

# Somatic Mutations Lead to an Oncogenic Deletion of Met in Lung Cancer

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## Abstract

**Activating mutations in receptor tyrosine kinases play a critical role in oncogenesis. Despite evidence that Met kinase is deregulated in human cancer, the role of activating mutations in cancers other than renal papillary carcinoma has not been well defined. Here we report the identification of somatic intronic mutations of Met kinase that lead to an alternatively spliced transcript in lung cancer, which encodes a deletion of the juxtamembrane domain resulting in the loss of Cbl E3-ligase binding. The mutant receptor exhibits decreased ubiquitination and delayed down-regulation correlating with elevated, distinct Met expression in primary tumors harboring the deleted receptor. As a consequence, phospho-Met and downstream mitogen-activated protein kinase activation is sustained on ligand stimulation. Cells expressing the Met deletion reveal enhanced ligand-mediated proliferation and significant *in vivo* tumor growth. A hepatocyte growth factor competitive Met antagonist inhibits receptor activation and proliferation in tumor cells harboring the Met deletion, suggesting the important role played by ligand-dependent Met activation and the potential for anticancer therapy. These results support a critical role for Met in lung cancer and somatic mutation-driven splicing of an oncogene that leads to a different mechanism for tyrosine kinase activation through altered receptor down-regulation in human cancer.** (Cancer Res 2006; 66(1): 283-9)

## Introduction

Met receptor tyrosine kinase (RTK) is activated by its cognate ligand hepatocyte growth factor (HGF) and receptor phosphorylation activates downstream pathways of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and phospholipase C $\gamma$  (1, 2). The major phosphorylation sites of Met Y1234/Y1235 in the kinase domain and Y1349/Y1356 in the multisubstrate docking site are known to mediate receptor activation (3–5). Receptor phosphorylation facilitates binding of SH2-containing proteins and activates downstream signaling pathways. An additional phosphorylation site in the juxtamem-

brane domain of Met, Y1003, is known to modulate receptor down-regulation. On receptor activation, phosphorylation of Y1003 serves as a direct binding site for the Cbl E3-ligase tyrosine kinase binding domain (6, 7). Cbl binding is reported to drive ubiquitination, endophilin-mediated receptor endocytosis, and subsequent receptor degradation (8, 9). This mechanism of receptor down-regulation has been previously described in epidermal growth factor receptor (EGFR) and HER2 that also harbor a similar Cbl binding site (10–12).

Signaling mediated by HGF/Met promotes biological activities such as proliferation (13, 14), motility (15), invasion (16–18), and angiogenesis (19, 20). Dysregulation of these activities leads to uncontrolled cell proliferation and oncogenesis. In fact, Met was originally identified as TPR-Met, an oncogene that exhibited constitutive kinase activation (21, 22). The most compelling evidence implicating Met in cancer is reported in familial and sporadic renal papillary carcinoma patients where mutations in the kinase domain of Met leading to constitutive activation of the receptor were identified (23). These mutations, when introduced into transgenic mice, lead to tumorigenesis and metastasis (24, 25). Ligand-driven Met activation has also been observed in several cancers. Elevated serum and intratumoral HGF are observed in lung cancer, breast cancer, and multiple myeloma (26–29).

In this study, we sequenced human colon and lung tumor specimens and cell lines to identify and characterize new Met mutations with a particular emphasis on the juxtamembrane region harboring the negative regulatory Cbl binding site. Somatic intronic mutations leading to exon 14 deletion were identified. We show that the Met deletion mutant, while displaying decreased Cbl binding, leads to prolonged protein stability, extended cell signaling on ligand stimulation, and increased tumorigenicity. Treatment with an HGF competitive anti-Met antibody, OA-5D5, inhibits Met activation and HGF-driven proliferation, suggesting that lung cancers harboring a juxtamembrane Met deletion may be ligand dependent and could potentially be targeted by an anti-Met therapeutic.

## Materials and Methods

**Tumor specimen analyses.** Frozen primary tumor tissue specimens were stained with H&E to confirm diagnosis and evaluate tumor content. Specimens exhibiting >50% tumor content were selected for DNA extraction. Met exons were PCR amplified from genomic DNA using a pair of nested primers (Supplementary Table S4). The internal pair of primers used in the amplification contained m13f or m13r primer sequences. After PCR, free nucleotides and excess primer were removed using ExoSAP-IT kit (U.S. Biochemical, Cleveland, OH); PCR products were sequenced in both directions using a m13f or m13r sequencing primer. PCR products were

**Note:** D. Wickramasinghe and R. Yauch contributed equally to this work. Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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cycle sequenced using BigDye Terminator Kit (Applied Biosystems, Foster City, CA). All sequencing products were resolved on a 3730xl sequencing machine (Applied Biosystems). Sequence trace files were analyzed using Sequencher (GeneCodes) and/or SeqScape (Applied Biosystems).

**Quantitative PCR.** Total Met transcript expression levels were assessed by quantitative reverse transcription-PCR (RT-PCR) using standard TaqMan techniques. Met transcript levels were normalized to the housekeeping gene,  $\beta$ -glucuronidase (*GUS*), and results are expressed as normalized expression values ( $=2^{-\Delta Ct}$ ). The primer/probe sets for *GUS* were forward, 5'-TGGTTGGAGAGCTCATTGGA-3'; reverse, 5'-GCACTCTCGTCGGTGA-CTGTT-3'; and probe, 5'-(VIC)-TTTGCCGATTTTCATGACT-(MGBNFQ)-3'. The primer/probe sets for Met were forward, 5'-CATTAAAGGAGACCT-CACCATAGCTAAT-3'; reverse, 5'-CCTGATCGAGAAACCACAACCT-3'; and probe, 5-(FAM)-CATGAAGCGACCCTCTGATGTCCCA-(BHQ-1)-3'. The Met amplicon represents a conserved region between wild-type (WT) and alternatively spliced Met transcripts.

**Immunohistochemistry.** Met DL-21 (Upstate, Lake Placid, NY) antibody was used in immunohistochemical analysis using the avidin-biotin complex method detection kit according to the instructions of the manufacturer (Vector Laboratories, Burlingame, CA).

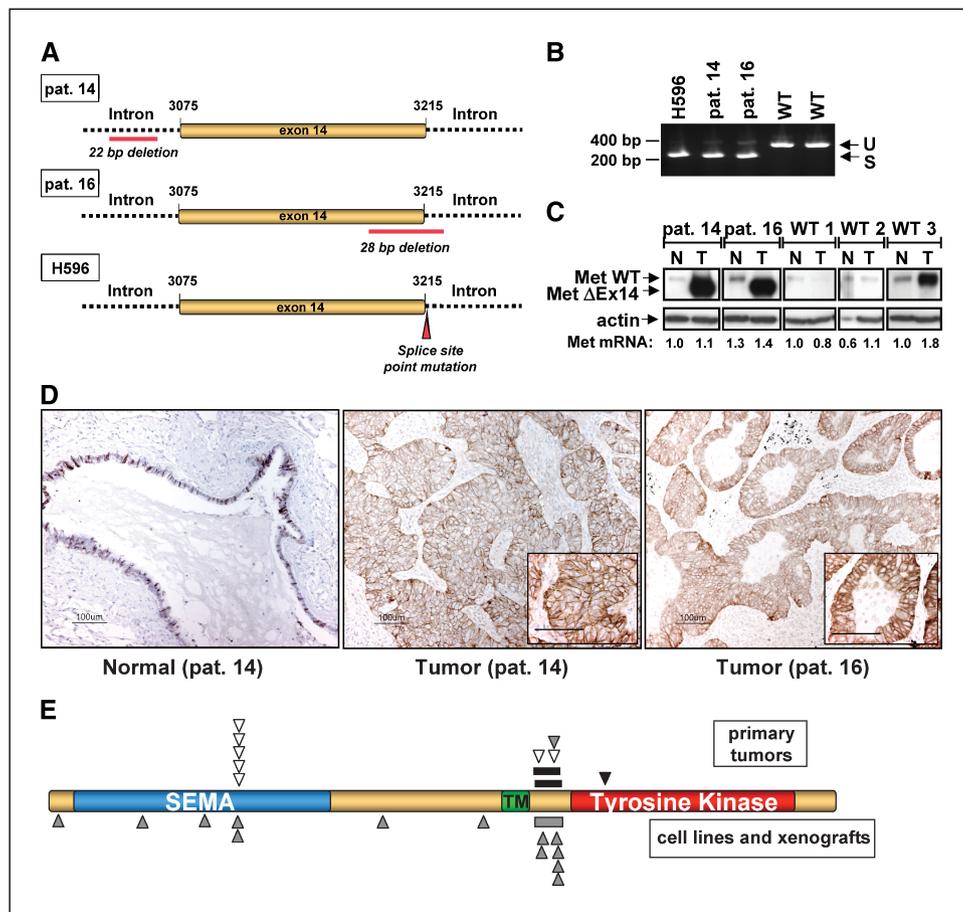
**Cell culture.** Cell lines were obtained from American Type Culture Collection (Manassas, VA), National Cancer Institute Division of Cancer Treatment and Diagnosis Tumor Repository, or Japan Health Sciences Foundation. Most cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO), penicillin/streptomycin (Invitrogen, Carlsbad, CA), and 2 mmol/L L-glutamine; 293 and Rat1a cells were maintained in high-glucose DMEM and supplemented as described.

**Plasmids and stable cell lines.** Full-length Cbl was kindly provided by Karen Ervin (Genentech, South San Francisco, CA) and subcloned into pflag5a vector (Sigma). Full-length Met WT-V5/His (30) served as the

template to produce Met Y1003F-V5/His using primers previously described (6) via QuikChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA). Exon 14 was deleted by creating new *NheI* restriction sites flanking amino acids 963 to 1011 via QuikChange, digesting with *NheI*, and religating. To generate Met Rat1a stable cell lines, 4  $\mu$ g each of pRK5TKneo, Met WT-V5/His, Met Y1003F-V5/His, or Met  $\Delta$ Ex14-V5/His linearized DNA were transfected into cells using Lipofectamine 2000 (Invitrogen). Cells were selected with 500  $\mu$ g/mL G418 (Sigma) for ~2 weeks before fluorescence-activated cell sorting. One cell per well was autocloned and expanded.

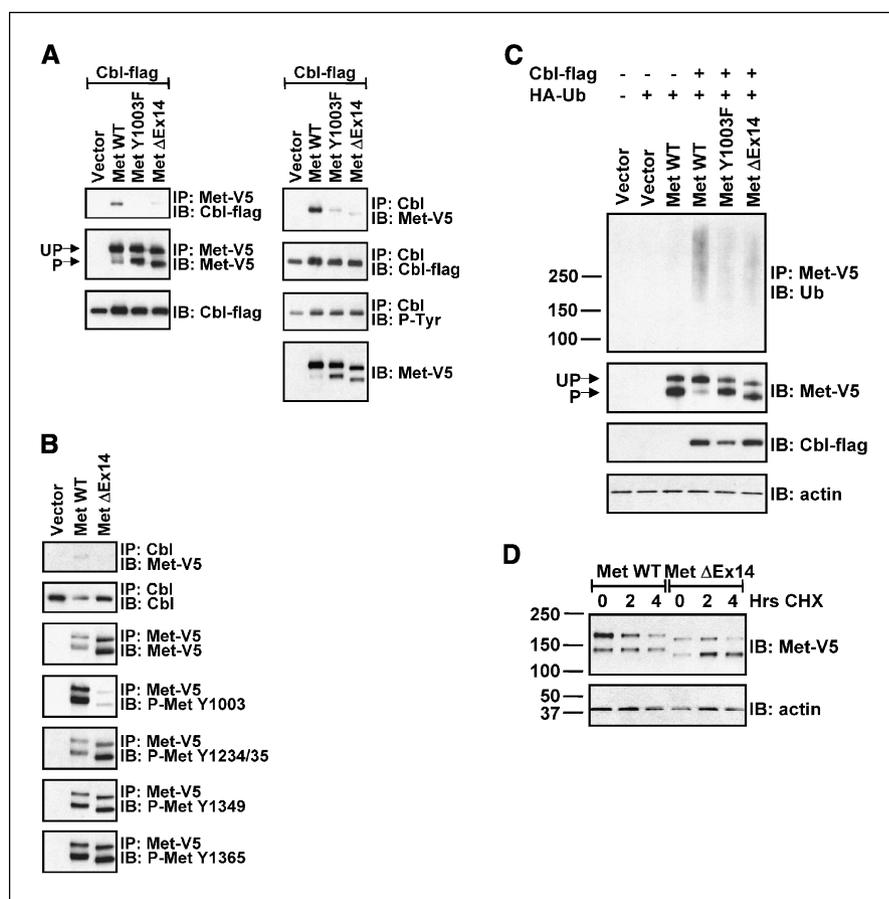
**Immunoprecipitation and immunoblot.** For protein expression analyses, frozen tissue specimens (~100 mg) were homogenized in 200  $\mu$ L of cell lysis buffer (Cell Signaling, Beverly, MA) containing protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktails I and II (Sigma), 50 mmol/L sodium fluoride, and 2 mmol/L sodium orthovanadate using a Polytron homogenizer (Kinematica, Cincinnati, OH). Samples were further lysed by gentle rocking for 1 hour at 4°C before preclearance with a mixture of Protein A Sepharose Fast Flow and Protein G Sepharose 4 Fast Flow (Amersham, Piscataway, NJ). Protein concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA). Proteins (20  $\mu$ g) were subsequently resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with Met (DL-21) or actin (I-19; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies.

In coimmunoprecipitation studies, 3  $\mu$ g of each Met construct and 3  $\mu$ g of Cbl-flag were transfected into 293 cells using FuGENE6 (Roche, Indianapolis, IN). The next day, cells were stimulated with 100 ng/mL rhuHGF for 30 minutes before harvest. Cell debris was centrifuged and 1 mg of lysates was immunoprecipitated with either 1.5  $\mu$ L V5 (Invitrogen) or 2  $\mu$ g Cbl (C-15; Santa Cruz Biotechnology) antibodies at 4°C with rotation overnight followed by incubation with Protein G or A beads for 2 hours. Samples were washed five times and 2 $\times$  sample buffer (Invitrogen)



**Figure 1.** Identification of tumor-specific, intronic mutations in Met leading to exon 14 splicing. **A**, schematic representation of Met exon 14 showing the position of nucleic acid deletions (red bars) or point mutations (arrowhead) with respect to the splice site junctions (based on RefSeq, NM\_000245.2). *H596*, cell line; *pat. 14* and *pat. 16*, patient tumor specimens. **B**, RT-PCR amplification of the RNA transcript encompassing exon 14 from specimens harboring either intronic mutations or Met WT. *U*, unspliced; *S*, spliced. **C**, lung tumors (*T*) and patient-matched, normal (*N*) tissue from patients were analyzed by SDS-PAGE and immunoblotted with Met antibody. **D**, immunohistochemistry staining of Met overexpression in tumor sections from patient 14 (middle) and patient 16 (right) in comparison with normal adjacent tissue from patient 14 (left). *Insets*, magnification,  $\times 40$ ; bar, 100  $\mu$ m. **E**, schematic representation of the Met protein showing amino acid alterations from either primary lung tumor specimens (top) or lung cell lines and xenograft models (bottom). *Bars*, amino acid deletions; *arrowheads*, amino acid substitutions. Genetic alterations were confirmed as somatic mutations (black bars/black arrowheads) or polymorphisms (white arrowheads) based on genomic DNA sequencing of patient-matched, nonneoplastic lung tissue. For specimens lacking matched normal tissue, mutation status could not be determined (gray bars/gray arrowheads).

**Figure 2.** Decreased Cbl binding and delayed down-regulation of the Met exon 14 deletion. **A, left,** 293 lysates cotransfected with Met constructs and Cbl were immunoprecipitated (IP) with V5 antibody and the membrane was immunoblotted (IB) with flag and V5 antibody to detect Cbl and Met expression, respectively. Lysates were also immunoblotted with flag antibody to confirm the presence of Cbl. **Right,** a reciprocal immunoprecipitation/blot of lysates immunoprecipitated with Cbl and immunoblotted with V5 antibody to detect Met. The membrane was immunoblotted with flag and P-Tyr antibody to confirm Cbl expression and phosphorylation, respectively. Lysates were also immunoblotted with V5 to detect Met expression. **UP,** unprocessed Met; **P,** processed Met. **B,** 293 lysates transfected with vector, Met WT, or Met  $\Delta$ Ex14 were immunoprecipitated with Cbl antibody and the membrane was immunoblotted with V5 antibody to detect Met expression followed by Cbl antibody. Additionally, lysates immunoprecipitated with V5 antibody for Met were immunoblotted with P-Met Y1003 antibody. The membrane was stripped and reblotted repeatedly with P-Met Y1234/35, Y1349, and Y1365 antibodies. **C,** 293 cells were transfected with the indicated constructs and immunoprecipitated with V5 antibody for Met. The membrane was immunoblotted with ubiquitin (Ub) antibody followed by V5 antibody. Lysates were also immunoblotted for Cbl-flag and actin. **D,** cells transfected with Met WT or Met  $\Delta$ Ex14 were treated with cycloheximide (CHX) for the indicated times before harvest. Lysates were immunoblotted with V5 and actin antibodies.



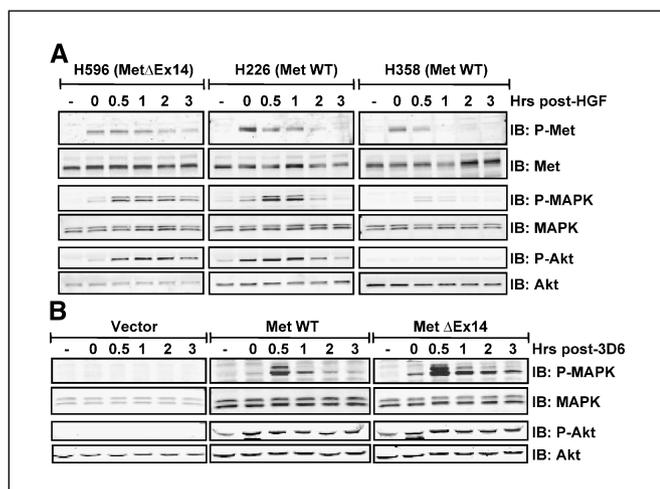
containing 20 mmol/L DTT (Sigma) was added. Boiled samples were loaded into 4% to 12% Tris-glycine gels (Invitrogen) and transferred to 0.45- $\mu$ m nitrocellulose membranes (Invitrogen). The membrane was blocked with 5% nonfat milk for 1 hour followed by immunoblotting. Immunoblots were probed with V5, flag polyclonal (Sigma), Cbl, or P-Tyr (4G10; Upstate) antibodies. Where indicated, blots were stripped with Restore stripping buffer (Pierce, Rockford, IL) and re probed with P-Met Y1003 (Biosource, Camarillo, CA), P-Met Y1234/Y1235 (Cell Signaling), P-Met Y1349 (Cell Signaling), or P-Met 1365 (Biosource) in 5% bovine serum albumin. For binding studies with endogenous Cbl, 293 cells were transfected with 6  $\mu$ g of each DNA construct per 10-cm plate using FuGENE6. Samples (1 mg) were immunoprecipitated with 2  $\mu$ g Cbl or 1.5  $\mu$ g V5 antibodies, followed by immunoblotting. For cycloheximide studies, 293 cells were transfected with 0.25  $\mu$ g of pRK5TKneo, Met WT-V5/His, Met Y1003F-V5/His, or Met  $\Delta$ Ex14-V5/His mutant using FuGENE6 in a six-well plate. Cells were stimulated with 100 ng/mL rhuHGF (Ralph Schwall, Genentech) 30 minutes before harvesting. Lysates (10  $\mu$ g) were analyzed on SDS-PAGE and immunoblotted with V5 or actin antibodies. All proteins were visualized by enhanced chemiluminescence plus (Amersham).

**Ubiquitination assay.** 293 cells were transfected with 3  $\mu$ g Met constructs, 2  $\mu$ g Cbl-flag, 1  $\mu$ g hemagglutinin-ubiquitin, and pRK5TKneo or pflag5a vectors for 6  $\mu$ g total DNA per transfection using FuGENE6. The next day, cells were treated with 25  $\mu$ mol/L MG-132 (Calbiochem, San Diego, CA) for 4 hours followed by 100 ng/mL rhuHGF stimulation 30 minutes before harvest. Cells were lysed in 1% NP40 lysis buffer containing inhibitors, 25  $\mu$ mol/L MG-132, and 10 mmol/L *N*-ethylmaleimide (Sigma). Lysates were immunoprecipitated with V5 antibody and immunoblotted with ubiquitin (P4D1, Santa Cruz Biotechnology) antibody, followed by stripping and re probed with V5 antibody.

**Cell signaling, proliferation, and inhibition studies.** To examine signaling, serum-starved cells were treated with 50 ng/mL rhuHGF or 5  $\mu$ g/mL agonistic anti-Met 3D6 (Genentech) for 5 minutes. Cells were

returned to serum-free media for the indicated times, lysed with SDS sample buffer, sonicated, boiled, and resolved by SDS-PAGE. To analyze inhibition of Met phosphorylation, serum-starved cells were treated with anti-Met OA-5D5 antibody for 30 minutes followed by treatment with 100 ng/mL rhuHGF for 15 or 30 minutes. Samples were processed as above and immunoblotted with P-Met (Y1230/Y1234/Y1235, Biosource), P-Met (Y1234/Y1235), Met (DL-21), P-MAPK (E10, Cell Signaling), MAPK (Cell Signaling), P-Akt (587F11, Cell Signaling), or Akt (Cell Signaling). Immunoblots were imaged and quantified using Odyssey (LICOR, Lincoln, NE). For cell proliferation assays, stable pools of Rat1a cell lines expressing vector, Met WT, Met Y1003F, or Met  $\Delta$ Ex14 were seeded at  $5 \times 10^3$  cells per well in six-well plates in 2% FBS. Each day for 5 days, cells were trypsinized and counted with a Z1 Coulter Counter in replicate samples. For cell viability assays, cells were plated in 0.5% FBS overnight and then treated with 50 ng/mL rhuHGF. After 72 hours, cell viability was measured using CellTiter-Glo (Promega, Madison, WI). Inhibition assays were carried out in a similar manner with either anti-Met OA-5D5 antibody or a control immunoglobulin added at the time of HGF stimulation.

**In vivo xenograft model.** Female athymic nude mice (Charles River Laboratories, Wilmington, MA) were inoculated s.c. with pools of Rat1a stable cell lines expressing Met WT, Met  $\Delta$ Ex14, or control vector (5 million cells per mouse;  $n = 10$ ). Seven days post cell inoculation, mice from these three groups were randomly assigned to two subgroups and stimulated with (a) 10 mg/kg anti-Met 3D6 agonist antibody (which preferentially activates the human Met receptor; ref. 31) or (b) vehicle alone, i.p. once weekly. Tumors were measured twice weekly using a digital caliper and tumor volumes were calculated using the following equation: tumor volume ( $\text{mm}^3$ ) =  $(\pi/6)(A)(B)(B)$ , where  $A$  is the longest width and  $B$  is the shortest width. Representative data from three independent studies are shown. All experimental procedures conformed to the guidelines and principles set by the Institutional Animal Care and Use Committee of Genentech.



**Figure 3.** Prolonged activation of Met and MAPK by Met exon 14 deletion mutant. **A**, H596, H226, and H358 non-small-cell lung cancer cells were stimulated with 50 ng/mL HGF for 5 minutes, washed, and harvested at the indicated times. Lysates were immunoblotted with antibodies to detect the phosphorylated form and total expression of Met, MAPK, or Akt. **B**, Rat1a stable cell lines expressing vector, Met WT, or Met  $\Delta$ Ex14 were stimulated with 5  $\mu$ g/mL 3D6 for 5 minutes, washed, and harvested at the indicated times. Lysates were immunoblotted with antibodies to detect both phosphorylated and total MAPK or Akt.

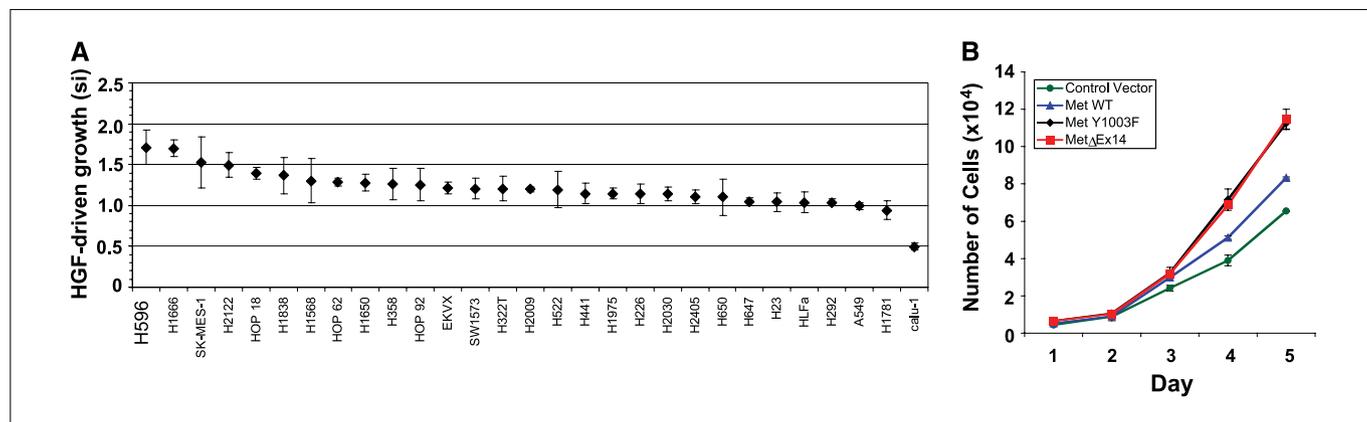
## Results

**Identification of somatic intronic Met mutations in non-small-cell lung cancer.** To assess Met mutations not limited to the kinase domain, we sequenced all coding exons of Met from a panel of lung and colon tumor specimens representing primary tumors, tumor cell lines, and primary tumor xenograft models. Previously unidentified somatic heterozygous mutations in primary lung tumors in the intronic regions flanking exon 14 were observed. The mutations mapped exclusively to the intronic region upstream of the 5' splice site or encompassed the 3' splice site junction and the surrounding intron at the 3' end (Fig. 1A; Supplementary Fig. S1). These deletions were tumor specific and were not identified in nonneoplastic lung tissue from the same individuals (Supplementary Fig. S2). In H596 cells, a non-small-cell lung cancer cell line,

we identified a homozygous point mutation in the 3p splice donor site (Fig. 1A).

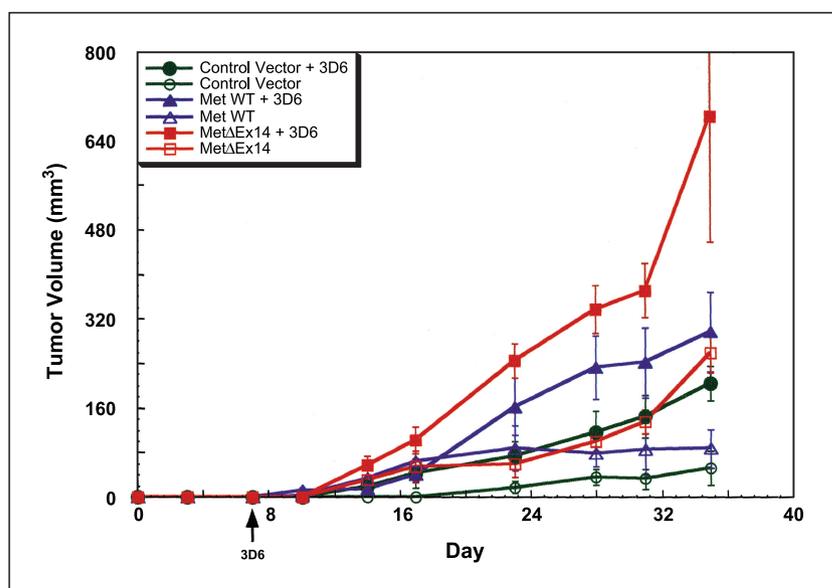
The presence of mutations within the dinucleotide splice site consensus and the upstream polypyrimidine tract of exon 14, combined with the observation that exon 13 and exon 15 remained in-phase, suggested that a potential Met transcript lacking exon 14 could still produce a functional Met protein. To address this, we did RT-PCR amplification of Met RNA from the mutant tumors and cell line. All three intronic mutations resulted in a shorter transcript compared with Met WT (Fig. 1B), consistent with deletion of exon 14. Sequencing confirmed the in-frame deletion of exon 14, removing amino acids L964 through D1010 in the juxtamembrane domain of Met. Interestingly, the deleted form of the receptor is expressed predominantly despite the tumor samples being heterozygous for the exon 14 deletion (Fig. 1B). Western blotting of patient 14 and patient 16 tumors confirmed the increased expression of deleted Met protein compared with tumors with Met WT (Fig. 1C), suggesting preferential expression of the variant transcript in patient tumors. In addition, immunohistochemical analyses reveal strong Met expression and distinct, membrane-associated staining in tumor samples compared with normal tissue (Fig. 1D). Interestingly, tumors harboring these intronic mutations were WT for K-ras, B-raf, EGFR, and HER2 in relevant exons sequenced (data not shown). In addition, the exon 14-deleted splice variant of Met was not observed in normal human lung specimens (data not shown). Taken together, these results potentially support the dominant nature of these Met intronic mutations in lung tumors. Overall genetic alterations in Met were identified in 13% and 18% of primary lung and colon cancer specimens, respectively, with a majority representing polymorphisms mapping to the semaphorin or juxtamembrane domains (Fig. 1E; refer to Supplementary Tables S2 and S3 for details).

**Decreased Cbl binding to exon 14-deleted mutant Met receptor.** We observed that the 47-amino-acid deletion of Met exon 14 (L964-D1010) removes the Y1003 phosphorylation site necessary for Cbl binding and down-regulation of the activated receptor. To confirm loss of Cbl binding to the mutant Met receptor, 293 cells were transfected with Met WT, mutant Met Y1003F (Met Y1003F), or exon 14-deleted Met (Met  $\Delta$ Ex14) and Cbl-flag. The data showed decreased Cbl binding to Met  $\Delta$ Ex14 and



**Figure 4.** Enhanced proliferative potential in cell lines harboring the Met exon 14 deletion. **A**, non-small-cell lung cancer cell lines were treated with 50 ng/mL HGF. After 3 days, cell viability was measured. *Points*, average values of three independent experiments depicted as stimulation index (*si*); *bars*, SD. **B**, Rat1a stable cells expressing vector, Met WT, Met Y1003F, or Met  $\Delta$ Ex14 were counted daily as replicate samples for 5 days. *Points*, mean; *bars*, SD. Representative of at least three independent experiments.

**Figure 5.** Ligand-dependent *in vivo* tumor growth of s.c. inoculated Rat1a stable cell lines expressing control vector, Met WT, or Met  $\Delta$ Ex14 in a xenograft model. Mice were injected with Met agonist 3D6 or vehicle once weekly starting at day 7 post cell inoculation (arrow). Points, mean tumor measurement from each group; bars, SD.



Y1003F in comparison with Met WT (Fig. 2A). Cbl tyrosine phosphorylations by Met WT and Met mutants were equivalent, suggesting that the Met mutations did not alter overall Cbl phosphorylation. Our data also indicate that endogenous Cbl coimmunoprecipitates with Met WT, but not with Met  $\Delta$ Ex14 (Fig. 2B), which is consistent with the observed data in coexpression studies of Met and Cbl. In addition, we examined tyrosine phosphorylation sites necessary for Met receptor activation. Our data indicate that phosphorylation of Y1234/Y1235 in the kinase domain, of Y1349 within the multisubstrate docking site, and of Y1365 is maintained in both Met WT and Met  $\Delta$ Ex14 (Fig. 2B). As expected, a loss of Y1003 phosphorylation in Met  $\Delta$ Ex14 was observed in contrast to Met WT (Fig. 2B). Residual P-Met Y1003 is detected in Met  $\Delta$ Ex14 (Fig. 2B, middle), suggesting that the antibody is not entirely phospho-Y1003 specific.

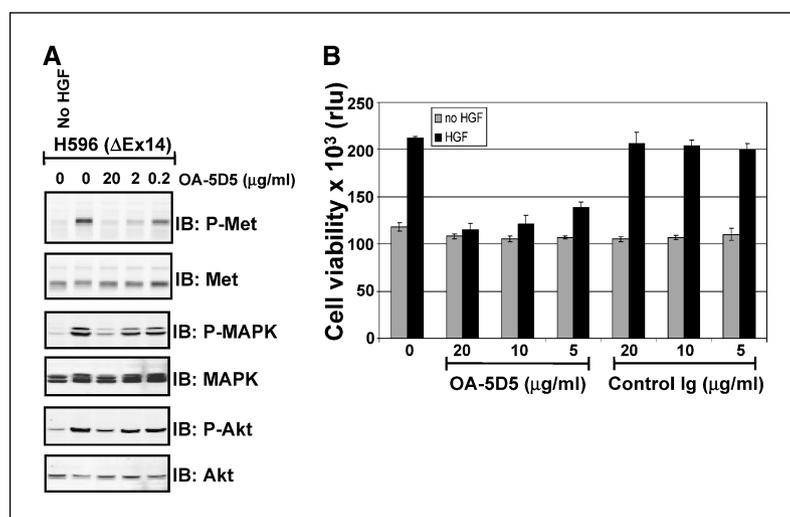
**Attenuated ubiquitination and degradation of exon 14-deleted Met receptor.** Because Cbl E3-ligase activity is reported to facilitate ubiquitin-mediated degradation of RTKs, ubiquitination assays were carried out on cells transfected with Met WT, Met Y1003F, or Met  $\Delta$ Ex14. Both Met  $\Delta$ Ex14 and Met Y1003F show decreased ubiquitination compared with Met WT in the presence of Cbl (Fig. 2C). Interestingly, less processed Met WT was detected with Cbl coexpression compared with the mutants or expression of Met WT alone (Fig. 2C) whereas unprocessed Met remained equivalent. These observations suggest that Met WT binds Cbl preferentially, leading to receptor ubiquitination and degradation, in contrast to Met  $\Delta$ Ex14.

To determine whether or not decreased ubiquitination of Met  $\Delta$ Ex14 alters receptor down-regulation, cells were transfected with Met constructs and treated with cycloheximide to block new protein synthesis. Following HGF stimulation, Met  $\Delta$ Ex14 showed delayed receptor down-regulation and accumulation of Met over time compared with Met WT (Fig. 2D). The Met Y1003F mutant showed similar results (data not shown). These results are consistent with the observed elevated expression of deleted mutant Met protein relative to Met WT (Fig. 1C) despite expressing similar levels of Met mRNA in primary tumors. Furthermore, immunohistochemistry analysis of Met expression in these exon 14-deleted patient tumors reveals strong membranous expression in all

neoplastic cells; in contrast, sporadic Met expression is observed in tumors with Met WT and in normal adjacent tissues (Fig. 1D; Supplementary Fig. S3).

**Exon 14-deleted Met receptor exhibits prolonged, ligand-dependent cell signaling *in vitro* and *in vivo*.** To determine if decreased down-regulation of Met  $\Delta$ Ex14 affected downstream cell signaling on HGF stimulation, Met, MAPK, and Akt phosphorylation levels were examined in non-small-cell lung cancer tumor cell lines harboring the Met exon 14 deletion (H596) or Met WT (H226 and H358). H596 cells show that both phospho-Met and phospho-MAPK levels are maintained up to 3 hours post-HGF stimulation whereas H226 and H358 cell lines exhibit a steady loss of phosphorylation over time (Fig. 3A). Akt, although activated, did not seem to be preferentially sustained in response to HGF over time. Because these tumor cell lines are derived from different genetic backgrounds, we generated stable cell lines in Rat1a cells with empty vector, Met WT, and Met  $\Delta$ Ex14 for comparison. Rat1a Met  $\Delta$ Ex14 showed prolonged MAPK phosphorylation but no discernible difference in Akt activation, compared with Met WT on stimulation with a Met agonist 3D6 which preferentially activates the human receptor (ref. 31; Fig. 3B; Supplementary Fig. S4). Similar prolonged MAPK activation was observed in Met Y1003F Rat1a cells as well (data not shown). These data corroborate our observations of MAPK activation in the non-small-cell lung cancer cell lines harboring WT and exon 14-deleted Met.

The consequences of sustained Met and MAPK signaling were examined in HGF-mediated proliferation of H596 cells which harbor exon 14-deleted Met. When compared with 28 additional non-small-cell lung cancer cell lines, H596 cells consistently exhibited the highest proliferative potential on HGF stimulation (Fig. 4A). Proliferation of Met WT, Met Y1003F, and Met  $\Delta$ Ex14 in Rat1a stable cell lines was also examined. Cells seeded in low serum were counted daily for 5 days. Increased cell proliferation was observed in both Met  $\Delta$ Ex14 and Met Y1003F Rat1a cells compared with Met WT (Fig. 4B). Moreover, to assess *in vivo* tumor growth of these cells, mice were inoculated with Rat1a stable cell lines expressing Met  $\Delta$ Ex14, Met WT, or vector control. Mice inoculated with each cell type were randomly assigned to two



**Figure 6.** Inhibition of ligand-dependent Met signaling and proliferation of H596 cells with anti-Met OA-5D5 antibody. **A**, H596 cells were treated with increasing concentrations of OA-5D5 antibody and stimulated with 100 ng/mL HGF. Lysates were immunoblotted with antibodies to detect the phosphorylated form and total expression of Met, MAPK, or Akt. **B**, cells were treated with OA-5D5 or control immunoglobulin (Ig) and inhibition of proliferation was determined by cell viability assay.

groups and stimulated with vehicle or 3D6 once weekly. Rat1a Met  $\Delta$ Ex14 cells were highly tumorigenic and developed larger tumors compared with those of Rat1a Met WT especially on stimulation with 3D6 (Fig. 5).

**Met activation and cell proliferation driven by the Met deletion mutant is inhibited by OA-5D5.** To determine whether Met antagonists inhibit signaling and proliferation in tumor cells harboring the Met deletion, H596 cells were treated with the HGF-competitive, anti-Met OA-5D5.<sup>6</sup> Met, MAPK, and Akt phosphorylation decreased with the addition of anti-Met OA-5D5 in a dose-dependent manner (Fig. 6A; Supplementary Fig. S5). In addition, H596 cells showed a dose-dependent inhibition of cell proliferation with OA-5D5 treatment in a ligand-dependent manner (Fig. 6B). These results suggest the possibility of targeting cancers expressing the juxtamembrane deleted receptor with a Met antagonist.

## Discussion

Our data support a role for somatic mutation-driven splicing in tumors to activate an oncogenic gene product. The involvement of somatic mutations in *cis*-acting regulatory splicing elements that drive splicing defects is rare; inactivation of the neurofibromatosis type 1 tumor suppressor protein provides the only known example of a splicing defect driven by somatic mutagenesis in cancer (32). The identification of multiple intronic somatic mutations that delete exon 14 highlights the relevance of such a mutagenic event in Met. Deletions within the juxtamembrane domain play an important role in the activation of RTKs by altering receptor conformation and activation of the kinase domain (33). For example, juxtamembrane deletions of KIT (34) and platelet-derived growth factor receptor  $\alpha$  (35) have been identified in gastrointestinal stromal tumors; internal tandem duplications within the juxtamembrane activate FLT3 in acute myeloid leukemia (36). However, this report identifies a different mechanism of RTK activation through somatic mutation-driven deletion that inhibits Met receptor down-regulation.

We show that loss of Cbl binding to the Met deletion mutant affects receptor ubiquitination and down-regulation, leading to sustained Met activation and oncogenesis. Previous reports of Met exon 14 splice variants in embryonic mouse and in human non-small-cell lung cancer were not functionally well characterized (37, 38). Interestingly, the highly oncogenic TPR-Met break point (22) excludes the juxtamembrane of Met and reintroduction of the juxtamembrane inhibited cell transformation (39). Negative regulatory sites within the juxtamembrane domain besides Y1003 have been reported, such as the protein kinase C binding site S985 (40, 41). We examined the S985A Met mutant in ubiquitination and cycloheximide studies and observed a profile similar to Met WT (data not shown). Although we cannot formerly exclude other negative regulatory sites, collectively our data strongly suggest that loss of negative regulation in the Met exon 14 deletion is mainly exerted through Cbl binding at Y1003.

A corresponding Cbl interacting site to phospho-Y1003 in Met is observed in the EGFR juxtamembrane region; phospho-Y1045 directly binds Cbl and leads to EGFR degradation (7, 42, 43). Interestingly, EGFR mutations have been identified in a subset of non-small-cell lung cancer patients that show clinical response to treatment with small molecule EGFR inhibitors such as erlotinib (Tarceva) and gefitinib (Iressa; refs. 44–46). The enhanced sensitivity to EGFR inhibition and the mechanism that drives EGFR activation in tumors harboring these mutations are the subject of much investigation (45, 47, 48). It is tempting to speculate that Cbl binding to mutant EGFR is attenuated leading to decreased receptor down-regulation in an analogous manner to the Met deletion mutant. Moreover, decreased Cbl binding to RTKs could be proposed as a common mechanism for enhanced oncogenic signaling in lung cancer.

Notably, Met juxtamembrane mutations were identified only in lung and not in colon cancers. Our analysis revealed that tumors with Met deletions were exclusive of EGFR as well as ras and raf mutations. A dominant role for activation of the Ras/MAPK pathway in lung cancer has been proposed and supported by several mouse models (49, 50). Collectively, our observations suggest that a subset of non-small-cell lung cancer cases may be driven exclusively by Met mutations that preferentially activate the MAPK pathway and we predict would be highly sensitive to anti-Met therapeutics. In fact, we show that the antagonistic anti-Met

<sup>6</sup> Z. Zhang and R. Schwall, unpublished data.

antibody OA-5D5 successfully inhibits Met activation and proliferation of H596 tumor cells carrying the exon 14-deleted Met receptor. Although Met amplification and mutation have been associated with distinct human cancers (1, 2), mutations that modulate ligand-mediated activation have not been previously described and suggest a novel strategy adapted by RTKs in driving neoplastic disease.

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## Somatic Mutations Lead to an Oncogenic Deletion of Met in Lung Cancer

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