Reduced Proteolytic Shedding of Receptor Tyrosine Kinases Is a Post-Translational Mechanism of Kinase Inhibitor Resistance

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Kinase inhibitor resistance often involves upregulation of poorly understood “bypass” signaling pathways. Here, we show that extracellular proteomic adaptation is one path to bypass signaling and drug resistance. Proteolytic shedding of surface receptors, which can provide negative feedback on signaling activity, is blocked by kinase inhibitor treatment and enhances bypass signaling. In particular, MEK inhibition broadly decreases shedding of multiple receptor tyrosine kinases (RTK), including HER4, MET, and most prominently AXL, an ADAM10 and ADAM17 substrate, thus increasing surface RTK levels and mitogenic signaling. Progression-free survival of patients with melanoma treated with clinical BRAF/MEK inhibitors inversely correlates with RTK shedding reduction following treatment, as measured noninvasively in blood plasma. Disrupting protease inhibition by neutralizing TIMP1 improves MAPK inhibitor efficacy, and combined MAPK/AXL inhibition synergistically reduces tumor growth and metastasis in xenograft models. Altogether, extracellular proteomic rewiring through reduced RTK shedding represents a surprising mechanism for bypass signaling in cancer drug resistance.

SIGNIFICANCE: Genetic, epigenetic, and gene expression alterations often fail to explain adaptive drug resistance in cancer. This work presents a novel post-translational mechanism of such resistance: Kinase inhibitors, particularly targeting MAPK signaling, increase tumor cell surface receptor levels due to widely reduced proteolysis, allowing tumor signaling to circumvent intended drug action. Cancer Discov; 6(4); 1–18. ©2016 AACR.
(ADAM) ADAM10 and ADAM17 are widely known as the principal “sheddases” of the cell surface responsible for shedding ectodomains of hundreds of transmembrane substrates, including many growth factors, cytokines, adhesion molecules, and metalloproteases involved in the processes described above. ADAM10 is required for activation of the Notch signaling pathway, whereas ADAM17 is needed for TNFα cleavage, and both Adam10−/− and Adam17−/− mice are not viable (12). ADAM10 and ADAM17 are particularly overexpressed in many cancers, including breast cancer and melanoma (13, 14), with activities governed by frequently dysregulated MAPK signaling (10, 15). Furthermore, ADAM10 and ADAM17 have been considered promising drug targets for their part in shedding EGF-family growth factor ligands from the surface of cancer cells, a process that mediates ERBB-family receptor mitogenic signaling in an autocrine manner (13, 16, 17). Unfortunately, metalloproteinase inhibitors, including a second-generation inhibitor with specificity toward ADAM10 and ADAM17 (INCB7839; Incyte), have failed in clinical trials despite promising initial results (18). These failures can largely be attributed to a poor understanding of how the broad activity of ADAMs, and metalloproteinasises in general, integrates to influence overall tumor behavior (19).

Here, we identify differential extracellular proteolytic shedding as a major post-translational mechanism of bypass signaling that complements other pathways of drug resistance. Proteolytic shedding of surface receptors, which can provide negative feedback on signaling network activity, is dramatically reduced upon inhibition of kinase pathways such as the MAPK pathway. Decreased RTK proteolysis consequently leads to surface RTK accumulation and increased signaling through other pathways that support mitogenesis. Thus, we hypothesized that RTK proteolysis could (i) be noninvasively monitored in patients; (ii) enable early detection of drug resistance in the clinic; and (iii) guide the design of combination therapies that forestall disease progression. Indeed, we found that circulating RTKs were detectable at elevated levels in a subset of patients and that shed RTK levels accurately predicted clinical MAPKi response better than mere RTK gene expression. We tested two strategies to enhance MAPKi efficacy: (i) modulating ADAM10 through neutralizing its cognate inhibitor tissue inhibitor of metalloproteinases 1 (TIMP1) and (ii) inhibiting the RTK AXL, a key protease substrate that we observed to be upregulated following MAPKi. In several orthotopic animal models, combination therapies exhibited synergistic effects on tumor growth, metastasis, and survival. Altogether, our findings demonstrate that extracellular proteomic rewiring through reduced proteolytic receptor shedding represents a significant and targetable mechanism for bypass signaling in acquired cancer drug resistance.

**RESULTS**

**MAPKi Causes a Global Reduction in Circulating RTKs**

RTK signaling mediates drug resistance (1, 7, 20), and the release of RTK ectodomains from the cell surface has become an increasingly appreciated regulator of signaling activity in contexts of cancer metastasis (21), antibody therapeutics (22), and in other invasive diseases (15). However, little is known regarding how extracellular RTK levels change in response to targeted kinase inhibitor treatment, and how such changes influence drug efficacy in cancer. To study these effects, we first measured how MEKi affects the supernatant accumulation of seven key RTKs that have been implicated in drug resistance: the four ERBB-family receptors (EGFR, HER2, HER3, and HER4); insulin-like growth factor receptor 1 (IGF1R); hepatocyte growth factor receptor (HGFR/MET); and the TAM family member RTK AXL. Using two MEK inhibitors, U0126 and PD325901 [Fig. 1A; Supplementary Fig. S1A validates reduction in phosphorylated ERK (pERK)], we examined a panel of 12 cell lines from several cancer types where bypass signaling has been implicated, including malignant melanoma (MM), TNBC, non–small cell lung cancer, and glioblastoma multiforme (GBM). We surprisingly found that only decreased (rather than increased) supernatant RTK accumulation was consistently and significantly observed across the panel of measurements (Fig. 1A and B). Most prominently, supernatant AXL and MET decreased by roughly 50% in nearly every cell line. We confirmed by ultra-centrifugation that AXL and MET were unassociated with supernatant microvesicles (Supplementary Fig. S1B). Supernatant accumulation of both RTKs could be blocked by broad-spectrum metalloproteinase inhibition (MPI) using batimastat (BB94), suggesting their accumulation resulted from proteolytic release off the cell surface (Supplementary Fig. S1C). EGFR, which is not a suspected metalloproteinase substrate, behaved in stark contrast to MET and AXL: In the supernatant, EGFR (i) did not consistently decrease in response to MEKi (Fig. 1A and B), (ii) was substantially associated with microvesicles (Supplementary Fig. S1B), and (iii) did not decrease in response to MPI (Supplementary Fig. S1C). Thus, we find that MEKi treatment induces significant changes in supernatant RTK levels, which contain a combination of free proteolytically shed soluble receptor (as with AXL and MET), and surface receptor on microvesicles (as with EGFR), with the significant effects of MEKi dominated by the former (Fig. 1A and B).

We next examined if reduced accumulation of extracellular RTK ectodomain was detectable in mice undergoing MAPKi therapy. We used two different xenograft tumor models: subcutaneous BRAF-mutant melanoma using LOX-IMVI cells and orthotopic TNBC using the BRAF/KRAS-mutant LM2 cells. With human-specific solution-phase immunoassays, we measured plasma levels of tumor-derived receptors in tumor-bearing mice following drug treatment. Indeed, MAPKi using combined MEKi (trametinib) and BRAFi ( vemurafenib) treatment in the melanoma model decreased circulating levels of tumor-derived AXL and MET (Fig. 1C). Similarly, MEKi significantly reduced levels of the circulating AXL and MET in mice with mammary fat pad tumors (Fig. 1D).

To test whether this RTK ectodomain accumulation may be relevant to clinical pathology, we measured circulating levels of AXL, MET, HER2, and HER4 ectodomains in patients with stage I and stage IV breast cancer along with healthy controls, and observed that AXL contributes to an overall pattern of increased RTKs in patients (Supplementary Fig. S1D–S1F). The combined level of these four RTKs in a given serum sample increased significantly in patients with breast cancer.
Drug Resistance via Reduced RTK Shedding

**Figure 1.** MEKi broadly reduces extracellular release of protein ectodomains and correlates with drug resistance in patients with melanoma. **A**, MEKi with U0126 and PD325901 reduces supernatant accumulation of soluble RTKs (measured by ELISA) in multiple cancer cell lines \((n \geq 2)\). **B**, change in supernatant RTKs after treatment with MEKi, averaged across all cell lines shown in **A** (pooled two-tailed \(t\) test; \(n = 22\)). **C** and **D**, circulating plasma levels of soluble AXL and MET decrease after \(10\) mg/kg vemurafenib +/− 1 mg/kg trametinib in LOX-IMVI melanoma xenografts \((P = 0.004, n = 4, \text{pooled two-tailed } t\) test), or \(1\) mg/kg PD325901 in orthotopic LM2 breast cancer xenografts \((P = 0.0036, n \geq 4, \text{pooled two-tailed } t\) test). **E**, plasma from patients with melanoma was assayed for soluble RTK levels before and on treatment with dual BRAFi/MEKi therapy; heatmap shows the average of six RTK levels (see Supplementary Fig. S1G for full dataset). As shown by shading, patients were ranked according to plasma RTK levels with MAPKi therapy initiation \((P = 0.005; \text{two-tailed log-rank test; total } n = 11)\). **G**, circulating RTK levels decrease in patients with melanoma with short PFS after MAPKi treatment [median ± interquartile range (IQR) of RTK levels, averaged as in **E**]. **H**, Kaplan-Meier analysis based on the change in RTK levels with MAPKi therapy initiation \((P = 0.005; \text{two-tailed log-rank test; total } n = 11)\).

who have not been exposed to the inhibitors shown here, compared with healthy controls (Supplementary Fig. S1D). In other words, roughly 50% of stage I (10/20) and stage IV (9/19) patient samples exhibited a “quadruple-positive” phenotype with increased levels of all four RTKs in circulation, compared with 5% (1/20) of healthy controls (Supplementary Fig. S1E). Among “quadruple-positive” samples, circulating RTK levels in stage IV patients were 40% higher than levels from stage I patients (Supplementary Fig. S1E; \(P = 0.03, \text{two-tailed } t\) test). The statistical significance of an elevated serum RTK signature was dependent upon inclusion of AXL measurements (Supplementary Fig. S1F), indicating it as the most vital among the four. These data confirm that stage I and especially advanced metastatic stage IV diseases are associated with changes in RTK shedding that are detectable circulating in human patients.

**Circulating RTK Levels but Not Their Mere Tumor Expression Predict MAPKi Resistance in Patients with Melanoma**

We next investigated whether reduced RTK ectodomain levels were observable in patients undergoing MAPKi therapy and if markers of RTK accumulation correlated with clinical outcomes. Plasma, rather than serum which was used in the breast cancer cohort, was collected from patients with melanoma who were undergoing treatment with a combination...
of trametinib (MEKi) and dabrafenib (BRAFi), both before treatment and while on treatment. As a surrogate marker of RTK shedding, we measured soluble levels of six RTKs known to be sheddase substrates, MET, HER2, HER4, and the three TAM receptors (AXL, MERTK, and TYRO3), using solution-phase antibody arrays (Fig. 1E; Supplementary Fig. S1G). With this blood-based test, we found that patients showing high levels of circulating RTKs before MAPKi treatment exhibited rapid disease progression (Fig. 1F). Motivated by the hypothesis that reduced RTK shedding may lead to MAPKi resistance, we next examined (i) if circulating RTK levels changed with MAPKi treatment, and (ii) if changes correlated with disease progression. We found that 5 of 11 patients, principally those with high initial circulating RTK levels, showed decreased circulating RTK levels upon initiation of MAPKi treatment (Fig. 1G), and that disease rapidly progressed in these patients (Fig. 1H). In fact, these circulating RTK changes were highly predictive of progression-free survival (PFS; $P = 0.005; n = 11$; two-tailed log-rank test; Fig. 1H). In contrast, initial tumor response as measured by RECIST failed to reliably predict PFS in a statistically significant manner ($P = 0.08; n = 11$; two-tailed log-rank test), as observed in other cancers (23), thus demonstrating the comparative superiority of circulating RTKs as predictive markers of disease progression.

We next examined whether mere RTK expression in the tumor, rather than levels of circulating RTKs, could similarly predict PFS in patients. In the same cohort used to assess circulating RTKs, total tumor AXL measured by immunohistochemistry of its intracellular C-terminus failed to correlate with PFS (Supplementary Fig. S1H). Furthermore, in an independent dataset of 21 patients with melanoma undergoing BRAFi therapy, RNA expression of the six RTKs measured here did not substantially predict PFS (Supplementary Fig. S1J). Upregulation of RTK RNA expression, measured by comparing matched gene expression before and after BRAFi therapy began, only mildly trended toward worse PFS, but the difference was not significant (Supplementary Fig. S1J; $P = 0.055$, two-tailed log-rank test). These results, combined with the aforementioned TNBC and melanoma xenograft studies, show that circulating RTK levels (i) can be non-invasively, quantitatively, and longitudinally monitored in patients undergoing MAPKi treatment, (ii) provide an early indication of MAPKi efficacy, (iii) are more predictive of MAPKi efficacy than mere expression in the tumor, and (iv) may consequently have utility as a patient selection criterion.

MEKi Increases Total and Phosphorylated AXL on the Cell Surface

We next investigated the relationship between AXL levels in circulation and within the tumors of patients with melanoma. We used samples from patients with similar initial tumor responses by RECIST (Supplementary Fig. S2A), yet very different plasma RTK patterns and times of disease progression (Fig. 2A). To simultaneously measure total tumor AXL and its ectodomain release from the tumor, we compared immunostaining by antibodies targeting either the AXL intracellular C-terminus (corresponding to total tumor AXL) or the N-terminus ectodomain (the latter was also used to detect circulating AXL in plasma). In one patient exhibiting relatively long PFS (patient #9), low plasma AXL levels (Fig. 2A, top row) corresponded to low tumor AXL levels before treatment (Fig. 2B, top row). A sustained increase in AXL and other RTKs was detected in plasma (Fig. 2A, top row) with MAPKi treatment initiation, and indeed C-terminus AXL expression was detected at higher levels in a subset of the tumor cells analyzed on treatment (Fig. 2B; Supplementary S2B). In contrast, another patient showing rapid disease progression (patient #4) exhibited high pretreatment AXL levels both in plasma and in the pretreatment tumor (Fig. 2A and B, bottom row), but tumor AXL appeared to be shed at high levels: although AXL C-terminus was high in this tumor, ectodomain staining was low by comparison. Even though plasma AXL levels declined with MAPKi therapy in this patient (Fig. 2B, bottom row), AXL C-terminus staining remained high in the on-treatment tumor biopsy, and tumor AXL ectodomain increased substantially (Fig. 2C). This evidence suggests that MAPKi-induced decrease in plasma RTK does not reflect decreased AXL expression in the tumor, but rather decreased release of the AXL ectodomain from the tumor.

Circulating tumor-derived extracellular vesicles (EV), including exosomes, can also be detected in patients, and surface proteins on such vesicles often correlate with membrane protein levels on the tumor (Fig. 2D; ref. 24). We examined whether changes in EV RTK levels could also explain the posttreatment decrease in circulating RTKs seen for patient #4. Using the recently developed nPLEX (nanoplasmonic exosome) assay to sensitively detect surface RTK ectodomains on EVs (24), we found that these EVs did not explain the overall circulating RTK decrease (Fig. 2E; Supplementary Fig. S2C). In fact, EV AXL and MET ectodomain levels increased with MAPKi in patient #4, which is consistent with the observed tumor histology (Fig. 2C) and likely reflects intact RTK ectodomain on the tumor cell surfaces. In sum, these data demonstrate the divergence between the levels of circulating, soluble RTKs, and RTK levels on cancer cell membranes following MAPKi in patients: Decreased circulating RTK levels do not simply reflect decreased expression in the tumor.

We next sought to understand how MEKi affects AXL surface levels and signaling activity in cultured melanoma (LOX-IMVI) and TNBC (MDA-MB-231) cells. Consistent with the clinical melanoma findings, we observed that although MEKi reduced total supernatant RTK for both cell lines (Fig. 1A), these changes did not correlate with exosomal RTK levels within that same supernatant (Fig. 2F and G). MEKi did not affect exosome production or size in MDA-MB-231 (Supplementary Fig. S2D), and previously discussed control experiments revealed that the majority of supernatant MET and AXL from MDA-MB-231 and LOX-IMVI cell culture was not exosome-anchored (Supplementary Fig. S1B). We next examined how MEKi influenced protein levels on the cell surface and lysate. Changes in both surface and phosphorylated AXL were detectable by 3 hours after treatment and further increased by 24 hours (Supplementary Fig. S2E). However, MEKi did not similarly increase RNA levels of AXL (Supplementary Fig. S2F and S2G), suggesting that surface AXL changes are not simply due to transcriptional regulation.

For a dose-response analysis of MEKi-induced AXL effects, we treated LOX-IMVI (Fig. 2H and I) and MDA-MB-231 (Fig. 2J and K) cells with increasing concentrations of the MEK inhibitors PD325901 and U0126; measured pERK1/2 as a
Figure 2. Decreased circulating AXL correlates with increased AXL surface levels in the tumor after MEKi. A, longitudinal plasma RTK levels monitored in patients with melanoma showing similar initial response to MAPKi (Supplementary Fig. S2A) but dramatically different PFS. B, immunofluorescence of AXL cytoplasmic domain (C-term) and ectodomain (N-term) from tumor biopsies from patients in A, C, schematic of AXL measurements and quantification of data from B. D, multivesicular bodies (MVB) generate exosomes containing membrane proteins resembling those on the cell surface. E, levels of MET, AXL, and the exosome marker CD63 on EVs isolated from plasma of patient #4 before treatment and after disease progression (nPLEX; *, P = 0.008, n = 8, two-tailed t test). F, levels of full-length AXL on exosomes isolated from LOX-IMVI cells in culture (Western blot; n = 3). G, levels of MET, AXL, and the exosome marker CD63 on exosomes isolated from MDA-MB-231 cells ± MEKi treatment (nPLEX; *, P = 0.01, n ≥ 2, two-tailed t test). H, LOX-IMVI lysate Western blot, probed with Abs for AXL ectodomain (N-term) and cytoplasmic (C-term) epitopes. I, quantification of cell count, pERK1/2, and RTK levels in LOX-IMVI cells (n ≥ 3). Sup., supernatant. J, MDA-MB-231 lysate Western blot, probed with Abs for AXL ectodomain (N-term) and cytoplasmic (C-term) epitopes. K, quantification of cell count, pERK1/2, and RTK levels in MDA-MB-231 (n ≥ 3). L, representative immunofluorescence of LM2 primary TNBC tumors 21 days after PD325901 treatment, showing upregulation of AXL ectodomain near the tumor edge. Scale bar, 100 μm. M, mean (thick line) ± SEM (shaded area) for staining intensity for MET and AXL measured within a 140-μm sliding window from the tumor edge (*, P = 0.013; n ≥ 3 tumors per group).
AXL Mediates MEKi Resistance, and Co-treatment with AXLi Synergistically Reduces Tumor Growth and Metastasis, and Extends Survival in Mice

We next asked how increased RTK surface accumulation following MEKi relates to overall drug response. Across 11 cancer cell lines, we observed a significant correlation between MEKi resistance and surface changes in MET and AXL: cells showing RTK accumulation following MEKi were insensitive to MEKi as measured by proliferation across a dose response (Fig. 3A and B). From these data, we hypothesized that MEKi-induced AXLi upregulation would also correlate with synergistic response to combined MEKi/AXLi treatment. To test this, we quantified MEKi/AXLi synergistic response using a model of Bliss independence across 10 cell lines and correlated the results to changes in surface AXL (Supplementary Fig. S3A-S3C). Consistent with our model, cell lines displaying synergistic responses to combined AXLi/MEKi treatment also showed corresponding upregulation of surface AXL following MEKi, in comparison with cell lines displaying non-synergistic responses (Fig. 3C; Supplementary Fig. S3C). Notably, cell lines displaying synergistic response were also enriched for RAS mutation, which is possibly related to a decreased reliance on signaling through proteolytically shed EGF ligands for MAPK activity, and which is clinically relevant due to the frequent association of RAS mutation in patient tumors (Fig. 3C; Supplementary Fig. S3D). Indeed, RAS-mutant MDA-MB-231 cells, among others, were resistant to treatment with an anti-EGFR antibody that blocks ligand binding (mAb225, nonhumanized cetuximab; Supplementary Fig. S3E), and KRAS mutation is a contraindication for cetuximab therapy in the colorectal cancer setting due to lack of efficacy in those patients (25).

We next tested the synergistic efficacy of dual MEKi/AXLi treatment in two mouse xenograft models, using cancer cell lines that showed increased surface AXLi following MEKi. First, we used orthotopic TNBC xenografts using the highly lung-metastatic derivative of MDA-MB-231, LM2. Combined MEKi/AXLi treatment reduced both tumor growth (Fig. 3D) and metastasis (Fig. 3E) more than either treatment alone, with significant superadditive synergistic effects in tumor growth reduction ($P = 0.015$; two-way ANOVA interaction term; $n \geq 7$). In melanoma LOX-IMVI xenografts, we hypothesized that MEKi + BRAFi (trametinib + vemurafenib) combined with the AXLi inhibitor R428 (AXLi) might extend PFS by compensating for drug resistance arising via AXLi-mediated bypass signaling. Using an in vitro proliferation/cytotoxicity assay, AXLi combined with either MEKi or BRAFi showed synergistic effects in LOX-IMVI (Supplementary Fig. S3F). In the LOX-IMVI xenograft, the addition of AXLi to the BRAFi/MEKi treatment regimen led to an enhanced initial tumor response (Fig. 3F), delayed tumor recurrence after the initial treatment course ended (Fig. 3G), and extended median overall survival by roughly 50% more than MAPKi treatment alone (Fig. 3H). In contrast, AXLi treatment by itself had no significant impact on overall survival, thus demonstrating that the interaction between MAPKi and AXLi is synergistic (Fig. 3H). Overall, these results provide evidence that AXLi mediates bypass signaling in response to BRAFi/MEKi treatment, contributes to drug resistance, and is therapeutically targetable using combination treatment regimens.

MEKi-Induced AXLi and MET Upregulation Is Consistent with Decreased Proteolytic Receptor Shedding

We next investigated the mechanism by which AXLi and MET accumulate on the cell surface following MEKi, while simultaneously decreasing levels in the supernatant and in circulation. We first took a proteomic approach to look for global patterns in how the tumor-derived extracellular proteome changes in response to targeted kinase inhibitor treatment, using antibody microarrays to screen 1,000 proteins for differential supernatant accumulation following MEKi in the MDA-MB-231 TNBC cell line. Gene set enrichment analysis (26) of the ~200 proteins exhibiting significantly altered levels in the supernatant indicated that MEKi reduced transmembrane receptor ectodomain abundance (Fig. 4A) while increasing secreted (not proteolytically shed) cytokine levels (Supplementary Fig. S4A). Among proteins that were depleted with MEKi from the supernatant, the top-ranked “transmembrane receptor activity” gene set (Fig. 4A) comprised various known sheddase substrates, including amyloid precursor protein (APP; ref. 27) and low-density lipoprotein receptor (LDLR; ref. 28). These results combined with the significantly decreased supernatant accumulation of RTKs, including known sheddase substrates MET and HER4 (Fig. 4A and B), collectively implicate reduced proteolytic activity as a potentially key effect of MEKi.

We next compared the supernatant effects of MEKi with broad-spectrum MPi using batimastat (BB94) and found similar patterns of widely decreased sheddase substrates amphiregulin (AREG), heparin-binding epidermal growth factor (HBEGF), tumor necrosis factor factor receptor 1 (TNFR1), and AXLi following inhibitor treatment (Fig. 4B). AREG, HBEGF, and TNFR1 have been largely associated with ADAM17 cleavage (13, 17), but all may also be shed by ADAM10 depending on the context (15, 29–32). Reduced sheddase substrate accumulation was not an exclusive effect of direct MEKi: Among 19 clinical and preclinical inhibitors targeting diverse signaling
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Figure 3. Combination MEKi and AXLi synergistically reduce tumor growth and metastasis in mouse models of melanoma and TNBC. A, cell counts and surface AXL and MET changes (measured by live-cell immunostaining; n = 3) after 24-hour treatment with MEKi in 11 different cell lines. B, correlation between cell count and surface RTK expression after MEKi treatment, ranked by order across cell lines in A (p = Spearman correlation, P value from exact permutation test). C, U0126 and PD325901 increase surface AXL (measured by live-cell immunostaining) more in cell lines showing synergistic inhibition of proliferation from dual AXLi/MEKi (P = 0.01, two-tailed t test). Dots represent data from 10 cell lines and two drug combinations (see Supplementary Fig. S3). Treatments in KRAS- or HRAS-mutant cell lines are shown in red and are significantly enriched among the synergistic responses (P = 0.02, Fisher exact test). D, dual AXLi/MEKi reduces tumor growth more than either treatment individually (1 mg/kg PD325901; 30 mg/kg R428), in LM2 TNBC xenografts (*, two-tailed t test; n ≥ 7). E, dual AXLi/MEKi reduces metastasis after 21 days of treatment, corresponding to D (*, two-tailed t test, n ≥ 7). F–H, AXLi (50 mg/kg R428) cotreatment synergistically increases efficacy of BRAFi/MEKi (1 mg/kg PD0325901 with 10 mg/kg vemurafenib) in LOX-MVI xenografts by enhancing initial tumor shrinkage (F, P = 0.031, two-tailed t test), delaying tumor recurrence (G, P = 0.002, two-tailed t test), and extending survival (H, P = 0.03, two-tailed log-rank test); n ≥ 8 animals per group for all. Note AXLi alone fails to significantly affect tumor growth and animal survival.

pathways and RTKs, roughly 80% (15/19) inhibited substrate accumulation to some degree. Nonetheless, MEKi exhibited some of the strongest effects (Fig. 4B). p38, PI3K, and c-JUN N-terminal kinase (JNK) inhibitors also significantly reduced sheddase-substrate accumulation, consistent with previous reports describing their effects on ADAM activity (15, 33). In contrast with supernatant decreases, surface levels of sheddase substrates AXL and TNFR1 correspondingly increased in response to several other inhibitors, but most significantly with MPI and MEKi (Supplementary Fig. S4B). Kinetics of surface and phosphorylated AXL following MPI (Supplementary Fig. S4C) closely resembled MEKi changes (Supplementary Fig. S2D), with observable increases by 3 hours after treatment and continued accumulation by 24 hours. To further compare the effects of MEKi and MPI, we profiled gene expression changes using RNA microarrays following either of the two treatments. Results show substantial overlap in the transcriptional responses arising from MEKi and MPI, suggesting a shared mechanism of action (Fig. 4C; Supplementary Fig. S4D). However, gene set enrichment analysis indicated that MEKi, but not MPI, induced growth arrest (Supplementary Fig. S4E); in fact, MPI did not elicit any significant gene set enrichment. Overall, these results show that reduced metalloproteinase activity is a surprisingly prominent effect of MEKi. Furthermore, these data implicate MAPK signaling as one of the key pathways broadly regulating supernatant sheddase-substrate accumulation, with effects similar to those seen when proteolytic shedding is directly inhibited.
**Figure 4.** MEKi-induced RTK changes are consistent with decreased proteolytic receptor shedding. **A,** top bar graph, differentially detected supernatant proteins from MDA-MB-231 treated with MEKi (PD325901) for 24 hours (q = FDR-corrected P value; n = 2). **B,** surface level changes of sheddase substrates following MPi or MEKi in two TNBC cell lines (live-cell immunostaining; q ≥ 2; ELISA), after normalizing to cell count. **C,** Venn diagram of differentially expressed genes (DEG) from RNA microarray analysis of MDA-MB-231 treated with PD325901 (PD) or BB94 for 24 hours (n = 4). **D,** enrichment score trace for top-ranked gene set of proteins depleted with MEKi; vertical bars below trace indicate location of proteins in the top-ranked gene set. **E,** diverse kinase inhibitors (labeled as drug target followed by drug name) affect supernatant (supe.) concentrations in MDA-MB-231 (n = 2; ELISA), after normalizing to cell count. **F,** full-length exosomal AXL and exosome markers CD63 and HSP70, in isolated MDA-MB-231 supernatant (n = 4). **G,** protein rank (rank 1/87; decreased with MEKi) and **H,** protein rank for shed full-length AXL fragment from immunoprecipitated supernatant after treatment (n = 3). For **E** to **H,** *P < 0.05, two-tailed t test. **I,** supernatant, lysate total, and pRTK levels following MPi; each dot represents one of 19 cell lines tested (see Supplementary Fig. 54K and 54L).
We next compared the effects of MPi and MEKi on RTK changes in levels in cell lysate, cell surface, and exosomes. Focusing on RTKs and key sheddase substrates identified in the Ab microarray, we first analyzed changes in surface levels of proteins (Fig. 4D). In two TNBC cell lines (MDA-MB-231 and MDA-MB-157), we measured a panel of 18 sheddase substrates on the cell surface in response to MPi and MEKi, and found significant correlation between changes with MEKi compared with MPi (Pearson’s correlation = 0.56; P = 0.0003, two-tailed t test). Results show AX1 as one of the most significantly upregulated membrane proteins (Fig. 4D). Across a panel of 12 cell lines, we observed a modest yet statistically significant correlation between changes in surface AX1 and MET in response to either MEKi or MPi, such that cells are more likely to exhibit increased surface AX1 or MET following MEKi if they also showed increased levels following MPi (Supplementary Fig. S4F). Gene expression helps explain why some sheddase substrates actually decrease on the cell surface following MPi; for example, HBEGF and AREG expression decreases following BB94 treatment, and their levels correspondingly decrease on the cell surface (Supplementary Fig. S4G). In contrast, AX1 expression does not significantly change with either MPi or MEKi, and in LOXIMVI (Fig. 4E) and MDA-MB-231 cells (Fig. 4F), treatment with both MPi and MEKi significantly increases levels of full-length intact lysate AX1 (see Supplementary Fig. S4H–S4I for confirming pERK reduction). Similarly, we found increased intact AX1 levels in CD63+ exosomes isolated from supernatant of the same cells (Fig. 4G). To confirm the hypothesis that this is due to changes in AX1 proteolysis, we measured levels of ectodomain AX1 receptor fragment in the supernatant of treated cells. Indeed, MPi and MEKi both decreased the accumulation of an AX1 ectodomain fragment in the supernatant (Fig. 4H), and full-length intact AX1 protein was not detectable to any similar degree compared with the ectodomain fragment in the supernatant (Fig. 4H). No substantial increases in cell death, apoptosis, or autophagy were observed with BB94 or PD325901 at the concentrations tested, suggesting these processes are not involved in the AX1 phenotype described above (Supplementary Fig. S4J). Taken together, these data provide further evidence that MEKi effects on RTK processing are highly consistent with reduced proteolysis by metalloproteinases.

To more broadly assess the impact of direct MPi on RTK signaling, we next examined MPi-induced changes in total, phosphorylated, and supernatant RTK levels across a panel of 19 diverse cell lines. MPi widely decreased AX1, MET, HER2, and HER4 in the supernatant, and increased total RTK and pRTK levels in many cases, most significantly and consistently for AX1 and MET (Fig. 4I; Supplementary Fig. S4K and S4L). Overall, these data show that proteolytic RTK shedding substantially affects RTK levels and signaling in many cells, especially for AX1.

MPi and MEKi Cause AX1-Dependent Bypass JNK/cJUN Signaling

We next tested how AX1 accumulation after MEKi or MPi leads to corresponding changes in downstream signaling events. At the RTK level, MEKi and MPi increase coimmuno-precipitation of AX1 with MET and HER2, which has been associated with RTK colocalization, signaling cross-talk, and AX1 transactivation (Supplementary Fig. S5A; ref. 34). Downstream of receptor signaling, we found that although MEKi reduced pERK, it increased phosphorylation in other signaling pathways (Supplementary Fig. S1A). Previous reports have implicated signaling of JNK and its substrate cJUN in MAPKi resistance (35), and here we found p-cJUN consistently increased with MEKi treatment more than 5 other canonical signaling pathways averaged across 8 cell lines; pAKT was also consistently elevated (Supplementary Fig. S1A). In response to MPi, JNK and cJUN phosphorylation were among the strongest correlates with fluctuating surface AX1 and pAX1 levels compared with 21 other signaling measurements across 13 cell lines (Supplementary Fig. S5B and S5C), suggesting that accumulation of cell surface AX1 following MPi leads to increased AX1 phosphorylation and downstream signaling through the JNK/cJUN pathway.

To test whether the observed JNK/cJUN signaling in fact depends on AX1 activity, we monitored pJNK following MPi or MEKi in the presence or absence of various AX1 perturbations. After 2-hour MPi treatment, a compensatory increase in phosphorylation occurs more in pJNK than in four other key phosphoproteins, and this increase can be blocked by cotreatment with AX1 using R428 (Supplementary Fig. S5D). After 3-hour treatment with either MPi or MEKi, we found that the compensatory increase in pJNK can be eliminated by siRNA targeting either AX1 or, to a lesser extent, MET (Fig. 5A; siRNA validation Supplementary Fig. S5E). By 24 hours after treatment, compensatory increases in p-cJUN and pAKT are likewise blocked by cotreatment with AX1 using R428 (Fig. 5B and C). Compensatory JNK signaling in response to MAPKi leads to a synergistic response to dual JNK and MAPK inhibition (35), and our data suggest that sheddase activity is a key feedback component driving this synergistic interaction. To test this model, we measured the degree of drug synergy as a response to combination MAPKi and JNKi treatment, in the presence or absence of MPi, and found that the degree of synergy between JNKi and MAPKi is strongly reduced in the absence of metalloprotease activity (Supplementary Fig. S5F and S5G).

MPi Drives AX1-Dependent Cell Proliferation and Blocks Response Synergy to Dual MEKi and AXLi

We next evaluated the direct effect of MPi on cell proliferation. MPi using BB94 caused a dose-dependent increase in cell growth in the absence of AX1i; however, in the presence of AX1i using R428, MPi actually caused a dose-dependent decrease in cell growth (Fig. 5D). Furthermore, MPi increased the mitotic index of two TNBC cell lines, and this increase was also blocked by R428 (Fig. 5E). Next, we directly tested the role of proteolytic activity in mediating MEKi/AXLi synergy. We treated cells with combinations of MEKi and AXLi in the presence or absence of MPi, and that fit the resulting cell growth measurements to a computational model of Loewe synergy, as done above with MAPKi and JNKi. This model fits the response data to a parameter α that quantifies the degree to which drugs act in a synergistic, superadditive manner where the effect of both drugs combined is greater than what would be expected if the effects were independent and additive (simulated in Fig. 5F). This analysis revealed that synergistic
interactions between MEKi and AXLi were substantially reduced in the absence of metalloproteinase activity (Fig. 5G and H), suggesting that proteolytic activity significantly contributes to AXL-dependent MEKi resistance.

**ADAM10 and ADAM17 Proteolytically Shed AXL to Downregulate Mitogenic Signaling Activity**

We next investigated which sheddases cleave AXL directly, focusing on ADAM17 as a principal sheddase and ADAM10 because AXL and MET shedding are closely correlated throughout our data, and MET is a previously known ADAM10 substrate (15, 21, 36). ADAM17 and ADAM10 knockdown using pooled siRNA (validation; Supplementary Fig. S6A) decreased supernatant AXL accumulation (Fig. 6A) and enhanced its levels on the cell surface in both MDA-MB231 (Fig. 6B) and MDA-MB157 cell lines (Supplementary Fig. S6B). Combined ADAM10 and ADAM17 knockdown yielded even greater effects, underscoring a role for both (Fig. 6A and B). Stable shRNA knockdown of either ADAM10 or ADAM17 also caused AXL accumulation on the surface of LOX-IMVI melanoma cells (Supplementary Fig. S6C and S6D) and decreased supernatant AXL accumulation (Supplementary Fig. S6E). ADAM10 inhibition with the ADAM10-selective inhibitor GI-254023X caused a dose-dependent decrease in supernatant AXL ectodomain (Fig. 6C). Similarly, treatment with the specific ADAM10 inhibitor proA10 (37) reduced supernatant AXL (Fig. 6D).

We examined the ability of recombinant ADAM10 to cleave purified AXL protein and showed by Western blot that ADAM10 cleaves recombinant AXL (Fig. 6E and F) in a dose-dependent manner into ectodomain fragments of roughly the same size (∼85 kDa) as found in cell supernatant (Fig. 4H). As a control, we confirmed that these cleavage products were reduced in the presence of MPi (Fig. 6G). We also confirmed the ability of ADAM17 to cleave both recombinant AXL (Fig. 6G) and full-length AXL that had been immunopurified from cell lysate (Supplementary Fig. S6F and S6G). Specific ADAM10 inhibition also affected cell proliferation in an AXL-dependent manner. Treatment with either GI-254023X or ADAM10 siRNA knockdown had minimal
impact on cell growth in the absence of AXLi (Fig. 6H), and stable ADAM10 knockdown using shRNA had minimal impact on the growth of LOX-IMVI xenograft tumors (Supplementary Fig. S6H). However, when AXL signaling was inhibited by R428, ADAM10 inhibition was effective in reducing cell growth (Fig. 6H). Similar trends were observed with ADAM17 siRNA knockdown (Fig. 6H). These combined results are consistent with BB94 effects on proliferation (Fig. 5D and E) and demonstrate AXL upregulation as significantly counteracting the antiproliferative capacity of metalloproteinase inhibitors.

**MEKi Dynamically Enhances Cell Surface TIMP1 Association with ADAM10, Thus Reducing ADAM10 Activity and Causing Drug Resistance**

We next investigated the mechanism through which MAPKi decreases AXL shedding. MEKi significantly reduced ADAM10 and ADAM17 catalytic activities in a live-cell assay (ref. 38; Fig. 7A) without affecting their cell surface levels (Supplementary Fig. S7A), suggesting direct regulation of the protease activities themselves. As further evidence of direct protease regulation, ADAM17 phosphorylation at a site previously associated with activity (33) decreased with MEKi (Supplementary Fig. S7B and S7C). The regulated binding of ADAMs with TIMPs, including TIMP1 and TIMP3, has been implicated as a mechanism for governing protease activity, and we next tested the role of TIMPs on AXL shedding. siRNA knockdown of TIMP1, but not TIMP3, mitigated inhibition (83% vs. 92%; 2) in MDA-MB-231. C, the specific ADAM10 inhibitor GI-254023X decreases supernatant AXL from MDA-MB-231 (*, P < 0.05; n = 3). D, the specific ADAM10 inhibitor proADAM10 reduces supernatant AXL compared with the vehicle control, measured by ELISA using MDA-MB-231 (*, P < 0.05; n = 2). E and F, recombinant ADAM10 cleaves recombinant AXL in a dose-response fashion, shown by AXL immunoblot (E) and corresponding quantification (F; *, P < 0.05; two-tailed t test). G, recombinant ADAM10 and ADAM17 cleave recombinant AXL, and cleavage is blocked by MMPi using BB94 (n = 2). H, in MDA-MB-231 cells, ADAM10 inhibition or knockdown by siRNA only decreases proliferation in the presence of AXLi using R428. ADAM17 siRNA only induces proliferation in the absence of AXLi. siRNA results were measured 24 hours after AXLi, 72 hours after transfection, and GI-254023X results were measured after 72-hour treatment (*, P < 0.05; n = 4; two-tailed t test).
**Figure 7.** MEKi reduces sheddase activity via increased TIMP1 association, and TIMP1 neutralization enhances MAPKi efficacy. **A,** MEKi reduces ADAM10 and ADAM17 catalytic activities in MDA-MB-231, directly measured using the live-cell PrAMA assay (*, P < 0.05; n = 4). **B,** live-cell immunostaining shows knockdown of TIMP1, but not TIMP3, reduces surface AXL in MDA-MB-231, 24 hours after PO325901 treatment and 72 hours after transfection (*, P < 0.05; n = 3; knockdown validation: Supplementary Fig. S7D). **C,** cotreatment with a TIMP1 neutralization antibody (T1-NAB) blocks tumor AXL accumulation following BRAFi/MEKi treatment in the LOX-IMVI melanoma xenograft model, shown by immunofluorescence quantification (*, P < 0.05; n = 3). **D,** 120-kDa ADAM10 dimerization band (39) and co-IP with rTIMP1 increases with 5-minute MEKi (*, P < 0.05; n = 3). **E,** significant co-IP of ADAM10-HA and rTIMP-fluor is only detected in bulk anti-HA IP lysate from cells transfected with ADAM10-HA and treated with U0126 (*, P < 0.05; n = 3; pooled two-tailed t test). **F,** the 120-kDa ADAM10 dimerization band increases surface TIMP1 in MDA-MB-231. **G,** flow cytometry shows 5-minute MEKi treatment increases rTIMP1-fluor binding to the cell surface (*, P < 0.05; n = 4). **H,** significant co-IP of ADAM10-HA and rTIMP-fluor is only detected in bulk anti-HA IP lysate from cells transfected with ADAM10-HA and treated with U0126 (*, P < 0.05; n = 3; pooled two-tailed t test). **I,** pretreatment [24 hours] with a TIMP1 neutralization antibody (T1-NAB) followed by cotreatment with PD325901 (MEKi) or vemurafenib (BRAFi) leads to enhanced reduction in cell count at 72 hours, normalized to the effect size of BRAFi or MEKi alone (*, P = 0.03, pooled two-tailed t test; n = 18 total reps). **J,** MEKi reduces AXL inhibition and MET phosphorylation following BRAFi/MEKi treatment in the LOX-IMVI melanoma xenograft model by enhancing initial tumor shrinkage (*, P = 0.014, two-way ANOVA interaction term, total n = 46) and delaying tumor recurrence (*, P = 0.04, two-tailed t test, n = 8 per group). **L,** overview schematic of RTK shedding as a mechanism of MAPKi resistance.
blocked tumor AXL accumulation after MAPKi treatment (Fig. 7C). Given that knockdown of TIMP3, which preferentially inhibits ADAM17, had no effect on AXL shedding, and because TIMP1 preferentially inhibits ADAM10, our data suggest a more prominent role for TIMP1 and ADAM10 in regulating MEKi-driven AXL shedding.

We found that MEKi continually enhanced the binding of TIMP1 to the cell surface from 30 minutes (Fig. 7D) to 24 hours (Supplementary Fig. S7E). In contrast, TIMP1 supernatant levels actually declined after MEKi (Supplementary Fig. S7F), indicating enhanced TIMP1 surface levels do not simply reflect enhanced TIMP1 secretion. We thus hypothesized that MEKi induced a rapid change on the cell surface that enhanced TIMP1 binding. To test this, we first treated cells for 5 minutes with MEKi, rinsed, and incubated cells on ice with a fixed amount of fluorescently tagged recombinant TIMP1 (rTIMP1-fluor). MEKi enhanced rTIMP1-fluor binding to the cell surface by nearly 50% (Fig. 7E), supporting the idea that MEKi treatment leads to increased accumulation of TIMP1 on the cell surface.

We next evaluated the role of ADAM10 in regulating MEKi-driven TIMP1 cell surface accumulation. Using siRNA, we found that TIMP1 accumulation on the cell surface was dependent upon ADAM10 expression (Supplementary Fig. S7G; knockdown validation in Supplementary Fig. S6A). To directly observe interaction between TIMP1 and ADAM10, we over-expressed transgenic HA-tagged ADAM10 by 2-fold (Supplementary Fig. S7H), treated cells for 5 minutes with MEKi, rinsed, and again incubated cells on ice with a fixed amount of rTIMP1-fluor. Proteins associating with ADAM10 were co-immunopurified (co-IP) using ethylene glycol bis(succinimidyl succinate) (EGS) cross-linking and anti-HA agarose resin. To sensitively detect rTIMP1-fluor co-IP, bulk immunoprecipitate was analyzed by fluorometry and found to exhibit detectable rTIMP1 fluorescence only after MEKi (Fig. 7F), further suggesting that MEKi induces ADAM10-driven cell surface TIMP1 accumulation.

We then investigated how MEKi dynamically influences interactions between ADAM10 and TIMP1. ADAM10 and ADAM17 dimerization has been associated with direct activity regulation (39, 40), and we hypothesized that MEKi elicited increased ADAM10 dimerization and association with TIMP1. Previously, immunoblots of EGS–cross-linked lysate have shown ADAM10 forms a dimerization band at roughly 120 kDa (39), and here we found that this band increases with MEKi and coimmunostains for TIMP1 (Fig. 7G and H), indicating TIMP1–ADAM10 interaction. Thus, MEKi broadly reduces proteolytic shedding by enhanced TIMP1 association with ADAM10, negatively regulating its activity.

We next examined the role of TIMP1 in influencing the therapeutic response to MAPKi. We co-treated cells with T1-NAB and either MEKi or BRAFi. Although T1-NAB had no detectable effect on cell growth in the absence of MAPKi, it enhanced MAPKi sensitivity by up to 50% (Fig. 7J; Supplementary Fig. S7I). For more clinical relevance, we next examined the effect of T1-NAB cotreatment in a melanoma xenograft model undergoing a combined MEKi/BRAFi inhibitor regimen similar to those used in the clinic. T1-NAB treatment alone did not significantly reduce tumor growth (Fig. 7J; P = 0.56, two-way ANOVA-independent effect term, total n = 46). In contrast, T1-NAB significantly enhanced the ability of MEKi/BRAFi to reduce tumor size by an additional 70% (Fig. 7J; note log-scale). Furthermore, T1-NAB extended the time to tumor recurrence after MEKi/BRAFi treatment had ended (Fig. 7K). Recurrent outgrown tumors from this study did not exhibit altered AXL levels (Supplementary Fig. S7J), which is in contrast to tumors analyzed while on drug treatment (Fig. 7C), and suggests that effects on AXL shedding largely reverted during the roughly 3 weeks after treatment ended. Nevertheless, these results provide evidence that reduced proteolytic AXL shedding via TIMP1 association leads to blunt MAPKi efficacy.

**DISCUSSION**

We have elucidated a new targetable mechanism of bypass cancer cell signaling with implications for the design and monitoring of cancer therapies (Fig. 7L). Inhibition of multiple signaling pathways, particularly ERK signaling through MEK1/2, reduces proteolytic RTK shedding and leads to enhanced mitogenic signaling through bypass kinase pathways including JNK. Numerous examples of increased RTK signaling activity have been observed following targeted kinase inhibitor treatment, often with little mechanistic explanation outside of transcriptional upregulation (1, 4, 5, 41). MEK and PI3K kinase inhibitions have been shown to enhance the signaling of sheddase substrates, including HER2 (3, 5) and AXL (1, 42), and we show here that inhibition of MEK, BRAF, and, to some extent, PI3K, p38, and JNK (as in Fig. 4B) reduces RTK shedding. The direct inhibition of RTKs also gives rise to bypass signaling. For example, enhanced AXL signaling mediates resistance to the EGFR/HER2 inhibitor lapatinib, even in the absence of AXL transcriptional upregulation (4). Although transcriptional and chromosomai reprogramming affects bypass signaling (2), these processes often fail to fully explain signaling network dynamics following drug treatment; this is especially true for AXL, which often exhibits little transcriptional upregulation despite sharply enhanced activity (1, 4). Here, we offer reduced proteolytic RTK shedding as a likely explanatory mechanism.

The promiscuous nature of metalloproteinases has made it difficult to anticipate the overall effects of perturbing their activities, especially in response to kinase inhibition (17, 19). Ectodomain shedding can be regulated through substrate-specific pathways (43), and in this work, we find that substrate-specific regulation, for example through differential substrate accumulation on the cell surface, can be explained only partially by transcriptional regulation (Supplementary Fig. S4G). Nonetheless, substantial evidence suggests that MEKi inhibits ADAM catalytic activities themselves (1, 4, 46). In contrast, T1-NAB significantly enhanced the ability of MEKi/BRAFi to reduce tumor size by an additional 70% (Fig. 7J; note log-scale). Furthermore, T1-NAB extended the time to tumor recurrence after MEKi/BRAFi treatment had ended (Fig. 7K). Recurrent outgrown tumors from this study did not exhibit altered AXL levels (Supplementary Fig. S7J), which is in contrast to tumors analyzed while on drug treatment (Fig. 7C), and suggests that effects on AXL shedding largely reverted during the roughly 3 weeks after treatment ended. Nevertheless, these results provide evidence that reduced proteolytic AXL shedding via TIMP1 association leads to blunt MAPKi efficacy.
partly due to unanticipated compensatory signaling feedback from unshed RTKs.

In general, we find that AXL and MET shedding down-regulates signaling activity by limiting the accumulation of full-length, signaling-competent RTK on the cell surface. Ligand-dependent receptor activation is an important signaling feature, particularly in the context of receptor shedding (15), and is relevant for future studies. Nonetheless, AXL and the other RTKs studied here exhibit significant ligand-independent activity (34, 41), which amplifies as they accumulate on the cell surface following protease downregulation. Clinically, AXL upregulation often occurs without apparent dysregulation of its ligand GAS6, and roughly half of observed AXL bypass signaling acts independently of GAS6 in drug-resistant cell lines (41). Receptor shedding also results in the generation of inhibitory “decoy” receptors that both compete for binding of free extracellular ligand and block cell-surface dimerization between signaling-competent receptors; decoy functions have been therapeutically exploited for multiple receptors, including MET (45) and AXL (46). Here, we show that kinase inhibition simultaneously increases full-length RTK on the cell surface while decreasing decoy receptor levels in the extracellular supernatant or in circulation.

Diminished RTK shedding likely complements other bypass signaling mechanisms. Many RTKs reported as transcriptionally upregulated in response to kinase inhibition are themselves sheddase substrates, including PDGFRβ (1), VEGFR2 (1), and CD44 (47). In the context of MAPKi, AXL repeatedly surfaces in genome-wide screens as a top candidate for rescuing drug sensitivity upon transgenic overexpression (48). Reduced RTK shedding has the potential to amplify the effects of transcriptional upregulation by increasing the fraction of total RTK that remains intact on the cell surface. Within the tumor microenvironment, stromal-derived growth factors, cytokines, and extracellular matrix contribute to drug resistance (20, 49–52). Here, we find that receptors affected by reduced RTK shedding are also implicated in tumor–stroma ligand interactions, with a prominent example being MET activation by stromal-derived HGF (51). Of note, extracellular HGF and GAS6 release occurs through secretion rather than metalloproteinase shedding. Consequently, reduced RTK shedding has the capacity to amplify prosurvival and prometastatic tumor–stroma interactions, and previous work has demonstrated RTK shedding as a modifier of ligand-dependent receptor activation (15).

The ability to assess RTK shedding in cancer patients using relevant biomarkers is essential for efficient clinical translation. A substantial proportion of molecular cancer diagnostics focuses on gene expression and/or genetic mutation analysis of tumors, is dependent on the presence of an accessible tumor, and is limited by factors such as tumor heterogeneity and post-translational regulation. We present RTK proteolysis as a mechanistic explanation for the discordance between gene expression and signaling activity, and provide evidence that receptor shedding can be noninvasively monitored in clinical samples following drug treatment. Although promising, the small cohort of patient samples examined here should be expanded to a study of both larger cohorts and a broader panel of sheddase substrates, such as adhesion receptors, cytokines, and cytokine receptors. Importantly, RTK proteolysis may also reflect drug toxicity in addition to drug efficacy, and multiple physiologic factors may influence altered circulating RTK levels. For instance, foretinib has been observed to cause elevated circulating MET in patients (53), which may be explained by its common liver toxicity (high aspartate and alanine transaminases) and the corresponding association of hepatotoxicity with elevated MET shedding (54). Changes may also be related to altered gene expression and known vascular effects of foretinib, including hypertension. Nonetheless, the value of a biomarker that can predict resistance and shed light on a next potential line of therapy cannot be underestimated. Ultimately, circulating RTKs hold the potential to stand alone and to complement other diagnostic biomarkers in guiding targeted combination therapies, monitoring drug response, and noninvasively detecting the emergence of drug resistance.

**METHODS**

Unless otherwise stated, reported replicates are from unique biologic samples, statistical tests used the two-sided Student *t* test for significance, and mean values are reported with error bars denoting SEM. With some explicitly stated exceptions, experiments used 10 μmol/L BB94, 3 μmol/L R428, 15 μmol/L PD325901, 5 μmol/L U0126, 10 μmol/L AZD6244, 15 μmol/L MP470, and 4 μmol/L pro-ADAM10. Please see Supplementary Experimental Procedures for further information regarding materials, experimental details, and computational analysis methods.

**Cell Lines**

The following cell lines were purchased directly from commercial or governmental repositories and immediately used for experiments in this article: MDA-MB-231 (ATCC; June 2012); LOX-IMVI (NCI-DCTD repository; March 2014); SUM149 and SUM102 (Astell and Biorepository; November 2011); HCCR27 (ATCC; January 2012); BT549 (ATCC; April 2010); and BT20 (ATCC; January 2012). Additional cell lines were procured from Massachusetts Institute of Technology (MIT), Harvard Medical School (HMS), and University of Michigan collaborating lab banks: MA2 and A375 cell lines (Richard Hynes, MIT, January 2013); BT474, Hs578T, HCC38, MDA-MB-436, and MDA-MB-468 (Mike Yaffe, MIT, April 2011); A172 (Leona Samson, MIT, January 2013); U87 (Dane Wittrup, MIT, May 2011); A549 (Linda Griffith, MIT, May 2011); SUM159 (Joan Brugge, HMS, January 2011); LM2 (ref. 55; Richard Hynes, MIT, January 2013); and SUM1315 (Stephen Ethier, University of Michigan, December 2009).

Cell lines were routinely tested for *Mycoplasma* (Lonza MycoAlert) within 3 months of use and were not authenticated. All cell cultures were performed according to the manufacturer’s guidelines.

**Melanoma Patient Samples**

Patients with metastatic melanoma containing *BRAF*V600E mutation (confirmed by genotyping) were enrolled on clinical trials for treatment with a BRAF inhibitor or combined BRAF + MEK inhibitor (dabrafenib 150 mg b.i.d.; trametinib 2 mg q.d.) at Massachusetts General Hospital and gave consent for blood and tissue acquisition as per Institutional Review Board-approved protocol, conducted in accordance with the Declaration of Helsinki. All patients (or legal representatives) gave written informed consent before enrollment. Blood was collected and tumor biopsies were performed before treatment (day 0), 10 to 14 days on treatment, and/or at time of progression if applicable. Multiple on-treatment blood samples were collected over the course of therapy as available. Plasma was isolated immediately from blood samples using BD Vacutainer CPT tubes with Sodium Citrate (BD 362761). Formalin-fixed tissue from each tissue biopsy was analyzed to confirm that viable tumor was present.
Drug Resistance via Reduced RTK Shedding

via hematoxylin and eosin staining. Clinical response was assessed by RECIST (see Supplementary Experimental Procedures).

In Vivo Tumor Growth and Metastasis Assays

All animal experiments and husbandry were approved by the MIT Division of Comparative Medicine in accordance with guidelines of the Institutional Animal Care and Use Committee of MIT. For orthotopic mammary transplant assays, 6-week-old female NOD/SCID-gamma mice (JAX) were anesthetized by i.p. injection of 125 to 250 mg/kg body weight of Avertin (reconstituted in PBS), followed by i.p. injection of 100 μL of 12 μg/mL buprenorphine for analgesia. A small incision was made on the right flank, and 2.5 × 10⁵ MDA-MB-231-LM2 cells in 25 μL of Hank’s Balanced Salt Solution (HBSS) were injected into the right #4 fat pad using a 25-μL Hamilton syringe. Mice received three additional i.p. injections of 100 μL of 12 μg/mL buprenorphine at 12-hour intervals following the surgery. Initial sample size was chosen based on previously published experiments with MDA-MB-231-LM2 xenograft models (55), as well as previously published data with the MEK and AXL drugs of interest (56, 57). Twenty days after surgery, when tumor size was palpable, mice were ranked by tumor size and were semirandomly divided into four groups of equal distribution in tumor size. Groups received one of four different drug treatments once daily for 21 days by oral gavage: vehicle (10% DMSO + 0.5% methylcellulose + 0.2% Tween-80 in water), AXL inhibitor R428 at 30 mg/kg, PD0325901 at 1 mg/kg, or a combination of both R428 at 30 mg/kg and PD0325901 at 1 mg/kg. Animals were sacrificed at the predetermined time of 21 days following initiation of drug treatment.

For the xenograft melanoma experiment, 7-week-old female athymic nude mice (Taconic) were injected with 1 × 10⁶ LOX-IMVI cells in 25 μL of Hank’s Balanced Salt Solution (HBSS) via hematoxylin and eosin staining. Clinical response was assessed by tumor size and were semirandomly divided into four groups of equal distribution in tumor size. Groups received one of four different drug treatments once daily for 21 days by oral gavage: vehicle (10% DMSO + 0.5% methylcellulose + 0.2% Tween-80 in water), AXL inhibitor R428 at 30 mg/kg, PD0325901 at 1 mg/kg, or a combination of both R428 at 30 mg/kg and PD0325901 at 1 mg/kg. Animals were sacrificed at the predetermined time of 21 days following initiation of drug treatment.

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Accession Numbers

RNA microarray expression data can be found in the GEO repository under accession number GSE77868.

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Drug Resistance via Reduced RTK Shedding

Reduced Proteolytic Shedding of Receptor Tyrosine Kinases Is a Post-Translational Mechanism of Kinase Inhibitor Resistance

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