A common p53 mutation (R175H) activates c-Met receptor tyrosine kinase to enhance tumor cell invasion

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Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive forms of human cancer with poor prognosis due to late diagnosis and metastasis. Common genomic alterations in ESCC include p53 mutation, p120ctn inactivation, and overexpression of oncogenes such as cyclin D1, EGFR, and c-Met. Using esophageal epithelial cells transformed by the overexpression of EGFR and p53^{R175H}, we find novel evidence of a functional link between p53^{R175H} and the c-Met receptor tyrosine kinase to mediate tumor cell invasion. Increased c-Met receptor activation was observed upon p53^{R175H} expression and enhanced further upon subsequent EGFR overexpression. We inhibited c-Met phosphorylation, resulting in diminished invasion of the genetically transformed primary esophageal epithelial cells (EPC-hTERT-EGFR-p53^{R175H}), suggesting that the mechanism of increased invasiveness upon EGFR and p53^{R175H} expression may be the result of increased c-Met activation. These results suggest that the use of therapeutics directed at c-Met in ESCC and other squamous cell cancers.

Introduction

Human squamous cell carcinomas are the most common type of epithelial cancers. One subtype, esophageal squamous cell carcinoma (ESCC), is an aggressive cancer with poor prognosis due to late diagnosis, local and distant metastases, and limited therapeutic options. The other subtype of esophageal cancer is esophageal adenocarcinoma, with the fastest rate of increase of any cancer in the US.[1] Risk factors for ESCC include cigarette-smoking, alcohol use, and certain mineral (e.g., selenium, zinc, molybdenum) and vitamin (A, C, E, and K) deficiencies.[2,3] The development of ESCC is a multistep, progressive process, and a number of genetic alterations in the tumor cells have been identified.[4,5] Among the oncogenes that are activated include cyclin D1 and EGFR, and the tumor suppressor genes that are inactivated include p53, p120ctn, and p16INK4a.[2] EGFR overexpression and p53 mutations are particularly common in premalignant lesions.[6-8] TP53 mutations result in the stabilization and accumulation of mutant p53 protein. Mutations fall into two categories: (1) mutations that directly block DNA binding by p53 (R273H mutation-archetype) and (2) mutations that alter the global conformation of the p53 protein (R175H mutation-archetype).

It has been shown to influence tumorigenesis in multiple ways, including loss of the gatekeeper functions of wild-type (WT) p53 by acting as a dominant negative directly on WT p53, and more recently appreciated, through WT p53-independent gain of function (GOF) mechanisms. In the latter context, the putative mechanisms involve abrogation of the effects of Tp63, an ortholog of Tp53, and direct DNA binding with activation of a different repertoire of genes by mutant p53. Clinically, tumors with p53 mutations carry a worse prognosis than those that are p53 null.[9,10] p53 null cells can be transformed by expression of mutant p53.[11] Tumors in p53$^{R172H/+}$ transgenic mice tend to be of more epithelial origin compared with p53$^{-/-}$ or p53$^{+/-}$ murine tumors and metastasize more than those from p53$^{+/-}$ mice.[11,12]

The c-Met tyrosine kinase receptor activates a signaling response program termed "invasive growth" that is necessary both during normal embryonic development and adult tissue repair.[13,14] It is activated upon binding its ligand, namely hepatocyte growth factor (HGF). Specifically, there is transphosphorylation of Tyr 1234 and Tyr 1235 on the intracellular domain of c-Met, induction of docking molecules, and the activation of diverse signaling pathways, such as Ras, PI3K, STAT, β-catenin, and Notch pathways.[15] Perturbed activation of c-Met signaling has been shown to be important for neoplastic transformation in

a wide variety of tumor types, including ESCC, largely through c-Met amplification or overexpression, although rare receptor mutations have been detected.[16,17]

There is limited evidence linking p53 mutation and c-Met signaling in cancer. Sarcomas arising in Li–Fraumeni patients have increased c-Met protein levels.[18] WT p53 was shown recently to regulate c-Met expression and influence cell migration and invasion in normal and transformed ovarian epithelium.[19] We have reported previously, using a model that recapitulates early genetic alternations in ESCC, that expression of mutant p53[R175H] and EGFR could transform immortalized human primary esophageal epithelial keratinocytes (EPC-hTERT), thereby dramatically increasing their migratory and invasive capabilities.[5] In this study, we report a previously unidentified direct connection between p53[R175H] and increased c-Met receptor activity that is linked directly to tumor invasion.

Results

Mutant p53[R175H] expression leads to c-Met activation in a HGF-ligand-independent manner. We have shown previously that c-Met phosphorylation was increased in EPC-hTERT-cells retrovirally infected to express p53[R175H] and its activation was further increased upon co-expression with EGFR.[20] This specific pattern of activation was not shared with other selected receptor tyrosine kinases as IGF1Rβ and EGFR phosphorylation (data not shown). In a certain context, EGF and HGF cooperate to promote cell proliferation, scatter and invasion in mouse mammary cells.[21] Certainly, EGFR inhibition has led to the evaluation of targeting c-Met for EGFR inhibitor resistant tumors.[22,23] Yet, inhibition of EGFR tyrosine kinase activity did not affect c-Met activation (**Fig. 1A**). That being said, our results are not inconsistent with reports of the lack of crosstalk between EGFR and c-Met in mouse adult liver oval cells.[24] In our experimental conditions, a c-Met inhibitor decreased c-Met phosphorylation, but interestingly, c-Met phosphorylation was not affected by an HGF blocking antibody (previously optimized for blocking efficiency[20]) in EPC-hTERT-p53[R175H] ± EGFR cells (**Fig. 1B and C**). This suggests that HGF was not responsible for inducing c-Met activation under these experimental conditions. Further support of this conclusion emerges from the finding that HGF was undetectable in cell culture media (keratinocyte serum free medium, KSFM) or in conditioned media collected from EPC2-hTERT-EGFR-p53[R175H] cells (data not shown). c-Met phosphorylation was reduced when EPC2-hTERT-EGFR-p53[R175H] cells were cultured in keratinocyte basal medium (KBM) or in KSFM without the added bovine pituitary extract (BPE) (**Fig. 1D**), but was constitutively phosphorylated in KSFM with BPE ± additional EGF (normal culture conditions). It is conceivable there is a factor(s) in BPE that influence Met phosphorylation. In order to determine if the p53[R175H]-mediated c-Met phosphorylation was the result of the potential expression and autocrine secretion of an unidentified ligand capable of activating c-Met, cells with low/absent c-Met phosphorylation (EPC2-hTERT-EGFR and EPC2-hTERT, respectively) were treated with conditioned media collected from EPC2-hTERT-EGFR-p53[R175H] cells

(**Fig. 1E**). No increase in c-Met phosphorylation was observed in either EPC2-hTERT-EGFR or EPC2-hTERT cells, thereby leading us to conclude that c-Met activation upon p53[R175H] expression is ligand-independent, either HGF or some other ligand.

Phospho-Met expression is specific to the p53[R175H] mutation. A survey of primary esophageal keratinocytes expressing additional p53 mutations (R273H, V143A, or R248W) described previously[5,25] revealed that the activation of the c-Met receptor upon p53 mutation was exclusive to R175H (**Fig. 2A**). Esophageal keratinocytes isolated from p53 null and WT mice with overexpression of the human p53[R175H] mutant (previously described)[26] did not have similar patterns of c-Met activation (**Fig. 2B**), which might point to species difference in the origin of the esophageal keratinocytes or the potential combinatorial effects of EGFR and p53[R175H].

Restoration of WT p53 function reverses p53[R175H] mutant activation of phospho-Met. In order to determine if the increased c-Met phosphorylation was linked directly to p53 mutation, three pharmacologic activators of WT p53 signaling were used. 5-iminodaunorubicin has been identified for its cytotoxicity independent of the cellular p53 status and does so by activating p53 family member p73 and downstream p21.[27] CP-31398 and PRIMA-1 have been shown to bind to mutant p53 and alter its conformation so as to restore proper DNA binding.[28,29] Treatment with either 5-ID or CP-31398 compound resulted in a loss of c-Met phosphorylation in EPC-hTERT-EGFR-p53[R175H] cells with a concomitant upregulation of p21 (**Fig. 3A and B**). PRIMA-1 treatment did not show an effect on phospho-Met or activation of p21 at 1 μM or 10 μM concentrations; treatments at higher doses proved to be toxic to the cells (**Fig. 3B**). Both 5-ID and CP-31398 compounds inhibited invasion of EPC-hTERT-EGFR-p53[R175H] cells as well as proliferation (**Fig. 3C–E**). These experiments give credence to the premise that the increased tumor cell invasion apparent with p53[R175H] mutation is mediated in part by phospho-Met.

Discussion

ESCC is typically inoperable and fatal when detected after tumor cells have metastasized. We have studied the genetic influences on esophageal tumor cell invasion, one of the initial critical steps of tumor dissemination and metastasis. We report a novel connection between mutant p53[R175H] expression and activation of the c-Met receptor tyrosine kinase that is HGF ligand independent and potentially independent of other ligands. The specific nature of the DNA contact inhibition by p53[R175] and how it activates c-Met tyrosine kinase activity is interesting. Mutant p53 has been demonstrated to result in enhanced integrin and epidermal growth factor receptor (EGFR) trafficking, which depends upon Rab-coupling protein (RCP) and results in constitutive activation of EGFR/integrin signaling.[30] To that end, mutant p53 may enhance c-Met signaling to promote cell scattering and invasion through both TAp63-dependent and -independent mechanisms;[30,31] however, this is dependent upon HGF ligand binding the c-Met receptor, a scenario not present in our results, suggesting that our specific interrelationship between mutant p53
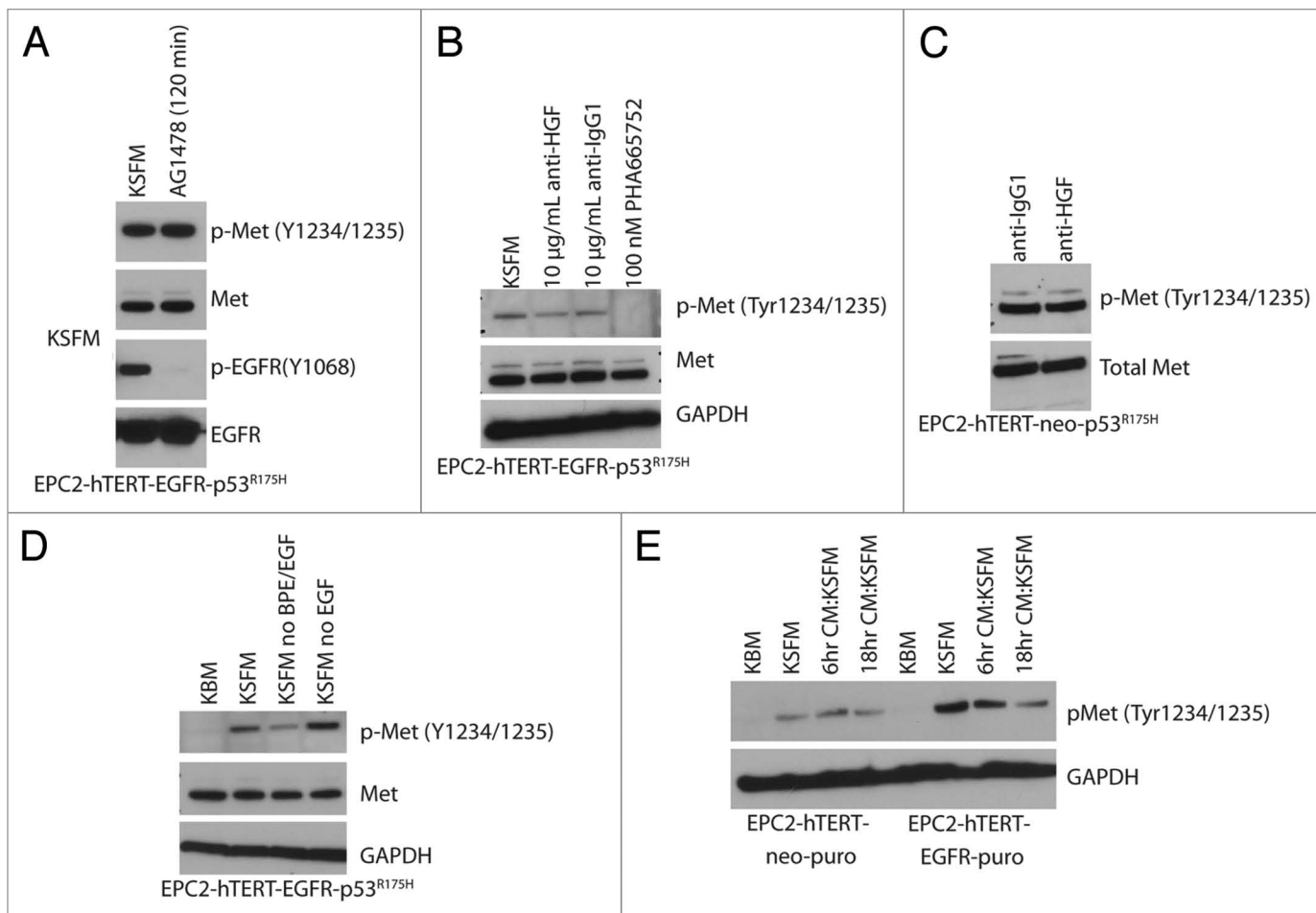
Figure 1. p53[R175H] expression leads to c-Met activation in an HGF-independent manner. (**A**) Inhibition with AG11478 EGFR tyrosine kinase inhibitor (1 uM) for 120 min. (**B and C**) Inhibition with anti-HGF antibody or the PHA665752 phospho-Met inhibitor. (**D**) Activation of phospho-Met in EPC2-hTERT-EGFR-p53[R175H] can be attenuated by using keratinocyte basal medium (KBM) or using keratinocyte serum-free medium (KSFM) without BPE (bovine pituitary extract) and EGFR supplements. (**E**) Treatment of cells with condition media from EPC2-hTERT-EGFR-p53[R175H] cells does not activate phospho-Met in EPC2-hTERT-neo-puro or EPC2-hTERT-EGFR-puro.

and c-Met is mediated through another mechanism. Another possibility is that mutant p53 may have a direct transcriptional effect upon c-Met by virtue of its GOF properties.[32] Recently, the miR34 family of microRNA was identified as a p53 transcriptional product and c-Met was confirmed to be a target of the miR34 family.[33-35] We did find that mir34a is decreased in the p53[R175H] cells (data not shown). However, we do not find that overexpressing mir34a had an effect on either c-Met expression levels or phospho-Met levels (data not shown).

ESCC is a difficult cancer to treat. Currently, neo-adjuvant or adjuvant therapy involves chemotherapy and radiation. Biologics have gained some attention, although EGFR inhibition is not standard. Since p53[R175H] is present in a subset of ESCC,[36] it would be intriguing to consider Met inhibition therapy in this context in future pre-clinical study.

Materials and Methods

Cell culture. Primary human esophageal keratinocytes, designated EPC2-hTERT, and their derivatives are described

previously.[37] The following mediums where used keratinocyte basal medium (KBM) from Lonza or using keratinocyte serum free medium (KSFM) (Invitrogen) without BPE (bovine pituitary extract) and EGFR supplements. Stable transduction of primary esophageal cells with retroviral vectors was described previously.[26,37,38] Briefly, pFB-neo retroviral vectors (Stratagene) containing the entire coding sequence for the human EGFR (pFB-neo-WT-hEGFR), pBabe-puro-p53[R175H] or pBabe-puro-p53[R273H] or pBabe-puro-[R248W] or pBabe-puro-[V143A] were transfected into Phoenix-Ampho packaging cells (gift of Dr Garry Nolan, Stanford University) using LipofectAMINE 2000 reagent (Invitrogen), according to the manufacturer's instructions. Culture supernatants from individual Phoenix-Ampho cells were used to infect EPC2-hTERT cells. Cells were passaged 48 h after infection and selected with G418 (300 μg/mL), puromycin (0.5 μg/mL), for a period of 7 d, resulting in generation of control EPC2-hTERT-EGFR-p53[R175H], EPC2-hTERT-EGFR-p53[R273H], EPC2-hTERT-EGFR-p53[R248W], EPC2-hTERT-EGFR-p53[V143A], EPC2-hTERT-neo-p53[R273H], and EPC2-hTERT-neo-p53[R248W] cells. Independent infections
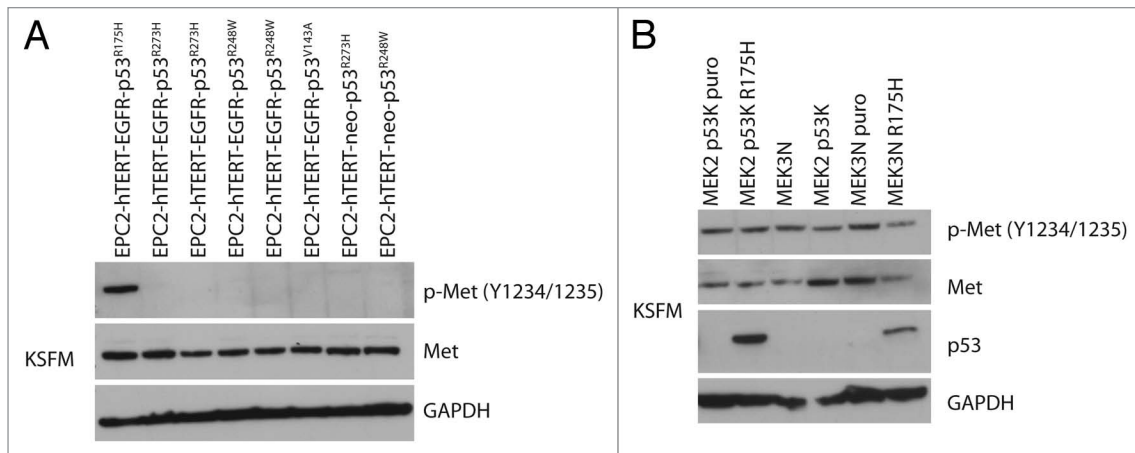
Figure 2. Phospho-Met expression is specific to the p53^{R175H}. (**A**) Panel of four different p53 mutations R175H, R273H, R248W, and V143A were analyzed by western blotting for phospho-Met expression. Only the cells expressing the p53^{R175H} mutant have increased phospho-Met expression. (**B**) Mouse esophageal keratinocytes (MEK) isolated from p53 null mice (MEK3N p53K puro, MEK2N p53K) and wild-type p53 mice (MEK3N, MEK3N puro) were transfected with human p53^{R175H}, in both the p53 null (MEK2 p53K p53^{R175H}) and wt p53 (MEK3N p53^{R175H}). The p53^{R175H} did not have the same effect on activation of phospho-Met as in human keratinocytes.

and selections were performed to generate two additional cell lines of each genotype. Mouse esophageal keratinocytes (MEK) isolated from p53 null mice (MEK3N p53K puro, MEK2N p53K) and wild type p53 mice (MEK3N, MEK3N puro) previously decribed,[38] were transfected with human p53^{R175H}, in both the p53 null (MEK2 p53K p53^{R175H}) and wt p53 (MEK3N p53^{R175H}). In both context the p53^{R175H} mutant did not have the same effect on activation of phospho-Met as in the human keratinocytes. The following reagents were used in cell culture studies: AG1478 (Sigma) EGFR tyrosine kinase inhibitor (1 µM) for 120 min; PHA665752 phospho-Met inhibitor (Tocris Bioscience); 5-iminodaunorubicin (5-ID) (gift of Dr Wafik El-Deiry) range from 0.1–10 µM; CP-31398[29] (50 ng/mL–5 µg/mL) (Tocris Biosciences) and PRIMA1[28] (1–10 µM) (Cayman Chemical).

Conditioned media treatment. Condition media of EPC2-hTERT-EGFR-p53^{R175H} cells grown in 2D cultures in KSFM medium, was collected after 48 h, collected medium was spun down for 5 min at 1000 rpm to remove any cellular debris. EPC2-hTERT-neo-puro or EPC2-hTERT-EGFR-puro cells (0.5 × 10^6 per plate) where plated 24 h before treatment. Condition medium was added after 24 h of growth, cells where harvested 24 h after for protein lysis previously described.

Western blot analysis. Cells were harvested in lysis buffer (50 mM Tris·HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, protease inhibitor tablet [Roche]). Thirty micrograms of protein were run on a 4–12% SDS/PAGE Bis-Tris gel (Invitrogen) and transferred to a poly (vinylidene difluoride) membrane (Immobilon-P; Millipore). Membranes were blocked in 5% nonfat milk (Bio-Rad Life Science) in PBS-T (1× PBS without Ca^{2+} and Mg^{2+} [Invitrogen] and 0.1% Tween 20) for 1 h at room temperature. Membranes were then probed with primary antibody diluted in 5% milk in PBS-T overnight at 4 °C, washed with PBS-T, and incubated with

anti-mouse or anti-rabbit secondary antibodies (1:5000 in PBS-T) for 1 h at room temperature and washed in PBS-T. The signal was visualized using an enhanced chemiluminescence solution (ECL Plus; GE Healthcare Life Sciences) and exposed to Blue Lite Autorad film (ISC-BioExpress).

Antibodies. Antibodies were obtained from the following sources: EGFR(Ab-12) (1:1000) (NeoMarkers/Thermo Fisher Scientific), phospho-EGFR (Tyr1068) (1:500) and phospho-Met (Tyr1234/1235) (1:500) Cell Signaling Technology, Met antibody (C-28) (1:1000) Santa Cruz Biotechnology, p53 (Ab-6) (1:1000) Calbiochem, GAPDH (1:15000) Chemicon/Millipore, Cell Signaling and p21 (WAF1 Ab-1) (1:1000) Oncogene. Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies were from GE Healthcare Life Sciences. Mouse IgG1 control antibody was from R&D Systems. 2B8 mouse anti-human HGF IgG1 generated from hybridoma (AVEO Pharmaceuticals) and mouse IgG1 control antibody from R&D Systems, where used in HGF blocking antibody experiment, as previously described.[20]

Cell proliferation WST-1 assay. Five thousand cells were plated in 100 µl of KSFM in a 96-well plate for 72 h before reading with WST-1 reagent (Roche). After 48 h of growth, we added 5-ID and CP-31398 reagents at described concentrations. After 24 h 10 ul of WST-1 reagent and incubated at 37 °C for 2 h. Following incubation plate was read using ELISA plate reader to measure absorbency. All experiments were performed in triplicate on three independent days.

Boyden chamber invasion assay. For invasion assays, insert plates (8-µm pore size, 24-well insert) coated with growth factor reduced Matrigel matrix were used (BD Biosciences). Inserts were placed in a 24-well plate containing DMEM + 10% serum to stimulate cell invasion. 1 × 10^5 cells in serum-free medium were placed in each insert (plus the 5-ID or CP-31398). Twenty hours later, the cells remaining inside the insert were removed with a cotton swab and the invading cells on the insert bottom
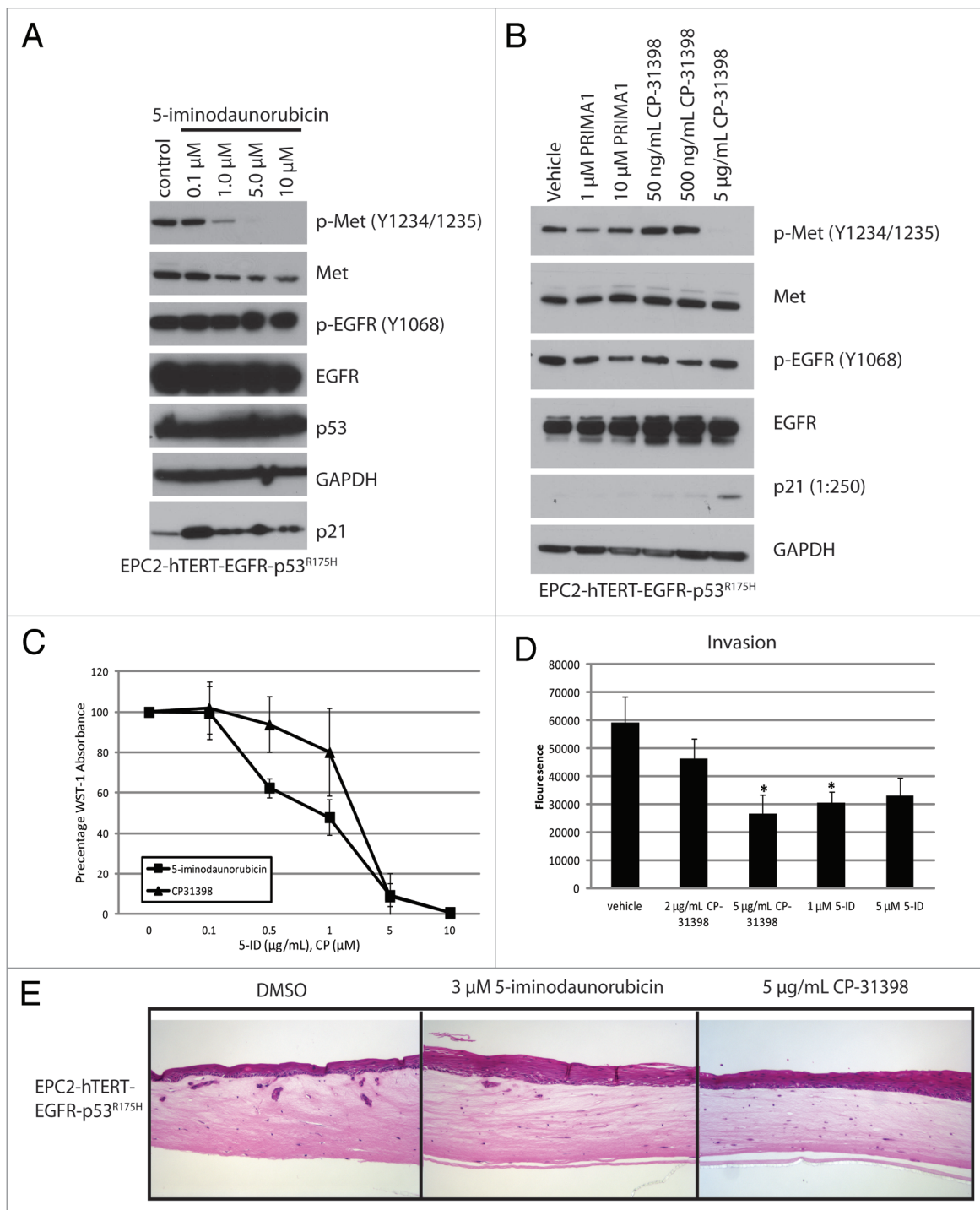
Figure 3. Restoration of WT p53 function reverses p53^{R175H} mutant activation of phospho-Met. (**A-B**) EPC2-hTERT-EGFR-p53^{R175H} cells treated for 24 h with 5-iminodaunorubicin (5-ID) in a range from 0.1–10 μM (**A**), CP-31398 (50 ng/mL–5 μg/mL) (**B**) show decrease in phospho-Met levels by western blotting; treatment with PRIMA1 (1–10 μM) (**B**) had no effect in the levels of phopho-Met. (**C**) Treatment with 5-ID (range 0.1–10 μM) and CP-31398 (range (0.01–10 μg/mL) inhibited cells growth of EPC2-hTERT-EGFR-p53^{R175H} in WST-1 assay (**C**). Error bars represent ± SEM, and the Student *t* test was used to determine significance (*, $P \leq 0.05$). (**D**) Treatment of EPC2-hTERT-EGFR-p53^{R175H} with 5-ID (1 uM and 5 uM) and CP-31398 (2 ug/mL and 5 ug/mL) caused a decrease in boyden-chamber invasion assay. (**C-D**) Error bars represent ± SEM, and the Student t-test was used to determine significance (*, $P \leq 0.05$). (**E**) H&E staining of organotypic 3D cultures show 5-ID (3 μM) and CP-31398 (5 μg/mL) treatment can reduce invasion, magnification 10×.

were labeled with 4 μg/mL Calcein AM dye (Invitrogen) in Hanks's balanced salt solution (HBSS) (Invitrogen) for 30 min at 37 °C. The labeled cells were then read on a Biotek FLX800 multidetection microplate reader (BioTek) at 485 nm excitation and 528 nm detection. All experiments were performed in triplicate on three independent days.

Organotypic culture. H&E staining of organotypic 3D cultures show 5-ID and CP-31398 treatment can reduce invasion. The employment of EPC cells and their derivative cell lines in organotypic culture was done as described previously.[39] The cultures were harvested and fixed for 1 h or overnight in 10% buffered formalin phosphate (Fisher) before being paraffin-embedded and sectioned (Leica RM2155 microtome; Leica Microsystems) for hematoxylin and eosin (H&E) staining.

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest to disclose.

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