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Targeting of the MET receptor tyrosine kinase by small molecule inhibitors leads to MET accumulation by impairing the receptor downregulation

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ABSTRACT

The MET receptor tyrosine kinase is deregulated primarily via overexpression or point mutations in various human cancers and different strategies for MET inhibition are currently evaluated in clinical trials. We observed by Western blot analysis and by Flow cytometry that MET inhibition by different MET small molecule inhibitors surprisingly increases in a dose-dependent manner total MET levels in treated cells. Mechanistically, this inhibition-related MET accumulation was associated with reduced Tyr1003 phosphorylation and MET physical association with the CBL ubiquitin ligase with concomitant decrease in MET ubiquitination. These data may suggest careful consideration for design of anti-MET clinical protocols.

Structured summary of protein interactions:
Cbl physically interacts with **Met** by anti bait coimmunoprecipitation (1, 2)

1. Introduction

Deregulated oncogenic activity of the receptor tyrosine kinase (RTK) MET for hepatocyte growth factor (HGF) is abundantly found in numerous types of human malignancies and is firmly correlated with diverse aspects of cancer pathogenesis and progression. In addition to MET receptor overexpression that is considered the major mode of ligand-independent deregulation of MET function, tyrosine kinase-activating mutations of MET have been described in papillary renal carcinomas as well as other human tumors [1–3]. The biologic significance of these mutations could be in the near future associated with resistance to MET targeted therapy similar to the accumulating experience with BCR-ABL, KIT and EGFR inhibitors.

Under normal conditions and following activation of the MET kinase, tyrosine residues at the receptor C-terminus are phosphorylated, creating docking sites for various MET downstream signaling molecules. Consequently, crucial biologic activities as cell proliferation, motility, invasion as well as receptor downregulation are tightly regulated. In that respect, phosphorylation leads to recruitment of the Grb2 adaptor protein, which recruits the E3 ubiquitin-protein ligase CBL [4–6]. If, in addition, tyrosine at position 1003 in the MET juxtamembrane domain is phosphorylated, a direct recruitment and activation of CBL through the CBL tyrosine kinase binding (TKB) domain is accomplished [5]. Upon binding and activation of CBL, MET is ubiquitinated [5]. This posttranslational modification is required for efficient recognition of MET during trafficking and leads to receptor degradation in the lysosome [7,8].

In cancer cells, there are different ways by which the receptor downregulation may be impaired. One example in this context is the TPR–MET fusion protein generated by carcinogen-induced chromosomal rearrangement that fuses a protein dimerization domain TPR to MET kinase domain, resulting in the deletion of the juxtamembrane Tyr1003 TKB site of MET. The lack of the juxtamembrane region containing Tyr1003 hinders the subsequent recruitment of CBL via its TKB domain and MET ubiquitination, and as a result, the receptor fails to degrade in the lysosome [9]. Aside of TPR–MET, naturally occurring MET variants that lack the CBL binding site have been identified in cancer tissues, showing the

Abbreviations: RTK, receptor tyrosine kinase; TKI, tyrosine kinase inhibitor; HGF, hepatocyte growth factor; wt, wild type; TKB, tyrosine kinase binding

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importance of CBL-mediated negative regulation of MET as a mechanism counteracting tumorigenesis. For example, in both non-small cell lung cancer cell lines and adenocarcinoma lung tumors, alternatively spliced mutants of MET that result in the excision of exon 14 containing the CBL TKB domain site (Tyr1003) have been identified [10–12]. These MET variants show enhanced stability and prolonged signaling and oncogenic capacity [11]. In addition, in the gastric cancer cell line Hs746T the amplified MET possesses a mutation that results in the loss of exon 14 [13], pointing to the fact that the loss of negative regulation by CBL may be selected also in cases of MET amplification.

Furthermore, in an experimental system in which the phosphorylation of Tyr1003 is abrogated by substitution of the CBL binding site Tyr1003 with a phenylalanine residue, CBL binding to MET is hampered, leading to a poorly ubiquitinated and stable MET receptor with a subsequent transforming potential both *in vitro* and *in vivo* [7].

Other observations in this respect were recently published by Joffre et al. who have characterized MET endocytosis of the wild type (wt) receptor and two activating MET mutants M1268T and D1246N, stably expressed in NIH3T3 cells [14]. The total abundance of the mutants was shown to be similar to that of the wt receptor; however, there was substantial basal accumulation of MET mutants in endosomal compartments. Analysis of MET trafficking within this study indicated not only that internalization of the active mutant was enhanced compared with wt protein but also that recycling of the M1268T mutant was more efficient. In these experiments especially the cell-surface abundance of M1268T receptor could be even further increased by expression of a dominant negative Rab11, which blocks the slow recycling pathway [15].

Because MET is considered an important molecular target in cancer therapy, various strategies to inhibit its activity have already proceeded to clinical trials. Among the most promising MET-inhibitory approaches are small molecule tyrosine kinase inhibitors (TKIs). In the present study we used two preclinical MET small molecule inhibitors, SU11274 and PHA665752 as well as two other MET inhibitors, the MET specific EMD1214063 and the non-specific MET TKI PF02341066 (Crizotinib), that have already entered clinical evaluations [16,17].

Here we show that MET inhibition by different small molecule inhibitors causes accumulation of the MET receptor on the cellular membrane most likely due to the impairment of the receptor downregulation. We propose that the phosphorylation inhibition of Tyr1003 by the small molecule TKI and consequent reduced CBL binding and ubiquitination of MET leads probably to a reduced lysosomal degradation of MET.

2. Materials and methods

2.1. Cell lines

The human lung adenocarcinoma cell line H1993 with MET wt overexpression was kindly provided by the lab of Dr. S. Giordano (Torino, Italy) and was maintained in the RPMI medium (GIBCO, Invitrogen Corp.) supplemented with 10% FCS (Sigma) and antibiotics-antimycotic (penicillin 100 U/ml, streptomycin sulfate 100 U/ml, amphotericin B as Fungizone 0.25 μg/ml; GIBCO). The NIH3T3 mouse fibroblast cell lines stably expressing activating MET-mutated variants H1112L, M1268T, V1110I, V1238I, L1213V or Y1248H were all obtained from Dr. L. Schmidt (NCI, Maryland, USA) and were cultured in DMEM medium (GIBCO) supplemented with 10% FCS, antibiotics-antimycotic and 0.5 mg/ml Geneticin/G-418 sulfate (GIBCO).

2.2. Drugs

The MET inhibitor SU11274 was obtained from SUGEN, Inc. (South San Francisco, CA, USA), EMD1214063 (MSC2156119J) from Merck-Serono (Darmstadt, Germany) and PHA665752 and PF02341066 (kind gift from Dr. A. Rothschild (University of Bern, Switzerland)) from Pfizer (La Jolla, CA). All MET inhibitors were dissolved in DMSO and working solutions were prepared freshly in the corresponding media.

2.3. Western blotting, immuno- and co-immunoprecipitations

Following 16 h of treatment with MET inhibitor, cells were lysed in a buffer containing 1% Triton X-100, 0.5% NP-40, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 10 mM Tris–HCl, 1 mM Na_3VO_4, 10 mM NaF, 1 mM $ZnCl_2$, 50 μM Na_2MoO_4, and a protease inhibitors cocktail (Roche; Basel, Switzerland) and cell extracts were cleared by centrifugation. Total protein concentration was determined using the BioRad protein quantification reagent (Bio-Rad Laboratories, Inc.). Fifty to 100 μg of total proteins was resolved by SDS–PAGE on 7% gels, transferred onto PVDF membranes and followed by incubation with indicated antibodies. Detection of secondary antibodies conjugated to horseradish peroxidase was performed using the ECL kit (Amersham Pharmacia Biotech, UK). Where indicated, quantification of ECL signals was performed using Quantity One software (BioRad).

To detect MET–CBL interaction as well as MET ubiquitination status, total protein lysates (0.5 mg) were incubated with 1 μg of corresponding antibody (CBL or MET) and subsequently, μMACS protein G Microbeads (Miltenyi Biotec, Germany) were added. After calibration, columns were loaded with samples and washed with high and low salt buffers. Beads were boiled with sample buffer and the immunoprecipitated complexes were subsequently analyzed by SDS–PAGE and probed for the presence of MET, CBL or ubiquitin by specific antibodies.

For standardization of the Ubiquitin-IP, total MET levels were measured by Western blotting, quantified using QuantityOne software (BioRad) and samples were adjusted to have equal amount of the MET protein.

To quantify the CBL–MET interaction, we measured the density of the bands of the pull-down sample as well as of the total lysate (this value was used for standardization) from 5 independent experiments, calculated the relative values between the untreated and treated samples, and used a paired *t*-test to obtain the *P*-value.

2.4. Antibodies

The rabbit polyclonal anti-phospho-MET (Tyr1234/1235) antibody was purchased from Cell Signaling Technology and the rabbit polyclonal anti-phospho-MET (Tyr1003) from Biosource. The rabbit polyclonal anti-MET and anti-CBL antibodies were purchased from Santa Cruz Biotechnology and rabbit anti-Actin antibody was from Sigma (Fluka, Buchs, Switzerland). The mouse mono- and polyubiquitinylated antibody was purchased from ENZO (Enzo Life Sciences Inc, Farmingdale, USA). The rat affinity purified anti-mouse c-Met antibody and the APC Donkey F(ab′)2 Fragment anti Rat IgG (H + L) Minimal reactivity to mouse IgG antibody was from eBioscience (eBioscience, Inc. San Diego, USA).

2.5. Flow cytometry

Following 16 h of MET inhibitor treatment, cells were washed with PBS and scraped from the culture plates. Unspecific binding was blocked by 30% donkey serum (SIGMA) in PBS and cells were

stained with an unlabelled anti-MET antibody and an APC secondary anti-rat antibody. Relative fluorescence intensities were measured with a BD LSRII (BD, Mountain View, CA). Data were analyzed using FlowJo™ software (Tree Star, Ashland, OR).

3. Results

3.1. MET inhibition by small molecule TKIs increases total cellular MET protein levels

Small molecule TKIs against MET RTK are chemical compounds designed to block MET activity by binding to the ATP-binding pocket and subsequently to restrict various MET-dependent biological features (reviewed in [18–21]). As such, we and others have shown that MET inhibition by small molecule TKIs affects MET tyrosine kinase autophosphorylation (Tyr1234/1235 residues), with consequent decreased cell proliferation, survival, anchorage-independent growth and changes in cellular morphology [12,22–25]. Here we show by Western blotting that the four various MET small molecule inhibitors, SU11274, PHA665752, EMD1214063, and PF02341066, efficiently inhibit MET autophosphorylation at kinase tyrosines 1234/1235 in five out of seven cell lines that have been tested, but, concomitantly, increase in a dose-dependent manner total MET levels (Fig. 1). We observe this phenomenon, surprisingly not previously reported, both in cells overexpressing the wt MET (human non-small cell lung cancer line H1993) as well as in NIH3T3 cells expressing activating MET mutated variants that overall respond to MET inhibition (MET H1112L, M1268T, V1110I, and V1238I mutations). Contrary, NIH3T3 MET L1213V and NIH3T3 MET Y1248H mutants, that have been previously shown to be resistant to MET small molecule inhibitors [22,23,26], do not respond to MET inhibition by decrease in tyrosine autophosphorylation and also do not display the increase in total MET protein upon treatment with any of the currently used MET inhibitors (Fig. 1). These observations suggest that efficient inhibition of the MET receptor by small molecule inhibitors is often correlated with moderate to considerable increases of total MET cellular protein levels.

3.2. MET accumulation upon receptor inhibition occurs on the cell membrane

In order to confirm this observation by an independent method and to determine whether the accumulation of the MET receptor upon treatment with small molecules leads to increased expression of the receptor on the cell surface or it is rather a result of its intracellular accumulation, we performed Flow cytometry experiments. We compared, by using an antibody that recognizes the extracellular portion of the receptor, the presence of the MET protein in MET inhibitor-treated versus untreated cells. The results suggest an accumulation of MET on the cell membrane in the case of the drug-sensitive NIH3T3 MET M1268T mutant cells whereas no change of MET protein levels on the surface of PHA665752-resistant NIH3T3 MET Y1248H mutant cells can be observed (Fig. 2).

3.3. Accumulation of the MET protein upon treatment with small molecules interferes with proper MET receptor downregulation

We further postulated that the MET kinase inhibition-associated upregulation of MET levels could be correlated with a decreased downregulation of the protein. Concerning a potential downregulation mechanism, it is currently well-established that the CBL proto-oncogene serves as a negative regulator of MET

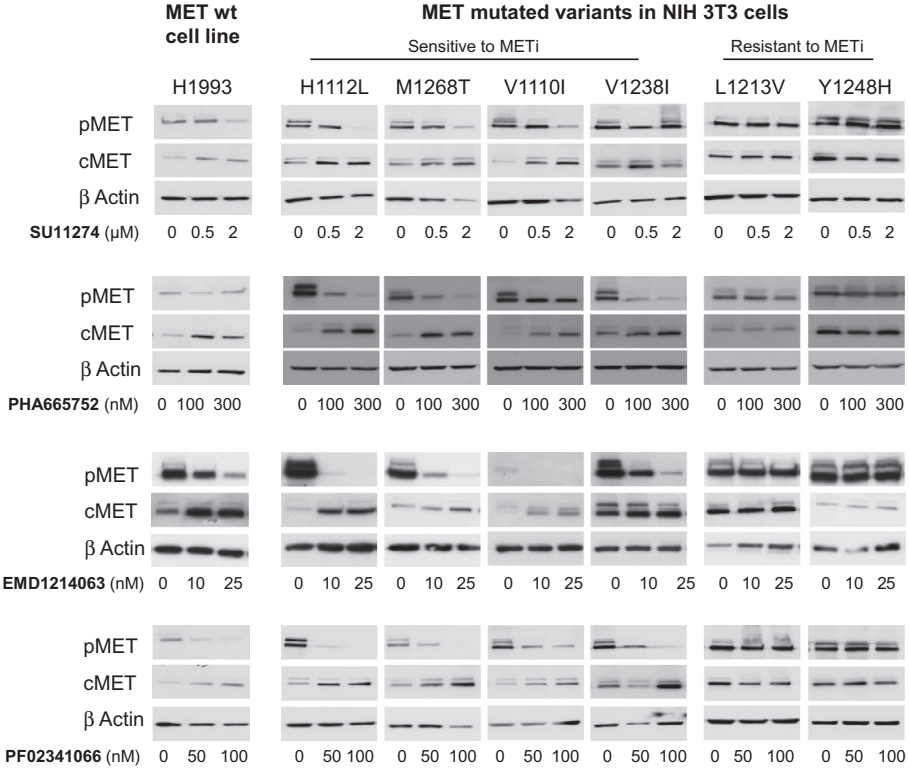

Fig. 1. Receptor inhibition by small molecules reduces the status of phosphorylated MET but leads to significant increase in total MET levels. Human lung cancer cell line H1993 and NIH3T3 cells expressing drug-sensitive or drug-resistant MET mutants were treated with the indicated concentrations of MET inhibitors for 16 h and MET protein levels and activation were analyzed by Western blotting. Experiments were performed in three repetitions, representative results are shown. (pMET – Tyr1234/1235 MET phosphorylation; βActin – loading control).

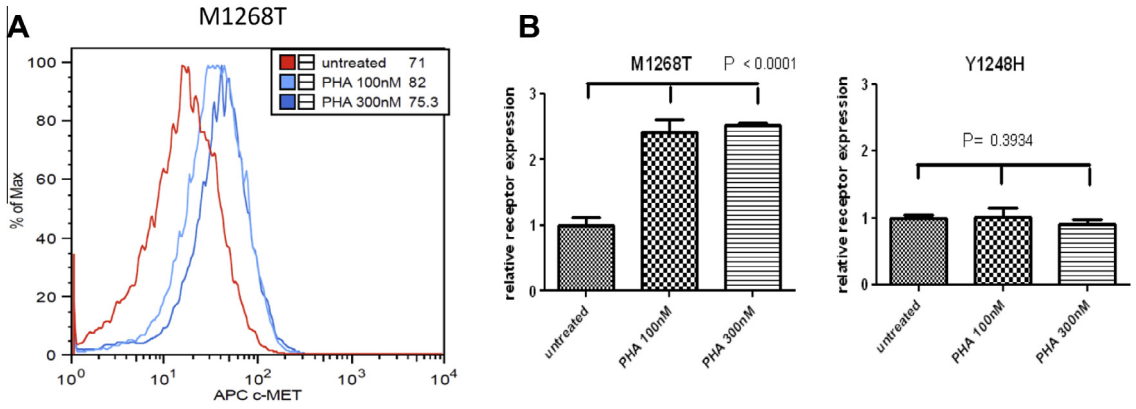

Fig. 2. Treatment by the TKI PHA665752 leads to increased membrane MET levels. Membrane MET levels in NIH3T3 MET M1268T and MET Y1248H cells (untreated cells versus cells treated by 100 or 300 nM of PHA665752 for 16 h) were determined by Flow cytometry. (A) Illustrative picture, membrane MET levels in NIH3T3 MET M1268T cells untreated (red) and treated by 100 or 300 nM PHA665752 (light and dark blue, respectively). (B) Quantification of the data obtained in three independent experiments. (*P*-values (for difference in the mean of treatment group, ANOVA was applied), $P < 0.05$ was considered statistically significant).

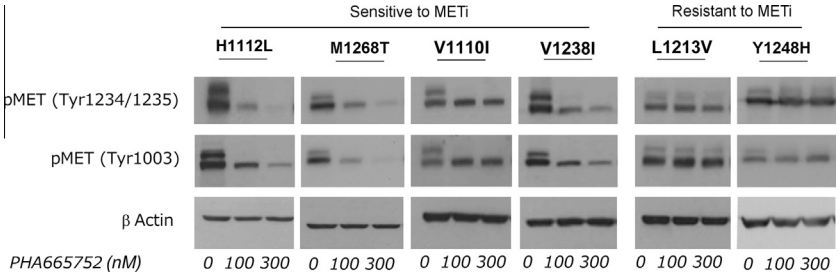

Fig. 3. The status of phospho-MET (Tyr1003) decreases following TKI PHA665752 treatment. Cells were treated with the indicated concentrations of PHA6675752 for 16 h and MET protein levels and activation were analyzed by Western blotting. Experiments were performed in three independent repetitions, data shown are representative results. (p – phosphorylation).

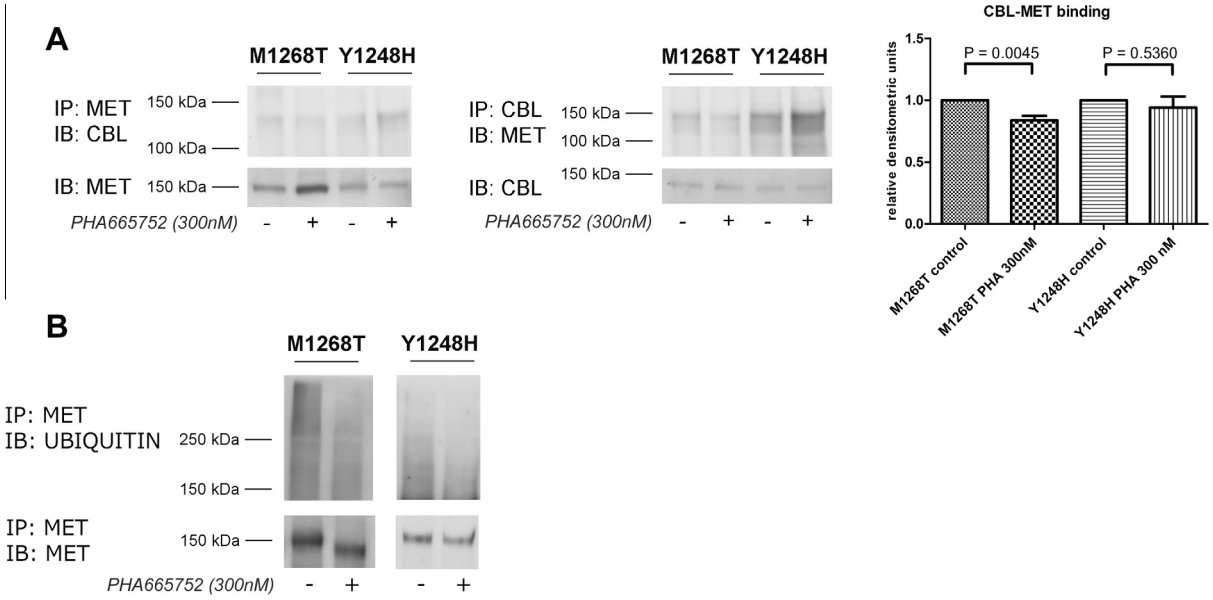

Fig. 4. TKI treatment leads to a decreased interaction of MET and CBL and subsequently reduces MET ubiquitination. (A) Cells were treated with PHA665752 (300 nM for 16 h before lysis) and the interaction between MET and CBL was determined by co-immunoprecipitation. Experiments were performed in five independent repetitions and MET–CBL interaction (Mean ± S.D.) was quantified as detailed in Materials and Methods. Blots shown are representative results. (B) Cells were treated with PHA665752 as in (A) and MET ubiquitination was determined by immunoprecipitation of the lysates with anti-MET antibody and subsequently stained for ubiquitin. Immunoblotting with anti-MET antibody was performed as an internal loading control. Data shown are representative results of at least three independent experiments.

and that the phosphorylation of Tyr1003 located in the juxtamembrane domain of the MET receptor is required for recruitment of the CBL TKB domain [20,27,28]. We report here that the phosphorylation of Tyr1003 is abolished upon anti-MET treatment by PHA665752 or SU11274 in a similar fashion as the phosphorylation of the tyrosines Tyr1234/1235 that constitute the MET autophosphorylation sites (Fig. 3 & Supplementary Fig. 1).

Further in this line, we show by co-immunoprecipitation experiments that PHA665752 treatment impairs the coupling of CBL to MET with a consequent and significant ($P = 0.0045$) decrease in MET–CBL association in NIH3T3 MET M1268T cells where MET activity was pharmacologically abrogated as compared to untreated controls (Fig. 4A). Since the MET–CBL binding is an indispensable step preceding MET ubiquitination, we next aimed to measure the impact of MET inhibition on the level of ubiquitination of the receptor. To that end, the samples were standardized to have equal total-MET amounts and ubiquitination was studied by immunoprecipitation. As shown in Fig. 4B, the observed disruption of CBL–MET association was indeed translated into decreased MET ubiquitination and hence could be the tentative reason for reduced MET receptor degradation through the lysosome, resulting in receptor accumulation in total and on the cell membrane.

As expected, and since the phosphorylation of the CBL-binding site, MET Tyr1003, in NIH3T3 MET Y1248H cells is not affected by PHA665752 or SU11274 (Fig. 3 & Supplementary Fig. 1), there are also no changes observed in the treated versus untreated cells in terms of MET–CBL association (Fig. 4A) and receptor ubiquitination (Fig. 4B & Supplementary Fig. 2).

Taken together, these data suggest that the impairment of MET downregulation by small molecule TKIs is restricted to MET oncogenic forms that respond to the given inhibitor and does not seem to display off-target activity on the non-responsive MET variants.

4. Discussion

Deregulation of the MET RTK is associated with the pathology of numerous human malignancies and partially predicts tumor aggressiveness as well as resistance to treatment strategies [29–31]. Aberrant MET activity that is unleashed by various molecular modes such as receptor overexpression, autocrine/paracrine mechanisms and activating point mutations, is considered as an important clinical target with anti-MET/anti-HGF antibodies and MET small molecules serving as the predominant targeting modalities (reviewed in [20]). MET small molecule inhibitors act by interference with the receptor autophosphorylation on Tyr1234/1235 and subsequently prevent the phosphorylation and activity of MET-downstream signaling adaptors and effectors [20]. Among these downstream signaling proteins, CBL leads to the downregulation of the receptor by ubiquitination and successive degradation in the lysosomes. The activation-dependent downregulation is important in keeping a steady-state of the receptor, balancing between active and non-active MET modes. Here we demonstrate that by inhibition of the receptor activation, also the MET activation-dependent downregulation might be hindered. As a consequence, the receptor accumulates on the cell membrane, where it potentially could be stimulated once the administration of the inhibitor is paused.

Based on our data, we propose that anti-MET small molecules inhibit the phosphorylation on CBL TKB binding site (Tyr1003), disrupting the interaction between MET and CBL and thus restricting ubiquitination of receptors that subsequently cannot be internalized and degradated in the lysosomes. Proper RTKs downregulation is a crucial step in regulating their activity and if not tightly controlled, may display oncogenic potential. This can be convincingly illustrated on the example of the TPR–MET fusion protein as described in a detailed way in the introduction. This oncoprotein is, similarly to cellular systems used in the current study (e.g., MET wt overexpression and MET activating mutations), constitutively active but due to the Tyr1003 deletion fails to bind CBL and to undergo ubiquitination [5]. Here we report that the same impairment of downregulation takes place upon inhibiting the activity of 'regular' (e.g., overexpressed or mutated) oncogenic MET forms by anti-MET small molecules, although in the current case the receptor accumulates initially in an inactive state. Importantly, by envision, in an *in vivo* system where the concentration gradients of drugs are common in tumors, our present finding could possibly translate into appalling consequences once the inhibitor drops below the inhibitory concentration in some cells within a tumor.

In summary, to our knowledge, this is the first report to describe that targeting of the MET RTK by small molecule inhibitors leads to its cell surface accumulation by potential interference with a downregulation-associated mechanism. This phenomenon might represent an important aspect to be considered following MET inhibition in clinical setups. It might also be important to further question as why similar observations have not been so far seen with other drugable RTKs. For example, EGFR downregulation via ubiquitination is also mediated through the CBL ubiquitin ligase, a process in which Y1045 of the EGFR [32] plays a very analogous role to that of MET Y1003. Nevertheless, comparable findings to those reported here following EGFR blocking with erlotinib or gefitinib have not been yet described. The molecular mechanisms underlying such differences may be related to potential variations in CBL-mediated downregulation of a wt receptor form compared with its mutated variants, an option that would have yet to be determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.12.025.

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