proximity of blood vessels. In addition, hypoxia enhances HGF signaling and cooperates with HGF in promoting branching morphogenesis and invasion (Pennacchietti et al., 2003).

Also ionizing radiations exert their effect on MET expression and activity. Indeed, ionizing radiations induce transcriptional up-regulation and catalytic activation of the receptor; increased MET activity delivers anti-apoptotic signals that prevent cell death induced by irradiation. Coherently, MET inhibition increases tumor cell radiosensitivity and prevents radiation-induced invasiveness (De Bacco et al., 2011).

Finally, only recently it has been clarified the control of the axis HGF/MET by DNA methylation in bone metastases from breast carcinoma. Authors observed that DNA methyltransferase blockade strongly reduced the expression of HGF/MET receptor axis together with impaired bone metastasis outgrowth and prolonged mice survival (Bendinelli et al., 2017). In fact, in the absence of DNA sequence modifications, the dynamic plasticity and opportunistic adaptations of cancers can largely depend on epigenetic mechanisms through the activation of alternative transcription-start sites and the release of loci from DNA-methylation repression, or by the blockade of transcription at methylated regions corresponding to tumor-suppressors (Vizoso and Esteller, 2015). During tumor progression, the aberrant DNA methylation seems to play a role in the switch from epithelial-mesenchymal transition (EMT) - the process that promotes invasion of primary tumors and dissemination of metastatic cells - to mesenchymal-epithelial transition (MET) - the process that contributes to the establishment of distant metastases (Bendinelli et al., 2015; Nickel and Stadler, 2015).

Definitely, it is clear that MET activation contribute not only to the emerging resistance to targeted therapies but also to the onset and progression of a number of cancers.

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2.6.4 MET targeted therapy and resistance to MET inhibitors

Due to its aberrant activation in cancer, MET is a very attractive therapeutic target. The strategies used to neutralize the receptor include: kinase inhibitors (ATP or non-ATP competitors), HGF inhibitors, antibodies and decoy. The first approach uses low molecular weight molecules, most of which are able to compete for the ATP binding site of the receptor and prevent receptor transactivation and recruitment of the downstream effectors. Some of these compounds comprise K252a, PHA-665752, SU11274 and JNJ-38877605 (Berthou et al., 2004; Christensen et al., 2003; Morotti et al., 2002; Smolen et al., 2006). More recently new inhibitors have been developed, among them Crizotinib, Foretinib, Cabozantinib (Cui et al., 2011; Kataoka et al., 2012; Katayama et al., 2015; Schwab et al., 2014; Shah et al., 2013; Yakes et al., 2011). HGF inhibitors are molecules designed to specifically prevent the ligand binding to the receptor and unable to activate the downstream signaling kinase inhibitors. NK4 is a molecule bearing the N-terminal region and the four kringle domains of HGF (Date et al., 1998). The molecule competes with the ligand for the receptor binding but fails to activate the receptor, thereby blocking the downstream pathways and the biological outcomes (Matsumoto and Nakamura, 2003). Monoclonal antibodies directed against MET receptor act blocking receptor activation. DN30 is a monoclonal antibody directed against the extracellular portion of MET that induces receptor shedding, prevents MET activation and abrogates its biological activity (Pacchiana et al., 2010; Petrelli et al., 2006; Spigel et al., 2013; Vigna and Comoglio, 2014).

Also for MET, the development of targeted therapy is followed by the spreading of resistances. A number of mechanisms of resistance to MET inhibitors have been described. Since 2010 it was shown that MET-amplified GTL16 in gastric cancer cell line exposed to increasing doses of two different MET inhibitors (PHA-665752 and

JNJ38877605) become resistant to MET inhibition via either KRAS amplification (Cepero et al., 2010) or activation of HER family members including EGFR, HER3 and downstream signaling pathways common to MET and HER families (Corso et al., 2010). More recently, it was described that in MET-amplified cells, in absence of qualitative or quantitative alterations of EGFR, MET inhibitors induced only tumor growth inhibition, whereas dual MET/EGFR inhibition led to complete tumor regression and completely prevented the onset of resistance due to EGFR phosphorylation and reactivation of the RAS/MAPK pathway (Apicella et al., 2016). A recent genomic and molecular screening on MET amplified lung cells resistant to MET inhibitors revealed that these cells acquire sensitivity to erlotinib thus supporting the known crosstalk between MET and the HER family of receptors (Gimenez-Xavier et al., 2017). The complex interaction of MET with other oncogenes is therefore of great interest to understand the basis of resistance to MET inhibitors and resistance to targeted therapy against MET partners.

2.7   Cancers of Unknown Primary site (CUPs)

MET has been found mutated at uncommonly high frequency in Cancers of Unknown Primary site of origin (CUPs). In CUPs, MET mutational incidence (30%) was significantly higher than the expected one (4%), in the absence of high mutational background thus suggesting the genetic involvement of this oncogene in the CUP syndrome (Stella et al., 2011). CUPs represent an extremely heterogeneous clinicopathological syndrome, constituting about 3–5% of cancers per year. CUPs present as multiple metastases affecting several organ in the absence of a clinically detectable anatomical primary site of origin (Greco and Hainsworth, 1992). An

appropriate diagnostic work-up aims at identifying the occult primary to allow patients to benefit from directed therapy. Standard diagnostic assessments comprise: a thorough physical examination, basic blood and biochemical analyses, and computed tomography (CT) scans of thorax, abdomen and pelvis. In addition, endoscopies are sign-, symptom- or laboratory abnormality-guided. Whole-body 2-deoxy-2-[18F]fluoro-D-glucose-positron emission tomography (FDG–PET)/CT is a powerful tool to search the primary tumor (Fizazi et al., 2011). Immunohistochemistry stains complement light microscopy in providing an 'ad excludendum' final diagnosis. Low molecular- weight cytokeratins (CK7 and CK20) are the most common immunostainings used in occult primary tumors to define subsets of carcinomas.

CUPs are not only of unknown origin, regarding the anatomic primary region, but also of unknown pathogenesis thus representing an unsolved clinical problem lacking effective therapeutic regimens (Briasoulis et al., 2009). Early metastatic dissemination, along with a very aggressive phenotype and unpredictable metastatic pattern are key features of CUPs (Greco and Hainsworth, 1992; Pentheroudakis et al., 2007). CUPs have a wide variety of clinical presentations and feature a poor prognosis in most patients. Life expectancy for CUP patients is very short and median overall survival is below one year (Hemminki et al., 2013; Pavlidis et al., 2003; Pavlidis and Pentheroudakis, 2012).

Recently, efforts have been directed to predict the tissue of origin (ToO) by means of gene expression (Greco et al., 2013; Hainsworth et al., 2013) or miRNA profiling (Søkilde et al., 2014), with the assumption that knowledge of the putative ToO could inform treatment decisions. The rationale for studying molecular profiling to define the site of origin in CUPs is that cancers from different sites of origin have specific gene expression profiles that match their normal counterpart (Bender and Erlander, 2009).

Nonetheless, it is now evident that cancers belonging to the same tissue/organ do not respond to treatment univocally; indeed, response to therapy appears to be mainly dictated by the genomic makeup of individual tumors rather than by tissue ancestry. Recently, 236 cancer-related genes were studied in 200 CUP cases by means of next-generation sequencing, in order to detect potential actionable alterations as therapeutic targets. At least, one genetic alteration was detected in 96% of CUPs, the most common including MET (Ross et al., 2015). Therefore, mutational profiles, rather than prediction of ToO, will be useful to reveal CUPs' vulnerabilities to be tackled by *ad-hoc* targeted therapies. The hallmark of CUPs is their precocious neoplastic spread. Acquisition of the invasive/metastatic phenotype requires the aberrant execution of the '*invasive growth*' genetic programs. Such programs orchestrate different biological activities and are driven by a number of oncogene products among which the tyrosine kinase receptor MET plays a leading role (Trusolino and Comoglio, 2002). In line with this, as already discussed above, a retrospective study showed that MET is frequently genetically altered in CUPs (Stella et al., 2011). In particular, MET mutations were found to occur with a remarkably higher frequency in a cohort of CUP patients (n = 47) compared to unselected cancers (30% versus 4%), and these mutations resulted to be activating. Within the limits of the small cohort analyzed, the relatively high incidence of MET mutations suggests a role of MET in sustaining premature occult dissemination of cancer cells in these tumors. However, the role of MET as driver of CUP progression has not been validated yet and the therapeutic potential of MET inhibition in CUPs has not been assessed, thus rendering urgently needed the study of the functional role of MET in this pathology.

2.7.1 Agnostos Profiling

In our Institute a non-treatment protocol -'Agnostos Profiling'- has been designed to study CUPs. Indeed, the exclusive biology of CUPs is still unknown and its study requires fresh biological specimens from patients. Agnostos Profiling is aimed to collect and screen biological specimens from CUP patients, to study the pathogenic mechanisms of CUPs, focusing on the role played by the MET oncogene. Fresh specimens are provided in a funnel-like model in which the enrolled patients with a suspect of CUP are deeply analyzed to confirm CUP diagnosis. However, only a small part of patients enrolled in the trial will undergo surgery or biopsy. This is a crucial point in obtaining fresh material to implant in mice. In fact, CUP patients are often diagnosed at very last stages of the disease when surgery is not recommended. In addition, most of them already received biopsy, useful for the diagnosis, thus reducing and tightening the neck of the funnel in a few cases of which one portion will be engrafted in mice to generate PDXs (Figure 4).
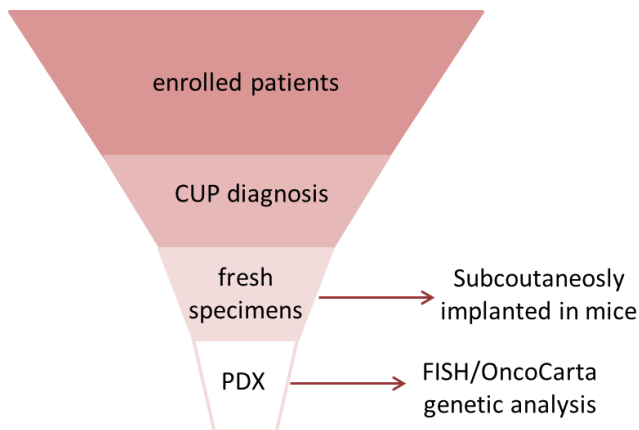

Figure 4: Funnel-like model in Agnostos Profiling

Upon receiving, each CUP sample is implanted in NOD/SCID mouse to generate CUP PDX platform. Patient-derived primary cell lines are isolated from PDX. Although PDXs are an invaluable resource to develop algorithms that accurately matches the drug

sensitivity to genomic abnormalities, xenopatient-based screening is resource-intensive. To overcome this limitation the integration of the *in vivo* trials with PDX derived cell lines enables functional studies on a larger scale, allowing genetic manipulation and mechanistic explorations (Figure 5).
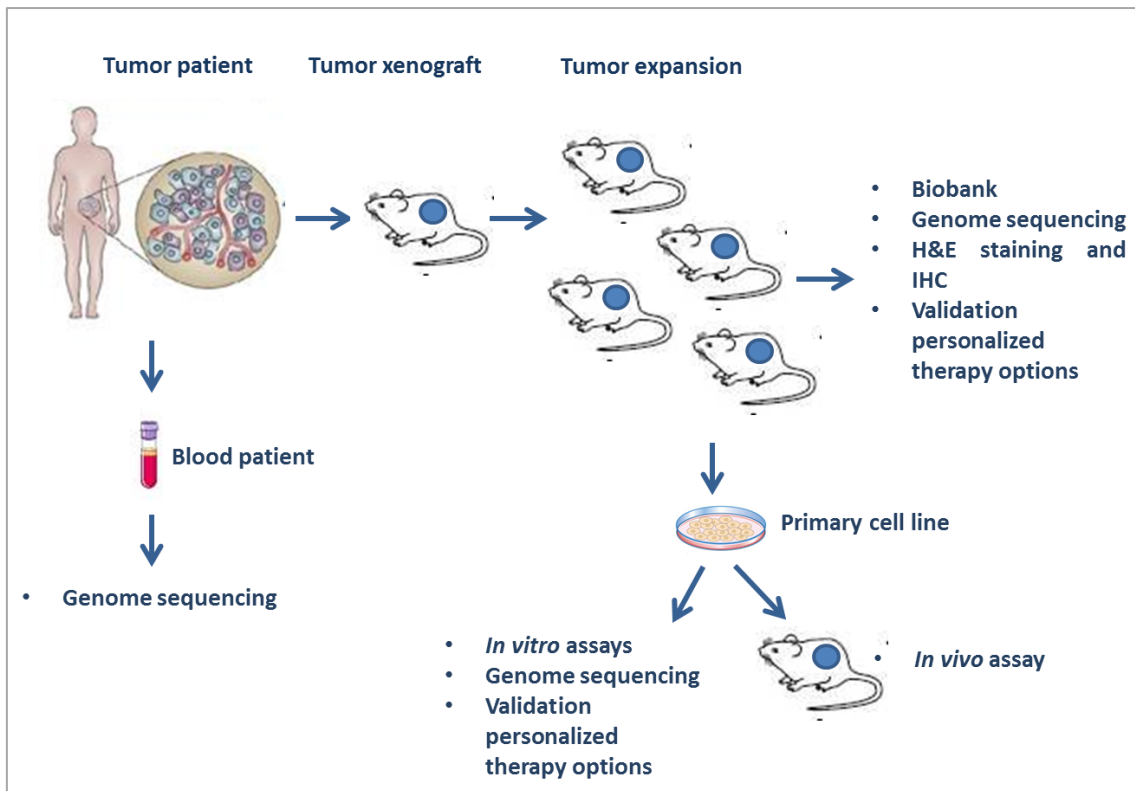

Figure 5: Study flow in Agnostos Profiling. One fragment from each CUP sample is implanted in one Non-Obese Diabetic/Severe Combined ImmunoDeficiency (NOD/SCID) mouse to generate the ancestor PDX while one fragment is paraffin-embedded for FISH and histological analysis and one fragment is used for genomic DNA extraction and subsequent genetic analysis. Once grafted in mice, tumors grow and can be 'serially passaged' leading to the generation of cohorts of mice bearing the same original tumor (Hidalgo et al., 2014). Patient-derived primary cell lines are isolated from PDX.

MET amplification is assessed by FISH analysis. The customized OncoCarta, Sequenom technology, is used to screen a panel of 'actionable' hotspot mutations (238 mutations across 19 oncogenes) (Figure 6).

GENE	MUTATION
ABL1	G250E, Q252H, Y253H, Y253F, E255K, E255V, D276G, F311L, T315I, F317L, M351T, E355G, F359V, H396R
AKT1	E17K, Q43X, V167A, E319G, L357P, P388T, V461L
AKT2	S302G, R371H
BRAF	G464R, G464V/E, G466R, F468C, G469A, G469E, G469R, G469S, G469V, D594V/G, F595L, G596R, L597Q, L597R, L597S, L597V, T599I, V600E, V600K, V600L, V600R, K601N, K601E
CDK4	R24C, R24H
EGFR	R108K, T263P, A289V, G598V, E709A/G/V, E709K/H, G719A, G719S/C, E746_A750del, E746_T751del, E746_T751>A, E746_T751>S, E746_T751>V, E746_S752>V, E746_S752>D, E746_S752>A, E746_S752>I, L747S, L747_E749del, L747_A750del, L747_A750>P, L747_T751del, L747_T751>P, L747_S752del, L747_P753>Q, L747_P753>S, A750P, T751A, T751P, T751I, S752F, S752_I759del, P753Q, M766_A767insAI, S768I, V769_D770insASV, V769_D770insCV, D770_N771>AGG/N771>GF, D770_N771insG, D770_N771insGF, D770_N771insGL, D770fs*61/D770_N771insAPW, N771T, P772_H773insV, H773N, H773_V774insNPH, H773_V774insPH, H773_V774insH, V774_C775insHV, T790M, L858R, L861Q
ERBB2	L755P, A775_G776insYVMA, G776S/LC, G776VC/VC, S779_P780insVGS, P780_Y781insGSP
FGFR1	S125L, P252T
FGFR3	G370C, Y373C, A391E, K650Q/E, K650T/M
FLT3	D835H/Y, I836del
HRAS	G12V/D, G13C/R/S, Q61H/H, Q61L/R/P, Q61K
JAK2	V617F
KIT	D52N, Y503_F504insAY, K550_K558del, P551_V555del, M552L, Y553_Q556del, W557R/R/G, K558_V560del, K558_E562del, V559del, V559D/A/G, V559I, V559_V560del, V560D/G, V560del, E561K, Y568D, Y570_L576del, L576P, D579del, F584S, P585P, K642E, D816H/Y, D816V, V825A, E839K
KRAS	G12A, G12C, G12D, G12F, G12R, G12S, G12V, G13V/D, A59T, Q61E/K, Q61H/H, Q61L/R/P
MET	R970C, T992I, Y1230C, Y1235D, M1250T
NRAS	G12C/R/S, G12V/A/D, G13C/R/S, G13V/A/D, A18T, Q61E/K, Q61H, Q61L/R/P
PDGFRA	V561D, S566_E571>K, T674I, F808L, D842_H845del, D842V, I843_D846del, I843_S847>T, D846Y, N870S, D1071N
PIK3CA	R38H, R88Q, N345K, C420R, P539R, E542K, E545K, Q546K, H701P, C901F, M1043I, H1047R/L, H1047Y
RET	E632_L633del, C634R, C634W, C634Y, A664D, M918T

Figure 6: OncoCarta Panel v1.0. mutational hotspots.

3 Aim of the work

Cancers evolve by repeating processes of clonal selection where acquired driver alterations contribute and collaborate in the adaptation and predominance of cancer cells in the tissue ecosystems. The coexistence of multiple alterations and their interactions are the cause of resistance to targeted monotherapy. The MET oncogene is a key player in interacting with other cellular surface receptors and intracellular proteins and in mediating resistance to targeted monotherapy. In the meantime, MET aberrant activation drives several features of tumor progression in particular in causing metastatization -eroding membranes, infiltrating stromal matrices and eventually colonizing new tissues-. MET has been found genetically altered at uncommon high frequency in Cancers of Unknown Primary site (CUPs), a syndrome characterized by early dissemination of multiple metastases without a primary tumor. CUPs are the prototype of the metastatic disease, likely characterized by activation of the invasive genetic program, in which MET has a key role. Thus in my thesis I investigated the role of MET as driver gene in a CUP sample and its interaction with other oncogenes. In particular, I focused on the specimen of a chemonaïve CUP patient harboring two concurrent alterations: MET amplification and BRAF (G469A) activating mutation. I studied the interactions between these two oncogenes in basal conditions and upon targeted therapies and I took advantage of the derived primary cell line to analyze a previously undescribed negative feedback between the two oncogenes. Moreover, I evaluated the potential involvement of phosphatase(s) in this feedback that may provide a way to restrict in time MET signaling in physiology with consequent implications in the use of combinatorial therapy in tumors harboring these activated oncogenes.

4   Results

4.1 Accrual of CUP1.13 patient

Fresh biological specimens from CUPs patients were obtained through Agnostos Profiling protocol. Based on previous diagnosis and clinical history, a total of 51 patients have been enrolled in the study. The enrolled patients with a suspect of CUP were deeply analyzed to approve the diagnosis (see Materials and Methods for details). CUP diagnosis was confirmed in 29 patients and it is still on hold for 4 patients. However, fresh tumor material was obtained from 10 patients. Among them, samples were obtained from biopsies for 8 patients while the remaining 2 patients underwent surgical resection. 6 specimens successfully engrafted in the recipient mice; engraftment failed for 2 samples while it has to be confirmed for the remaining implanted tumors (Figure 7).
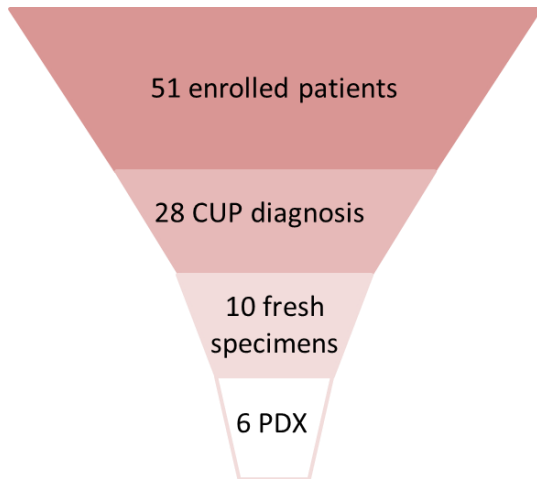
Figure 7: Funnel-like model for PDX platform generation from CUP patients.

Fifty-one patients with the suspect of CUP, were enrolled (dark pink rectangle). A scrupulous diagnostic work-up was performed to exclude non-CUP patients (see M&M for details). CUP diagnosis was confirmed for 28 patients. Fresh specimens were obtained from 10 patients (light pink rectangle), 2 from surgery and 8 from biopsy. Fresh tissues were used to generate PDXs, with engraftment success of 60% (white rectangle).

4.2 CUP1.13 harbors MET amplification but MET targeted therapy fails in reducing tumor growth *in vitro* and *in vivo*

Among the 6 cases for which PDX models were successfully obtained, we focused our attention on CUP1.13. This was derived from a 64-year-old, male patient that was diagnosed with multiple lesions involving metastases to the bone and the liver, together with hilar and mediastinal lymphadenopathy. When the patient underwent a liver biopsy, a tumor fragment (CUP1.13) was subcutaneously implanted in a NOD/SCID mouse and, after tumor growth, expanded to create a xenograft line (PDX1.13). Histological characteristics of PDX1.13 were evaluated and closely recapitulated those of the original tumor CUP1.13 (Figure 8a). Based on PDX-derived material, a primary tumor cell line (L1.13) was also established. Prompted by the high frequency of MET genetic alterations in CUPs (Stella et al., 2011), we decided to perform FISH analysis on PDX-derived material to evaluate MET copy number. FISH analysis on FFPE sections performed with probes for MET (red) and CEP7 (chromosome seven α-centromeric, green) estimated that the signal corresponding to the probe spanning MET gene ranged from 5 to 24 and signal corresponding to CEP7 probe ranged from 1 to 3 (mean values of 17,6 and 2,05 for MET and CEP7, respectively), indicating MET fold-amplification of 8,6. It was then retrospectively validated in CUP1.13 patient and in L1.13 (Figure 8b). MET gene copy number was confirmed by Real-Time (RT) PCR performed on DNA extracted from PDX1.13 and L1.13 and compared to gene copy number of GTL16 MET-amplified cells; EGFR (located on the same chromosome of MET) was used as reference control to rule out polysomy (Figure 8c).

MET expression was therefore evaluated in L1.13 (Figure 8d). FACS analysis displayed higher MET expression in L1.13 (red line) compared to A549 cells (black line) expressing normal level of MET, and A2780 cells (grey line) that do not express the

protein. The expression level of MET was comparable to that of GTL16 cells (blue line).

GTL16 cells are addicted to MET activity. To assess whether this was the case for L1.13, we treated cells with the specific MET inhibitor JNJ-38877605. The treatment with the MET inhibitor did not affect cell viability in L1.13 (Figure 8e) compared to GLT16 cells used as control (Figure 8f).

These results were confirmed also *in vivo* (Figure 8g). In this experiment, once tumors established (average volume 250/300 mm^3), PDX1.13-carrying mice were randomized in 2 cohorts and treated with vehicle or MET inhibitor JNJ-38877605 based on a schedule previously optimized in our institute, which demonstrated high efficacy against MET-amplified PDXs from colorectal cancer (Bardelli et al., 2013). Tumor growth was monitored once a week by evaluating volumetric changes.

Also in this case, treatment with JNJ-38877605 failed in reducing tumor growth in mice.
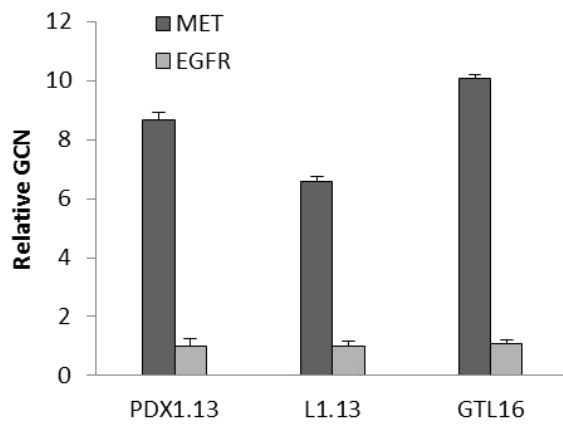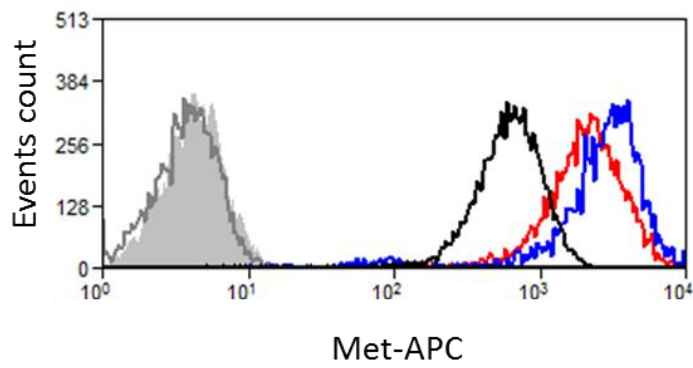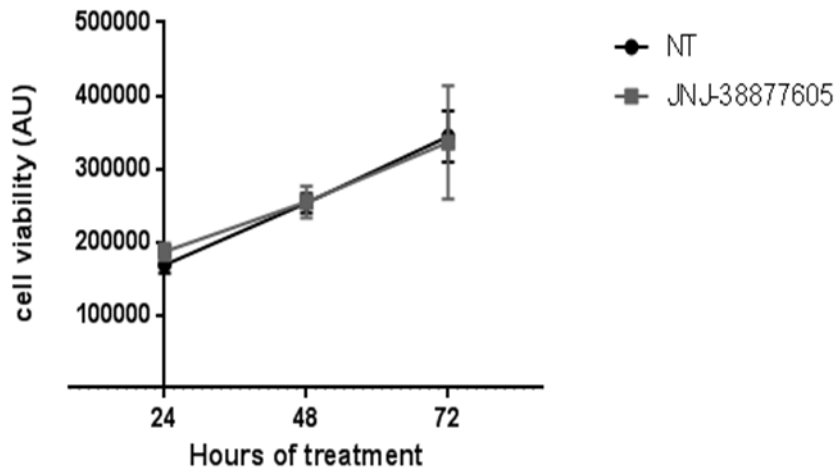
a

CUP1.13   PDX1.13

b

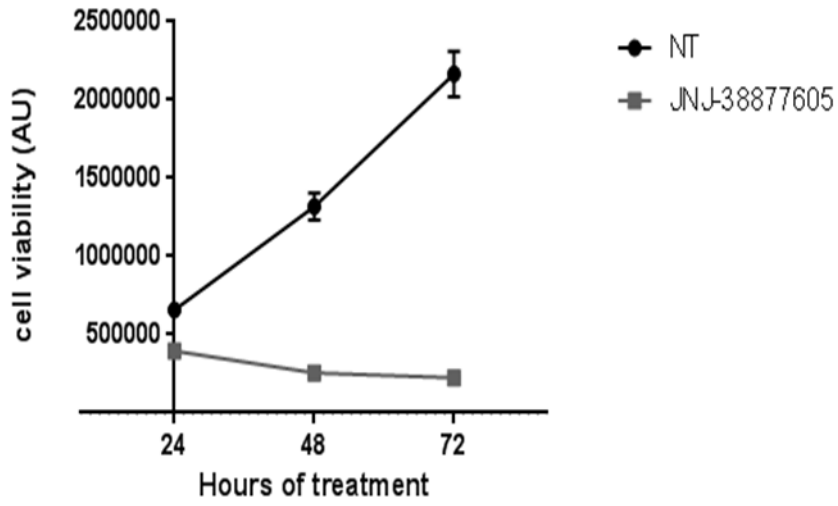CUP1.13   PDX1.13   L1.13
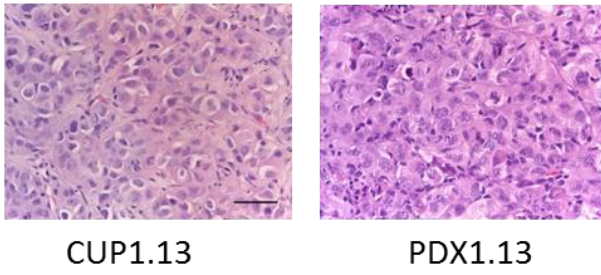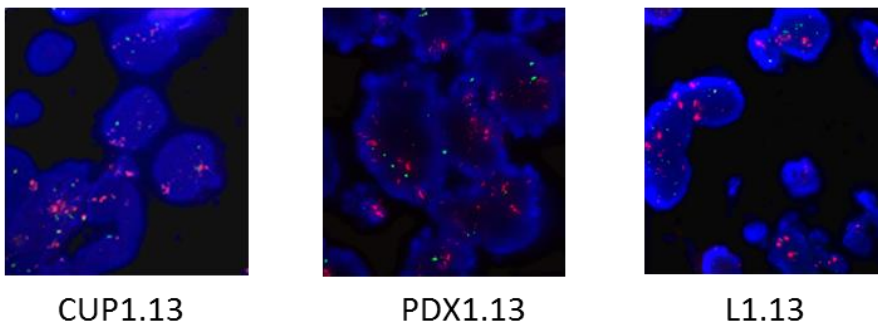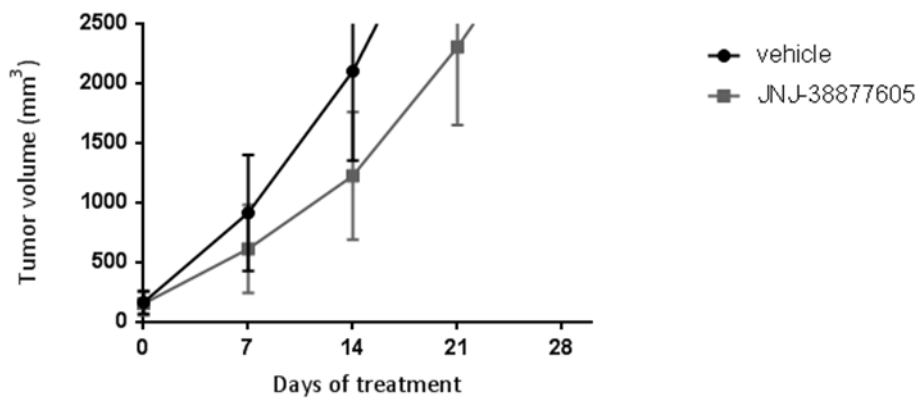
c

d

e

L1.13

f

GTL16

g

Figure 8: MET inhibition is not effective in CUP1.13, although MET amplification

a) PDX1.13 retains the histopathologic characteristics of the original sample. 40X magnification. Scale bar 50μm.

b) FISH analysis of the original tumor (CUP1.13), PDX tumor (PDX1.13) and PDX-derived cells (L1.13) performed with MET (red) and chromosome 7 centromere (green) specific probes. The pictures show multiple red signals due to gene amplification. 100x magnification.

c) Gene copy number (GCN) analysis of MET (black) and EGFR (grey) in PDX1.13, L1.13 cells and GTL16 cells performed by qRT-PCR. The number of MET and EGFR copies were compared to diploid A549 cells. EGFR was used as reference gene on Chr.7 to exclude the polysomy of the entire chromosome.

d) Flow cytometric analysis of MET expression in L1.13 cell line (red), compared to a MET negative cell line (A2780, grey), a cell line expressing normal level of MET (A549, black) and a cell line expressing high level of MET (GTL16, blue). PMT voltages were set using an unstained sample of L1.13 (fill grey).

e) Cell viability assay performed on L1.13 cells, upon treatment with MET inhibitor (JNJ-38877605) for the indicated time.

f) Cell viability assay performed on GTL16 cells, upon treatment with MET inhibitor (JNJ-38877605) for the indicated time.

g) Tumor growth curve of PDX1.13 cohorts treated with placebo (vehicle) and MET inhibitor (JNJ-38877605) as indicated. N=6 mice for each treatment arm. Mice were treated daily for 21 days. Tumor size was evaluated weekly by caliper measurements. Each symbol represents the average tumor volume at the indicated time point. Bars represent the standard deviation.

4.3 CUP1.13 bears a downstream activating mutation in BRAF and only combinatorial MEK and MET inhibition reduces cell viability *in vitro*

To shed more light into the genetics on CUP1.13, the OncoCarta analysis was performed on genomic DNA from PDX1.13. OncoCarta is a custom mutation analysis able to detect 238 hotspot mutations across 19 common oncogenes. It showed a missense mutation (c.1406G) within exon 11 of the BRAF leading to the change of a glycine to an alanine in position 469 (G469A) (Figure 9a). This mutation, falling in the kinase domain of the protein, is known to constitutively activate BRAF kinase, increasing MEK and ERK activation (Davies et al., 2002). Unfortunately the mutation couldn't be validated in CUP1.13 patient due to scarcity of tumor material. However, it was confirmed in L1.13 by Next-Generation Sequencing on a larger number of genes (241 genes) together with the presence of in-frame indels in BRCA1, NOTCH4 and NCOA3 and R726Q mutation in SMO. Importantly, we verified the coexistence of the two alterations in the L1.13 cells by single cell dilution assay (data not shown).

The constitutive activation of BRAF, which is downstream MET in the signal transduction cascade, could explain the failure of anti-MET therapy. Therefore, we treated L1.13 cells with two different drugs targeting BRAF and its downstream effector MEK. To inhibit BRAF, we took advantage of TAK-632, a pan-RAF inhibitor (Okaniwa et al., 2013). We did not consider other well-known inhibitors of the BRAF kinase, such as Vemurafenib (Tsai et al., 2008) and Dabrafenib (Kainthla et al., 2014), since they proved to be ineffective against BRAF G469A mutations (Porcelli et al., 2015).

To inhibit MEK, we used AZD-6244 (Selumetinib), a potent and highly selective MEK 1/2 inhibitor, which has demonstrated significant efficacy in several tumors carrying BRAF mutations (Solit et al., 2006).

Interestingly, the treatment of L1.13 cells with either TAK-632 or AZD-6244 did not affect cell viability (Figure 9b), while it was effective against sensitive HT29 cells used as control (Figure 9c). To test the combinatorial effect of the two drugs, we combined MEK inhibitor AZD-6244 to the specific MET inhibitor JNJ-38877605. While monotherapy did not affect cell viability, the combinatorial therapy induced a higher effect on viability inhibition in L1.13 (Figure 9d).

Although the combinatorial treatment (AZD-6244+JNJ-38877605) induced only a modest decrease in L1.13 viability, the moderate effect prompted us to evaluate CUP1.13 pharmacological response *in vivo*.

a
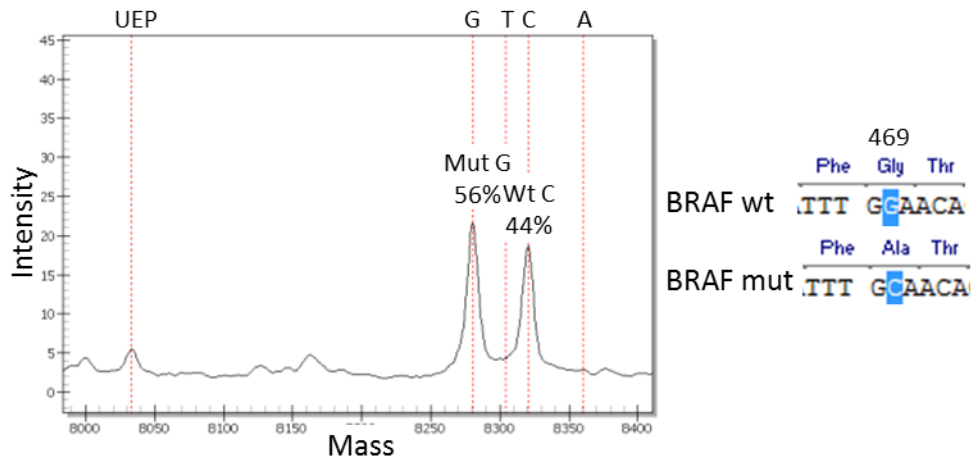
b
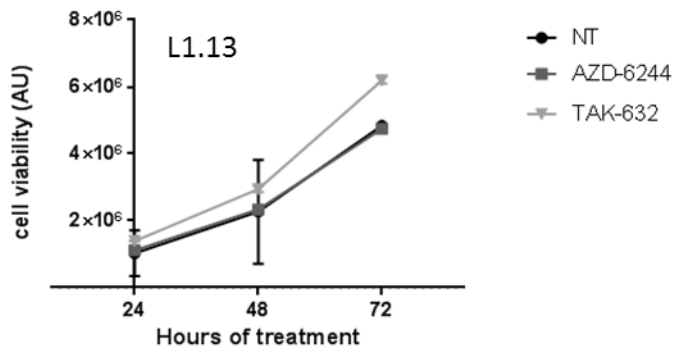
c

d

L1.13
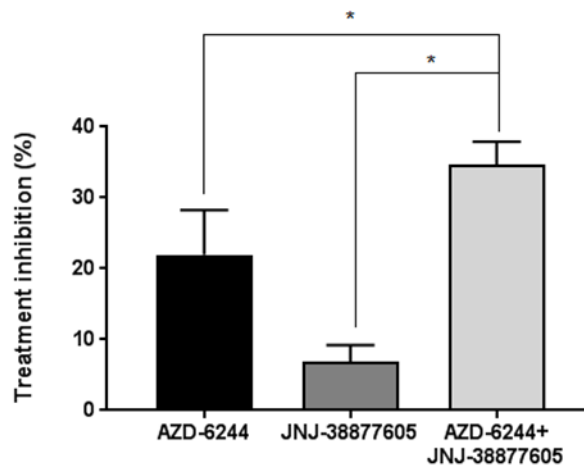
Figure 9: CUP1.13 harbors BRAF G469A mutation and L1.13 cells display sensitivity to combinatorial inhibition of MEK and MET but not to monotherapy.

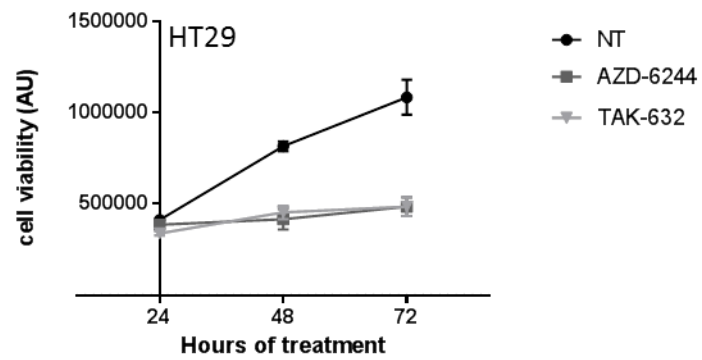a) Genetic analysis of PDX1.13 by OncoCarta (Panel v1.0) revealed a BRAF missense mutation (c.1406G, enlighten on the right panel) falling in exon 11, encoding the kinase domain. Left panel shows MassArray analysis. The expected positions for the UnExtended Primer (UEP), and the extension products (mutant and WT) are indicated. The proportion of peak areas and the specific base are also shown.

b) Cell viability assays performed on L1.13 cells, upon treatment with BRAF inhibitor (TAK-632) and MEK inhibitor (AZD-6244) for the indicated time.

c) Cell viability assays performed on BRAF/MEK inhibitor-sensitive HT29 cancer cells, upon treatment with BRAF inhibitor (TAK-632) and MEK inhibitor (AZD-6244) for the indicated time.

d) Inhibition rate on L1.13 cells, treated with MEK inhibitor (AZD-6244), MET inhibitor (JNJ-38877605) and the combination of the two drugs for 72 hours. Statistical significance is indicated: $*<0.05$; $**<0.01$; $***<0.001$; $****<0,0001$. Two-way ANOVA.

4.4 BRAF pathway inhibition restores sensitivity to MET inhibitor *in vivo*

To assess the effect of BRAF signaling pathway and MET inhibition on CUP1.13 *in vivo*, we carried out a preclinical trial on PDXs. Due to the difficult manageability of TAK-632 for *in vivo* treatment, we used the MEK inhibitor AZD-6244 for these experiments (Okaniwa et al., 2013). Once palpable tumors were established (average volume 250/300 mm^3), PDX1.13-carrying mice were randomized in 4 cohorts and treated with (i) vehicle, (ii) MEK inhibitor AZD-6244, (iii) MET inhibitor JNJ-38877605, and (iv) the combination of MEK and MET inhibitors. Tumor growth was monitored once a week by evaluating volumetric changes. In accordance with *in vitro* data, the treatment of mice with MET inhibitor was ineffective; the treatment with MEK inhibitor was unsuccessful as well, resulting in mild growth inhibition. In contrast, the combination of the two drugs elicited a complete growth arrest (Figure 10a).

These data were confirmed measuring the percentage of cells entering S-phase by EdU incorporation, a thymidine analogue that is incorporated in the DNA of duplicating cells. The percentage of cells entering S-phase was dramatically reduced in mice treated with the combinatorial therapy, while each drug was ineffective when used alone (Figure 10b).

In search for a mechanistic explanation for this synergistic effect, we performed immunohistochemical staining on FFPE PDX tumors explanted from mice not treated or treated with each inhibitor and the combination of both. The analysis showed that MEK inhibition unexpectedly induces Tyr-phosphorylation of the MET receptor, while significantly quenched phosphorylation of the MEK substrate ERK (Figure 10c). Combined treatment with AZD-6244 and JNJ-38877605 abrogated phosphorylation of both MET and ERK. Interestingly, MET was not phosphorylated in basal conditions,

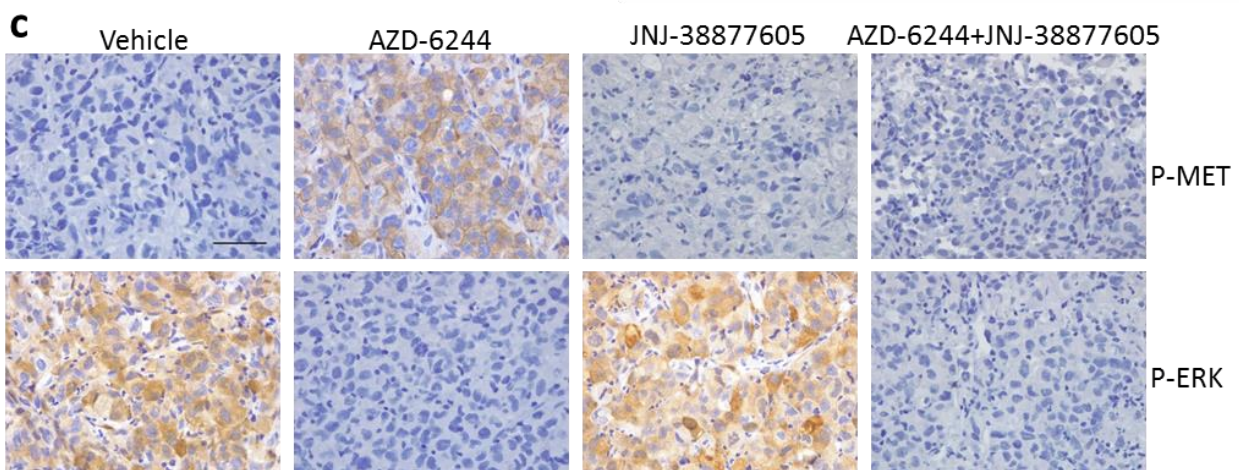which suggests the existence of a previously unrecognized negative feedback loop between oncogenic BRAF and MET.
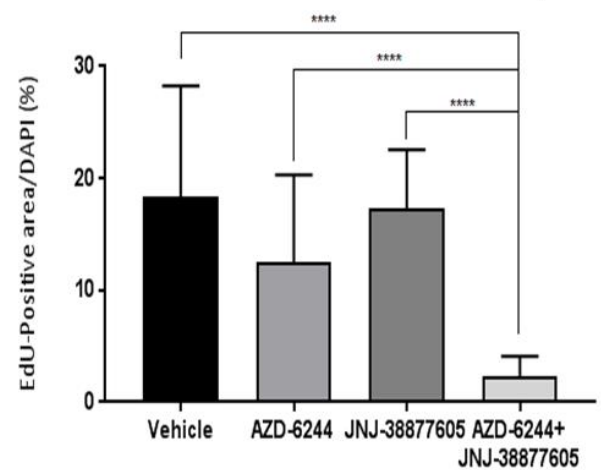
45
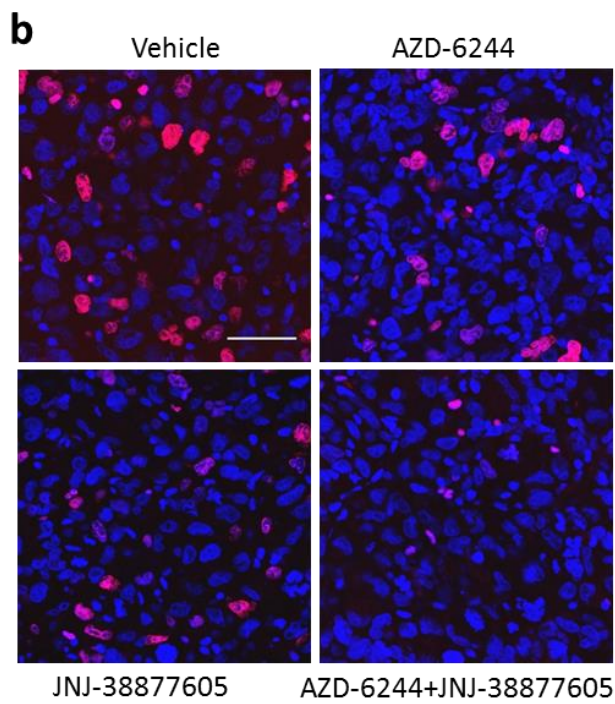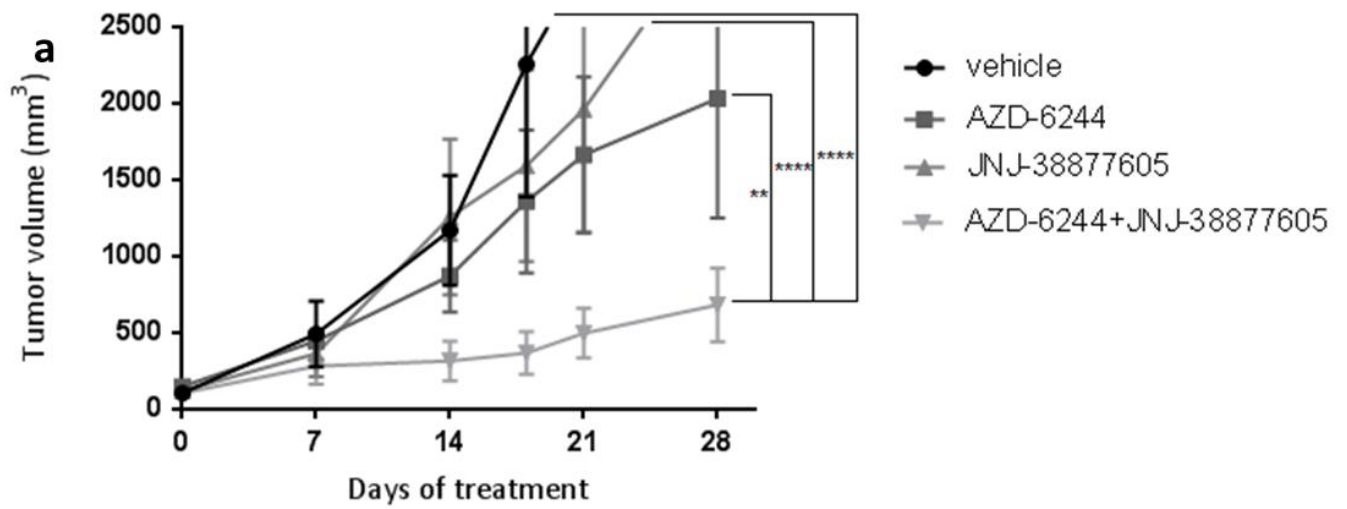
Figure 10: Inhibition of the BRAF pathway restores sensitivity to MET inhibitor *in vivo*.

a) Tumor growth curves of PDX1.13 cohorts treated with placebo (vehicle), MEK inhibitor (AZD-6244) and MET inhibitor (JNJ-38877605) alone or in combination, as indicated. N =6 mice for each treatment arm. Mice were treated daily for 28 days. Tumor size was evaluated weekly by caliper measurements. Each symbol represents the average tumor volume at the indicated time point. Bars represent the standard deviation. Statistical significance is indicated: *<0.05; **<0.01; ***<0.001; ****<0,0001. Two-way ANOVA.

b) Representative merged confocal images of tumors described in a, showing the nuclei in blue and the EdU-positive cells in red, on the left. The percentage of cells that entered the S-phase of the cycle was estimated by injecting EdU 24 hours before harvesting the tumors. 40x of magnification. Scale bar 50 µm. Analysis of the rate of proliferative cells on the right panel. Bar graph shows the percentage of EdU-positive area normalized for DAPI (means ± SEM; 15 fields/tumor were quantified). Statistical significance is indicated: *<0.05; **<0.01; ***<0.001; ****<0,0001. Two-way ANOVA.

c) Representative IHC images of tumors derived from the mice shown in a, untreated (vehicle) or treated with the indicated drugs. Slides have been probed with the listed antibodies. 40x of magnification. Scale bar 50 µm.

4.5 MEK inhibition selectively enhances MET phosphorylation

To further investigate the mechanistic underpinning of the crosstalk between BRAF and MET, we treated L1.13 cells with MEK inhibitor or MET inhibitor alone or in combination. Also in this case MET was not phosphorylated in presence of DMSO, despite the high expression of the protein, and AZD-6244 treatment induced MET phosphorylation. Again the concomitant inhibition of MEK and MET quenched the signal (Figure 11a). This effect was not restricted to JNJ-38877605 but was exerted also by another MET kinase inhibitor PHA-665752 (Figure 11b).

To check the specificity of the feedback loop, we screened 49 tyrosine kinase receptors by antibody-based phosphoproteomics in cells not treated or treated with AZD-6244. In a panel of 49 RTKs (out of 58 existing RTKs -(Blume-Jensen and Hunter, 2001)-) beside a slight effect on EGFR, MEK inhibition unbridles kinase activity of MET specifically (Figure 11c). Of interest none of the other 47 analyzed RTKs was basally phosphorylated and, most importantly, none of them became phosphorylated upon inhibition of MEK pathway.

This indicates that this mechanism of phosphorylation of MET tyrosine kinase receptor is extremely specific in L1.13.
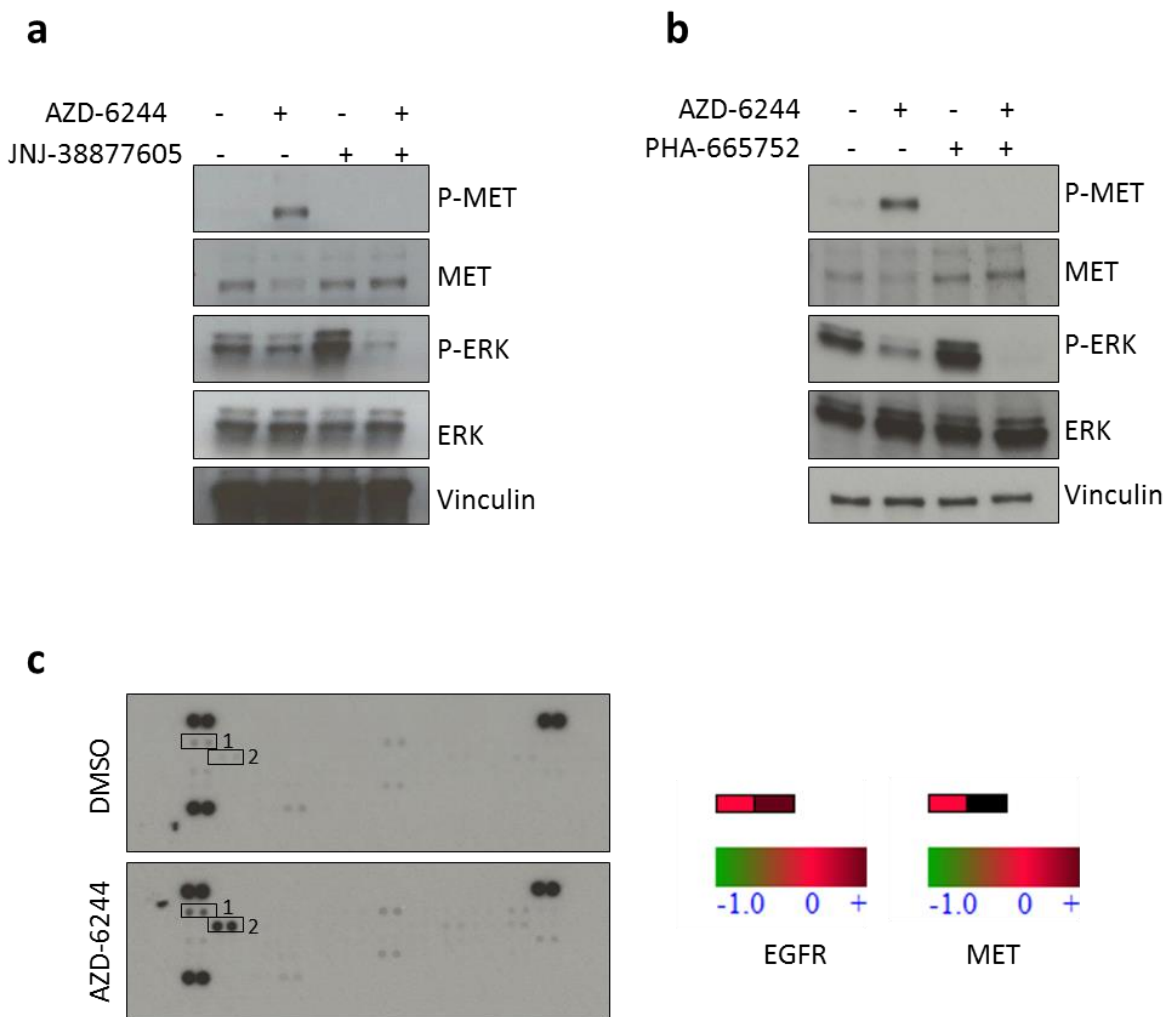
Figure 11: Inhibition of MEK pathway selectively enhances tyrosine phosphorylation of MET

a) Western blot analysis of L1.13 cells, untreated or treated with AZD-6244 (500nM) or JNJ-38877605 (250nM) alone or in combination for 30 minutes. Blots have been probed with the indicated antibodies. Vinculin was used as loading control.

b) Western blot analysis of L1.13 cells, untreated or treated with AZD-6244 (500nM) or PHA-665752 (250nM) alone or in combination for 30 minutes. Blots have been probed with the indicated antibodies. Vinculin was used as loading control.

c) Modulation of phosphorylation levels of 49 RTKs proteins upon treatment with AZD-6244. Whole-cell lysates from L1.13 cells treated with DMSO or AZD-6244 (500nM) for 1h were subjected to "human phospho-Receptor Tyrosine Kinase

Array" and representative array images are presented. Rectangle 1 indicates p-EGFR, rectangle 2 indicates p-MET. The pairs of spots in the three corners of each array are references. On the right, quantification of p-EGFR and p-MET spots.

4.6 The mechanisms underlying BRAF-MET feedback involve post-translational modifications

To get further insights in the mechanism(s) underlying this phenomenon, we considered the relatively fast kinetics of MET phosphorylation and focused on post-translational mechanisms. In fact, L1.13 cells treatment with AZD-6244 caused a peak of MET phosphorylation at 30 min, followed by plateau at 2 h and drop after 6 h of treatment (Figure 12a). Similar, BRAF inhibition led to a peak of MET phosphorylation after 30 minutes and decreased after 6 hours (Figure 12b). Coherently, phosphorylated ERK decreases. MEK inhibition activates MET to the same extent as BRAF inhibition, indicating that MEK acts downstream of BRAF to mediate the feedback regulating MET. In addition, the relatively short kinetics suggests that post-translational modifications are likely responsible for regulating MET kinase activity.
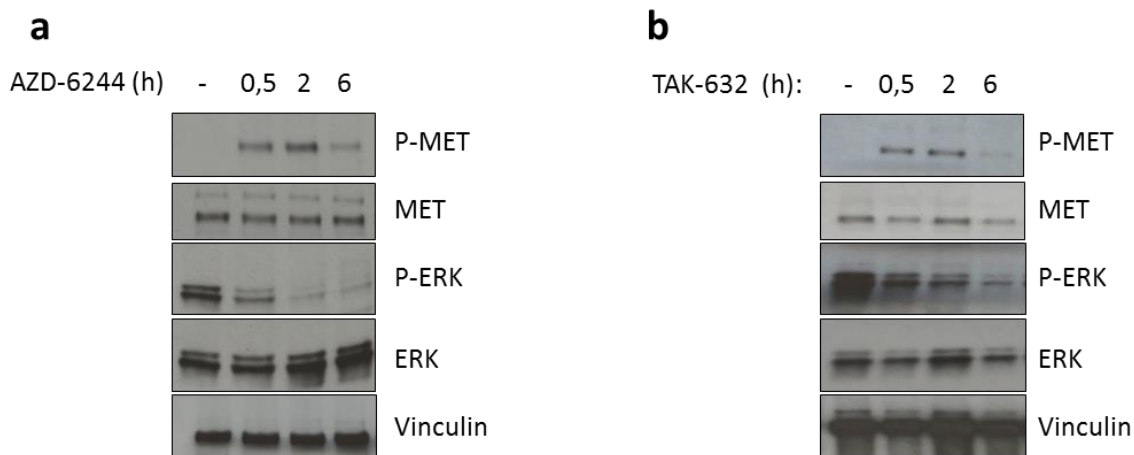
Figure 12: Both BRAF and MEK inhibitions induce MET phosphorylation

a) Western blot analysis of L1.13 cells, untreated or treated with AZD-6244 (500nM) for indicated times. Blots have been probed with the indicated antibodies. Vinculin was used as loading control.

b) Time-course treatment of L1.13 cells with the specific BRAF inhibitor TAK-632 (500nM). Blots have been probed with the indicated antibodies. Vinculin was used as loading control.

4.7 BRAF-MET negative feedback loop is mediated by Protein Phosphatase-2A

Receptor tyrosine kinases are positively or negatively regulated by feedback loops which enforce or attenuate their kinase activity, and such regulatory mechanisms have been reported also in the case of MET (Trusolino et al., 2010). MET kinase negative regulation occurs though direct dephosphorylation of tyrosines of the kinase domain by the protein tyrosine phosphatases PTP1B, TCPTP and LAR (Machide et al., 2006; Sangwan et al., 2008). In addition, the critical residue Ser985 of the juxtamembrane domain of the protein acts on kinase domain activation; MET Ser985 when phosphorylated inhibits MET kinase tyrosine phosphorylation, while its dephosphorylation unleashes MET activity (Gandino et al., 1994). Intriguingly, a recent phosphoproteomics study demonstrated that AZD-6244 reduces the phosphorylation of Ser985 in MET (Kim et al., 2016).

In order to assess the involvement of Ser985 activation in the feedback loop from BRAF to MET, we treated L1.13 cells with a standard Ser-Thr phosphatases inhibitor, Sodium Fluoride -NaF-, alone or in combination with AZD-6244. As expected, AZD-6244 induced MET Tyr-phosphorylation, while the presence of NaF impaired AZD-6244 triggered MET Tyr-phosphorylation, suggesting that Ser985 dephosphorylation is necessary for MET activation upon MEK inhibition (Figure 13a).

In literature, it is reported that MET Ser985 is dephosphorylated through the recruitment of Protein Phosphatase-2A (PP2A) (Hashigasako et al., 2004), prompting us to investigate a possible role of this phosphatase in the feedback loop. To this aim, we silenced PP2A in L1.13 cells. Two siRNAs matching different sequences of the protein (referred to as siRNA58 and siRNA59) were used to minimize potential off-target effects; a non-targeting (SCR) siRNA was used for mock transfectants. Both siRNAs

almost abolished PP2A expression (Figure 13b, right panel). PP2A suppression completely inhibited the feedback loop and fully prevented MET re-phosphorylation by AZD-6244 (Figure 7b, left panel). We thus validated our findings in the cell model and we observed that MEK inhibition, in the case of PP2A suppression, drastically decreased cell viability, compared to the Non-targeting siRNA (Figure 13c). It could be translated in a scenario in which in absence of PP2A, MEK inhibition alone is sufficient to inhibit cell growth. In this context, PP2A silencing, preventing MET kinase activation, supplies MET inhibition by JNJ-38877605.

Even if other mechanisms may be envisaged, taken together these data provide the mechanistic explanation for the negative feedback loop between BRAF and MET, via MEK inactivation of PP2A.
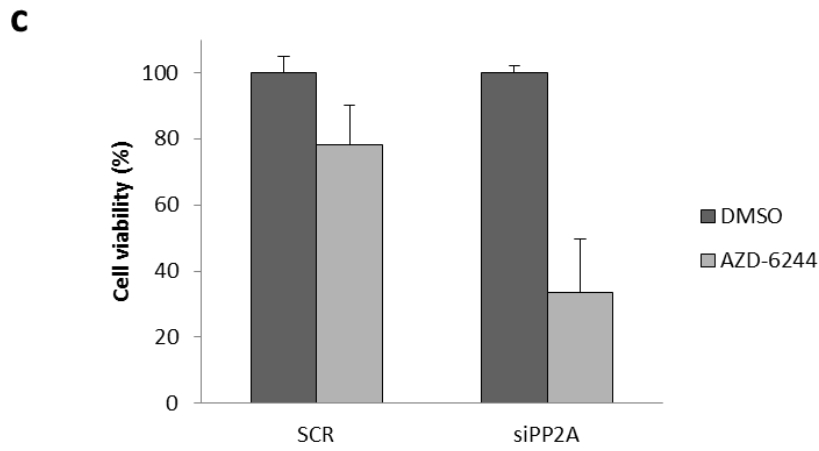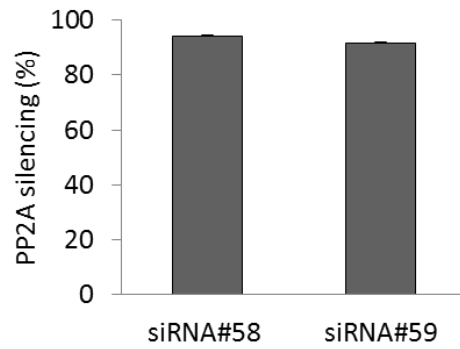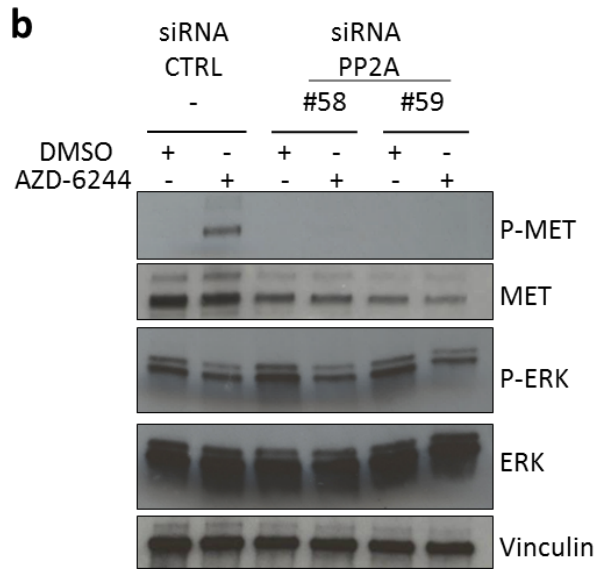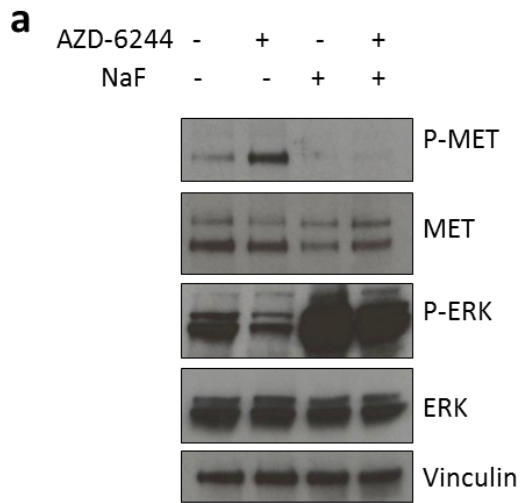
Figure 13: Mechanistic explanation of the feedback loop between MEK cascade and MET

a) Western blot analysis of L1.13 cells treated with phosphoseryl and phosphothreonyl phosphatases inhibitor Sodium Fluoride -NaF- (25mM) alone or in combination with AZD-6244 (500nM), for 30 minutes. Vinculin is used as a loading control

b) Western blot showing that silencing of PP2A Ser/Thr phosphatase, by two siRNA targeting different sites (#58 and #59), prevents AZD-6244 induced MET phosphorylation. Scrambled siRNA was used as control (SCR). DMSO was used as control vehicle. On the right, RT-PCR on mRNA expressing the percentage of PP2A silencing for each siRNA.

c) Cell viability assay performed on L1.13 cells, upon PP2A silencing treated with MEK inhibitor (AZD-6244) for 48 hours, compared to control cells (DMSO). SCR siRNA is used as control.

5 Discussion

Cancer is a complex disease that involves genetic mechanisms such as overactivation of driver oncogenes, as well as non-genetic mechanisms, such as epigenetic factors that promote intrinsic diversity and phenotypic plasticity (Lee et al., 2014). In this study, we focused our attention on the genetic mechanisms that characterize the tumor. In fact, it is widely accepted that cancer is a genetic disease and several tumors depend on overactivation of a single driver oncogene for their sustained survival and growth (Vogelstein et al., 2013). In such tumors, the inactivation of such driver oncogene leads to cancer cells growth arrest and apoptosis. This is defined as 'oncogene addiction' (Sharma and Settleman, 2007). These tumors are responsive to therapy targeting the constitutively hyperactive form of the molecule, which derives from point mutations, gene amplification or chromosomal translocation (Herbst et al., 2004; Sharma et al., 2007). However, in most cells multiple oncogenes coexist. In this scenario, oncogene addiction can be bypassed by the interplay among activated oncogenes. Here, we report a CUP patient (CUP1.13) harboring two concomitant alterations acting on the same pathway, as MET amplification and BRAF activating mutation. In this case, the downstream mutation (BRAF) hides the effect of the upstream receptor (MET) alteration. In fact MET, although amplified, is not phosphorylated at basal conditions and it get phosphorylated only upon inhibition of BRAF signaling cascade. Quenching the downstream mutation restores sensitivity to the drug targeting MET thus reviving oncogene addiction to MET driver gene. Indeed, the mutation affecting BRAF, bypasses MET amplification but it does not engender sensitivity to BRAF (or MEK) inhibitors. We found that this is due to a previously unrecognized negative feedback loop between BRAF and MET, where the BRAF pathway restricts MET activity, likely by impinging on the negative regulatory serine 985 located in MET juxtramembrane domain. A

similar regulatory feedback was observed by Prahallad and colleagues in the EGFR pathway, in which the inhibition of EGFR by the antibody drug or small molecules was strongly synergistic with BRAF V600E inhibition in multiple BRAF V600E mutant colon cancers. Mechanistically, they found that BRAF V600E inhibition causes a rapid feedback activation of EGFR, given by the inhibition of MEK and ERK kinases, which in turn reduced CDC25C phosphatase activation; the increase in p-EGFR supports continued proliferation in the presence of BRAF V600E inhibition (Prahallad et al., 2012). A similar mechanism likely applies to our scenario. Indeed, silencing of Protein Phosphatase-2A (PP2A) in CUP1.13 prevents MET tyrosines phosphorylation triggered by BRAF cascade inhibition. This is in line with the proposed model in which BRAF constitutive activation leads to PP2A inhibition and consequently to serine 985 phosphorylation and MET inhibition (Figure 14a). Accordingly, it is known that PP2A interacts and dephosphorylates MET serine 985 (Hashigasako et al., 2004) and it has been demonstrated that CIP2A (Cancerous Inhibitor of PP2A), able to inhibit PP2A activity, is positively regulated by MEK/ERK signaling pathway activation (De et al., 2014). Therefore, although amplified and highly expressed, MET receptor is inactive. In our model, the blockade of the BRAF signaling cascade unleashes PP2A activity, leading to the dephosphorylation of the inhibitory serine 985 and the consequent reactivation of the MET kinase, reviving the transformed phenotype (Figure 14b). This loop, fixed in cancer cells harboring an activating mutation of BRAF, may operate as well in physiological conditions, providing a mechanism to restrict in time MET signaling. Indeed, during development and homeostasis maintenance, MET plays a critical role in orchestrating cellular organization patterns, including tissue morphogenesis and function, that need tight spatial and temporal controls and aberrations in phosphatases and kinases that fine-tune MET activities result in

compromised cellular processes (Casaletto and McClatchey, 2012; Trusolino et al., 2010). Our data provide the mechanistic explanation for the negative feedback loop between BRAF and MET, via inactivation of PP2A. If on one side this could be reminiscent of physiologically relevant regulation circuits, our findings also imply non-obvious therapeutic implications in oncology. Indeed, at first sight the evidence that MET amplification can cause resistance to BRAF inhibitors is counterintuitive, being BRAF a well-known downstream effector of MET-driven signals and one of the major transducers of MET activity. However, our results indicate that the phosphatase-mediated suppression of MET inhibition that follows BRAF blockade can revive proliferative and survival cues independent of BRAF itself. Therefore, only the synchronous and combined inhibition of both kinases lead to an effective tumor inhibition. These findings could have broader clinical implications extending beyond the specific context of CUPs. In fact, bioinformatics analysis (http://www.cbioportal.org/) showed that, although with low frequency, other tumors feature the coexistence of MET amplification and BRAF oncogenic mutations, enlarging the number of patients that could benefit from combinatorial MET/BRAF inhibition. For example, it has been reported that 18% of chemonaïve patients affected by BRAF V600E mutated colorectal cancers display subclones featuring both MET amplification and BRAF V600E mutation (Pietrantonio et al., 2016). Of course, as mentioned above, the genetics of a tumor is just one of the aspects to take into consideration. To get a broader view, for example, it has been shown that HGF from the stroma mediates innate resistance of BRAF-mutant melanoma to RAF inhibitor. Here, HGF activates MET, reactivating signaling pathways and resistance to RAF inhibition (Straussman et al., 2012). In a reductionist picture, our case reports the same circumstance in a cell-autonomous system.

The current study holds some limitations. We could not observe direct evidences in phosphorylation changes of the serine residue in basal conditions and upon BRAF/MEK inhibition but a phosphoproteomics approach could be used for a deeper investigation. Similarly, we have no direct evidences of PP2A activation status changes in the feedback, but it can be examined through direct observation of phosphorylation/dephosphorylation of its tyrosine 307, in basal conditions and upon MEK inhibition. To further confirm the role of PP2A, additionally to the *in vitro* data, it could be evaluated the response to PP2A inhibition in PDX1.13 alone or in combination with MEK inhibitor AZD-6244. In addition, the feedback loop has been studied in only one case (CUP1.13) and further evaluations, in other tumors, are necessary to sustain the general value of the identified mechanism. Finally, tumor cells are complex adaptive systems ruled by nonlinear dynamics and, genetic or non-genetic, multiple mechanisms that regulate phenotypic plasticity and adaptive evolution, exist. In this setting, therapy approach and response can be influenced by all these mechanisms.

However, even considering all such limitations, our data, together with demonstrated predictive power of PDX-based therapeutic predictions (Hidalgo et al., 2014), provide strong evidence indicating potential clinical efficacy of combined inhibition of MET and BRAF in a subset of patient that lack viable therapeutic options.
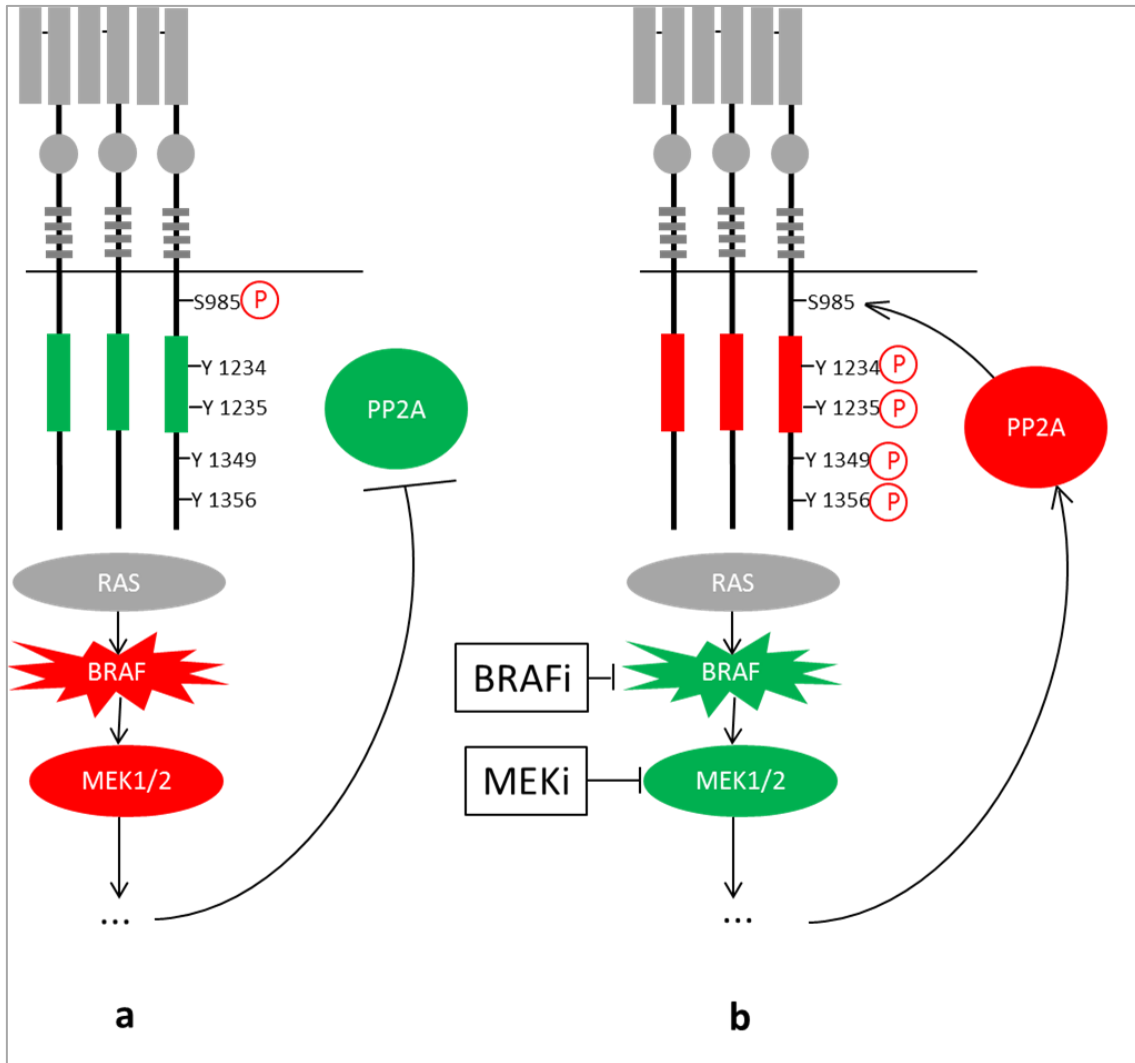
Figure 14: Proposed model elucidating the negative feedback loop between BRAF pathway and MET in CUP1.13.

CUP1.13 is characterized by the concomitant alterations affecting MET gene copy number and BRAF sequence. MET is 8-fold amplified while BRAF is mutated in its P-loop of the kinase domain, rendering it constitutively active. MET inhibition is ineffective due to an unexpected non-constitutive activation; inhibition of BRAF pathway is unsuccessful as well. However, concomitant targeting of the two proteins significantly impairs tumor growth. This phenomenon is explained by a re-phosphorylation of MET upon MEK cascade blockade.

a) BRAF constitutive activation inactivates PP2A, known to dephosphorylate MET Ser985. MET Ser985 is a regulatory residue that, in its phosphorylated status, leads to MET kinase activity shut-down.

b) BRAF and MEK inhibitions induce the activation of PP2A. Dephosphorylation of Ser985 leads to the re-phosphorylation of kinase domain (Tyr1234/1235).

6 Materials and methods

6.1 Patient and tumor sample

Tumor specimen was obtained through protocols approved by the Candiolo Cancer Institute. The patient provided informed consent, sample was procured and the study was conducted under the approval of the Review Boards and Ethical Committees of the Institution.

6.2 Selection criteria for CUP diagnosis

The final decision about CUP diagnosis is based on ad-excludendum evaluation of immunohistochemical stainings. More in general, Agnostos project guidelines consist of an initial evaluation of patients with a suspected metastatic CUP that include a complete clinical history and physical examinations, with attention to and review of past biopsies or malignancies, removed lesions, spontaneously regressing lesions, and existing imaging studies. Routine laboratory studies (CBC, electrolytes, liver function tests, creatinine, calcium) are performed, comprising occult blood stool testing and symptom-directed endoscopy. Serum assessment of α-fetoprotein (AFP), human chorionic gonadotropin (hCG), plasma chromogranin A and PSA is suggested in male patients to exclude potentially curable extragonadal germ cell tumors, neuroendocrine tumors and prostate cancers amenable to hormonal treatment (Greco et al., 1986). Also imaging plays an integral role in the multidisciplinary diagnostic evaluation of patients with CUP sites. PET scans and combination PET/CT scans are 2 of the most frequently used imaging modalities in the management of CUP patients. The final methods to exclude non-CUP patients use immunohistochemistry. Immunohistochemistry stains

complement light microscopy in providing an 'ad excludendum' final diagnosis (Figure 15). Indeed, staining for cytokeratins CK7 and CK20 may provide indications of a possible carcinoma primary site, and staining for chromogranin A and synaptophysin is needed to profile neuroendocrine differentiation. In fact, CK7 is mainly found in tumors of the lung, ovary, endometrium, thyroid, and breast while CK20 is usually expressed in gastrointestinal, urothelial, and Merkel cell carcinomas (Chu and Weiss, 2002).
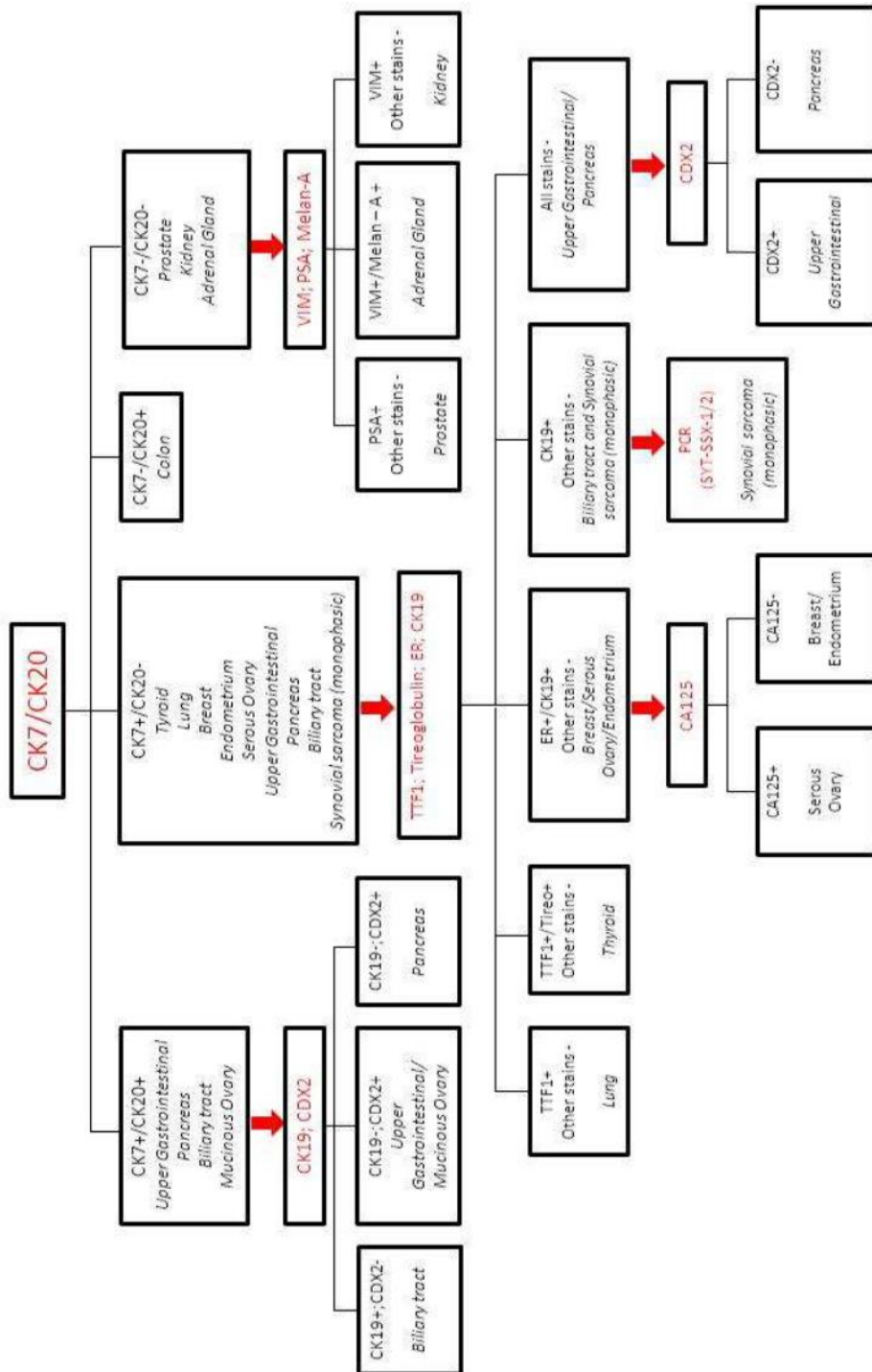
Figure 15: Ad-excludendum immunohistochemistry panel in CUP patients

6.3 Cell cultures and inhibitors

Primary cell line L1.13 was derived from PDX. The tumor was explanted, and immediately digested with Collagenase 1 (Sigma-Aldrich, St Louis, MO, USA) for 1 h at 37°C in a shaking incubator, then centrifuged. The pellet was resuspended in L-15 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich). Cells were incubated for 5 min with DNase, washed in L-15 medium, centrifuged and the pellet plated in a cell culture plate. L1.13 cells were cultured in RPMI medium 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), penicillin/streptomycin solution (Sigma Aldrich) and 2 mM L-glutamine (Sigma Aldrich).

A549, HT29 and A2780, cells were purchased from American Type Culture Collection (ATCC). The cells were cultured according to the instructions from cell banks using the appropriate medium, supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), penicillin/streptomycin solution (Sigma Aldrich) and 2 mM L-glutamine (Sigma Aldrich). GTL16 (gastric cancer) is a laboratory batch obtained from limiting dilutions of MKN45 (Giordano et al., 1988). L1.13 cells genetic identity tumor has been identified by short tandem repeat profiling (Cell ID, Promega, Madison, WI, USA).

AZD-6244 was purchased from Sequoia Research Products (Pangbourne, UK). TAK-632 and PHA-665752 were purchased by Selleck chemicals. The MET inhibitor JNJ-38877605 was kindly provided by Janssen Pharmaceutica NV (Beerse, Belgium).

6.4 RNA silencing

In the RNAi experiments, siRNA oligo duplexes from Ambion were transfected with Lipofectamine RNAiMAX reagent from Invitrogen (according to manufacturer's instructions) and harvested 72h after transfection.

PPP2CA Assay ID: s10958, s10959.

Silencer™ Select Negative Control No. 1 siRNA.

Silencer™ Select GAPDH Positive Control siRNA.

6.5 Nucleic acid extraction

mRNA and gDNA were extracted with Maxwell® RSC miRNA Tissue Kit and Maxwell RSC Blood DNA Maxwell® RSC Whole Blood DNA Kit (Promega) respectively according to the manufacturer's protocol.

6.6 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. Copy Number Assay for MET (assay ID Hs04993403_cn) and for EGFR (assay ID: Hs04942325_cn). RNase P was used as endogenous reference gene (TaqMan™ Copy Number Reference Assay, human, RNase P).

For gene expression analysis DNA was transcribed using iScript RT Super Mix (Bio-Rad) following the manufacturer's instructions. Then expression assay for MET (assay

ID: Hs01565584_m1), PPP2AC (assay ID: Hs00427259_m1) and the TaqMan Copy Number Reference Assay GUSB.

6.7 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. Briefly, the samples were deparaffinized with xylene and subsequently enzymatically digested with a commercial kit (Histology FISH Accessory kit, DAKO, Glostrup, Denmark). The tissue sections and the MET (7q31) / SE 7 dual color probe (Kreatech, Leica Biosystems Nussloch, Germany) were incubated at 75°C for 5 minutes for codenaturation and then placed in a humidified chamber at 37°C overnight for the hybridization step. After post-hybridization washes, carried out according to manufacturer's protocols, the samples were counterstained with DAPI I (Vysis-Abbott Molecular, Downers Grove, IL, USA). The FISH analysis was performed with the fluorescence microscope BX61 (Olympus, Tokyo, Japan) and the automated FISH imaging platform Bioview (Abbott Molecular). An average of 100 non-overlapping interphase nuclei with intact morphology was analyzed using H&E-stained sections as hysto-topographic reference. MET gene was considered amplified when the MET/CEP 7 Ratio was >2 (Jurmeister et al., 2015).

6.8 Mutational analysis

240 ng of DNA undergoes PCR amplification and primer extension with the OncoCarta Panel v1.0 reagents. The extension products are distributed onto a SpectroCHIP® Array and detected via MassARRAY® MALDI-TOF mass spectrometry. The calls and mutation are provided by an automated software report with a confidence score. Next

Generation Sequencing on 241 genes was performed using Illumina MiSeq. A custom pipeline was used for NGS, to call somatic variations when supported by at least 1% allelic frequency and 5% Fisher's test significance level.

6.9 Flow-cytometric analysis

Cells (2×10^5) were incubated with the APC conjugated mouse anti-MET (FAB3582A, clone 95106; R&D Systems Inc.) and were analyzed in a CyAn ADP and analyzed in a CyAn ADP 9 colors (Beckman Coulter, Brea, CA). Data were analyzed using Summit 4.3 software (Beckman Coulter, Brea, CA).

6.10 Western blot analysis and Phospho-Kinase Array

Cells were incubated for indicated times with 500 nM AZD-6244 or TAK-632 or for 30 minutes with 25 mM NaF.

Cells were lysed in EB buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA and 2 mM EGTA) in the presence of 1mM Na_3VO_4, 100mM NaF and a mixture of protease inhibitors and quantified using the BCA Protein Assay kit (Pierce, Rockford, lL, USA). Primary antibodies: anti-MET (sc-10) and anti-Actin (sc-1616) were from Santa Cruz Biotechnology, antibodies against phosphorylated MET (Tyr1234/1235) (Clone D26), phosphorylated ERK (Thr202/Tyr204) (Clone D13.14.4E), total ERK were from Cell Signaling. Antibody against Vinculin (V9131) was from Sigma. Secondary antibodies were from Amersham. Detection was performed with ECL system (Amersham, UK).

The Phospho-Kinase Array Kit (Human Phospho-Receptor Tyrosine Kinase Array Kit, R&D Systems) was used according to the manufacturer's instructions.

6.11 Biological assay

Cell viability was assessed by ATP content using CellTiter-Glo luminescent assay (Promega). Cells cultured in complete medium supplemented with 10% serum were plated in 96-well plates (1000 cells/well) and treated for indicated times. Cell quantity was determined by measuring the absorbance at 485 nm using Victor X Multilabel Plate Readers (Perkin Elmer). Each experimental point was performed in triplicate.

6.12 *In vivo* experiments

Human tumor sample was placed in medium 199 supplemented with 100 μg/ml levofloxacin. The biopsy specimen was coated in Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and implanted in a subcutaneous pocket produced in the right flank of one female NOD (NonObese Diabetic)/SCID (Severe Combined Immunodeficient) mouse, purchased from Charles River Laboratories. Tumors were then passaged and expanded until production of a cohort of the necessary number of mice, some of the pieces were incubated overnight in RNA later (Invitrogen, Carlsbad, CA, USA) and then frozen at − 80 °C for molecular analyses. Once tumors established (average volume 250/300 mm^3), mice were randomized and divided in cohorts (6mice/cohort) and treated daily by gavage with (i) vehicle, (ii) JNJ-38877605 (50 mg/kg) or (i) vehicle, (ii) AZD-6244 (25 mg/kg), (iii) JNJ-38877605 (50 mg/kg) and (iiii) combination of the two drugs. Tumor size was evaluated weekly by caliper measurements and approximate volume of the mass was calculated using the formula

$4/3\pi(D/2)(d/2)2$, where d is the minor tumor axis and D is the major tumor axis. Tumor volumes are plotted as mean ± SEM. All animal procedures were approved by the Ethical Committee of Candiolo Cancer Institute and by the Italian Ministry of Health.

6.13 Immunofluorescence and Immunohistochemistry

At day 28, mice were injected intraperitoneally with 75 μg/mouse of EdU in 200 μL of PBS. Twenty-four hours later, mice were sacrificed and tumors were extracted, formalin-fixed, embedded in paraffin, and sectioned. Tumor slices were deparaffined with Xylene, then washed twice with BSA 3% in PBS for 5 minutes, permeabilized in PBS, 0.1% Triton X-100 (20 minutes at room temperature), and 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.

For immunohistochemistry, tissue sections of 5 μm thickness were obtained from FFPE tissues and stained with hematoxylin and eosin. Immunohistochemistry was carried out on 5 μm paraffin tissue sections, dried in a 37°C oven overnight. Slides were deparaffinized in xylene and rehydrated through graded alcohol to water. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 30 minutes. Microwave antigen retrieval was performed using a microwave oven (850 W for 5 minutes) in 10 mmol/L citrate buffer, pH 6.0. Slides were incubated with individual primary antibodies overnight at 4°C inside a moist chamber. Anti-rabbit secondary antibody (Dako Envision+System-horseradish peroxidase–labeled polymer, Dako) was added and then incubated for 1 h at room temperature. Immunoreactivities were revealed by incubation in DAB chromogen (DakoCytomation Liquid DAB Substrate Chromogen System, Dako) for 10 minutes. Slides were counterstained in Mayer's hematoxylin, dehydrated

in graded alcohol, cleared in xylene, and the coverslip was applied by using DPX. A negative control slide was processed with secondary antibody, without primary antibody incubation. The following antibodies were used: phospho-p44/42 MAPK (ERK1/2) rabbit mAb (Thr202/tyr204, clone D13.14.4E; Cell Signaling Technology); P-MET (Tyr1234/1235) antibody AF2480 from R&D Systems (Minneapolis, MN, USA). Images were captured with a 40× objective, and representative images were been acquired.

6.14 Statistical analysis

Statistical significance was done using the Two way Anova. Error bars represent the SEM. All experiments, except the *in vivo* trials and Phospho-Kinase assay, were repeated at least three times. Figures show one representative experiment, reporting the average of the technical replicates. Statistical significance: *<0.05; **<0.01; ***<0.001; ****<0,0001.

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