Loss of MIG6 Accelerates Initiation and Progression of Mutant Epidermal Growth Factor Receptor–Driven Lung Adenocarcinoma

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INTRODUCTION

Lung cancer is the leading cause of cancer mortality in the United States, accounting for about 27% of cancer-related deaths. EGFR and KRAS are among the most commonly mutated genes associated with the initiation and maintenance of lung adenocarcinomas. The most prevalent EGFR mutations associated with lung cancer are two hotspot mutations, a leucine to arginine substitution at position 858 (L858R; 40–45%) and an in-frame deletion mutation eliminating the conserved sequence LREA in exon 19 (e.g., Del E746–A750; 45%; refs. 1–4). These mutations render the EGFR protein–tyrosine kinase constitutively active. Lung adenocarcinomas harboring these mutations are sensitive to EGFR-directed tyrosine kinase inhibitors (TKI), such as erlotinib and gefitinib. Unfortunately, patients undergoing TKI treatment eventually develop acquired resistance. A mutation in the gatekeeper residue, T790M, accounts for 50% to 60% of acquired drug resistance (5, 6). Other mechanisms of resistance to TKIs include MET amplification (9), CRKL amplification (10), and low-frequency mutations in ERBB family members, including HER2 (12, 13); small cell lung cancer transformation (12, 13); NF1 loss (11); and epithelial–mesenchymal transformation (EMT; refs. 14–16). It is therefore important to understand the signaling pathways activated downstream of mutant EGFRs in TKI-sensitive and TKI-resistant lung adenocarcinoma cells.

Aberrant EGFR signaling that leads to activation of downstream signaling components, such as AKT and ERK, is associated with increased cellular proliferation and development of cancer (19–21). Recently, several groups, including ours, have performed global phosphoproteomic profiling of lung adenocarcinoma tumor tissue from patients and in cell lines, particularly TKI-sensitive lung adenocarcinoma cell lines, and have identified a large number of sites that are tyrosine phosphorylated (22, 23). We previously employed stable isotope labeling with amino acids in cell culture (SILAC) and quantitative phosphoproteomics to elucidate the differences in use of phosphorylation targets of wild-type and mutant EGFRs in isogenic human bronchial epithelial cells (24). One of the candidates that was hyper-phosphorylated on tyrosines in cells expressing mutant EGFRs was MIG6 (gene symbol ERRFI1, also known as RALT; Gene 33), an immediate early response gene that is induced by growth factors, including EGF and stress stimuli (25, 26). MIG6 functions as a negative feedback regulator of ERBB family members, including EGFR and ERBB2 (27). Ablation of MIG6 in mice leads to tumors of various tissues, including lung, implicating MIG6 as a potential tumor suppressor gene (28–30). Several studies have reported that MIG6 inhibits EGFR by blocking downstream signaling components, such as AKT and ERK, is associated with increased cellular proliferation and development of cancer (19–21). 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its kinase activity, as well as by promoting its degradation (29, 31, 32). It has also been demonstrated that Mig6 RNA is increased in EGFR-mutant lung adenocarcinoma cell lines (33). These observations raise questions about whether Mig6 is a tumor suppressor for mutant EGFR–driven lung adenocarcinoma and, if so, how mutant EGFR induces lung adenocarcinomas in the presence of Mig6.

In this study, we sought to establish whether Mig6 deficiency would accelerate tumorigenesis induced by the common mutant alleles of EGFR, thus demonstrating its tumor-suppressive role. We generated doxycycline-inducible mutant EGFR transgenic mice on different Mig6 genetic backgrounds and demonstrate that Mig6 deficiency accelerates the initiation and progression of mutant EGFR-driven tumorigenesis in vivo. Mig6 also functions as a haploinsufficient tumor suppressor in this model. To further examine the mechanisms of tumor suppression by Mig6 and to elucidate how mutant EGFR can circumvent Mig6 function in human lung tumors, we studied the consequences of tyrosine phosphorylation of Mig6 in human cancer cell lines. Using global quantitative mass spectrometry–based phosphoproteomics, we identified Y394/Y395 as constitutively phosphorylated sites on Mig6 in lung adenocarcinoma cells expressing mutant EGFRs; these sites are inhibited by erlotinib in TKI-sensitive lung adenocarcinoma cells but not in drug-resistant cells. Increased phosphorylation of Mig6 increases the interaction of mutant EGFRs and Mig6. However, contrary to its effects on wild-type EGFR, Mig6 does not promote degradation of mutant EGFR. We propose a model in which mutant EGFR may circumvent the tumor suppressor function of Mig6 by constitutively phosphorylating Y394/Y395. However, the attenuated inhibitory function of Mig6 in the context of mutant EGFRs is still sufficient to delay tumorigenesis in a mouse model of mutant EGFR–driven lung adenocarcinoma.

RESULTS

Ablation of Mig6 Accelerates Formation of Mutant EGFR–Induced Adenocarcinomas and Decreases Survival of Transgenic Mice Expressing Mutant EGFR

Tissue-specific knockout of Mig6 increases EGFR signaling and the proliferation of epithelial cells in mouse lungs, suggesting that Mig6 is essential for lung homeostasis (34). Deletion of Mig6 in mice also promotes adenomas and adenocarcinomas in the lung, gallbladder, and bile duct, albeit at low penetrance (30). However, the role of Mig6 in mutant EGFR–driven lung tumorigenesis has not been studied. To test this, we crossed Mig6 heterozygous mice (Mig6+/−; ref. 30) with doxycycline-inducible mutant EGFR transgenic mice (tetO-EGFRmut; ref. 35) and CCSPrtTA mice (36). The resulting tetO-EGFRmut/Mig6+/− and CCSPrtTAMig6+/− were further bred to generate transgenic mice with conditional, doxycycline-inducible expression of EGFRLSRR or EGFRDL in type II lung epithelial cells in Mig6+/−, Mig6+/−, and Mig6−/− backgrounds. After induction of transgenic EGFR mutants, we monitored mice for the appearance of lung tumors by serial MRI CC10rtTA/EGFRLSRR/Mig6+/− mice developed tumors earlier than CC10rtTA/EGFRLSRR/Mig6+/− mice (Fig. 1A; Supplementary Fig. S1A). The same was true for CC10rtTA/EGFRLSRR/Mig6−/− mice (Supplementary Fig. S1B). The Mig6−/− mice carrying mutant EGFR transgenes were euthanized earlier than mice without EGFR transgenes because of progressive disease. The Mig6−/− mice without the EGFR transgene had to be euthanized between 3 and 6 months of age, not due to lung tumor formation, but because of osteoarthritis affecting food intake (data not shown).

Although there were transgenic line–specific differences, histopathology of the tumors at the survival endpoint indicated a higher incidence of adenocarcinoma in Mig6−/− mice compared with Mig6+/− mice (Fig. 1B and Supplementary Fig. S1C, Table). Lungs of CC10rtTA/EGFRLSRR/Mig6+/− mice showed only pulmonary adenomas or adenomas with infrequent adenocarcinomas. There were no signs of invasion. The surrounding alveolar compartment showed type II cellular hyperplasia and variable amounts of macrophages (Supplementary Fig. S1D; A–C). The neoplastic lesions induced by both EGFR mutants in Mig6+/− or Mig6−/− mice were more advanced with features of adenocarcinoma (Supplementary Fig. S1E; A–C). Lungs were often completely effaced with hyperplastic and dysplastic type II epithelial cells and had intense infiltration of macrophages and other inflammatory cells. These mice also demonstrated marked abnormalities of the airway lining epithelium with Clara cell hyperplasia or dysplasias and proliferation at bronchioalveolar duct junctions of the terminal bronchioles (Supplementary Fig. S1E; D and E).

To examine the effect of Mig6 deletion on the survival of mice harboring mutant EGFRs (EGFRLSRR and EGFRDL), we generated tumors by doxycycline induction of mutant EGFR transgenes and euthanized the mice when they displayed specific criteria related to lung tumor burden, such as labored breathing, weight loss, and failure to thrive. We performed Kaplan–Meier survival analyses in two separate transgenic lines of each of the mutant EGFRs in Mig6+/−, Mig6−/−, and Mig6−/− backgrounds (Fig. 1C–F). In all the tested lines of mutant EGFR mice, the survival time of Mig6−/− mice was significantly shorter than that of Mig6+/− mice. The median survival of CCSPrtTA/TetO-EGFRLSRR/Mig6+/− mice after doxycycline induction was 13 days (both lines) compared with 60 line 57) to 100 days (line 56) for CCSPrtTA/TetO-EGFRLSRR/Mig6−/− mice (Fig. 1C and D). The median survival of CCSPrtTA/TetO-EGFRDL/Mig6+/− mice after doxycycline induction was 16 (line 9) to 45 days (line 11) compared with 143 (line 9) to 337 days (line 11) for CCSPrtTA/TetO-EGFRDL/Mig6−/− mice (Fig. 1E and F). Interestingly, the median survival of Mig6−/− mice expressing EGFRDL in EGFRDL L9 (line 9) was only 34.5 days after doxycycline induction and was significantly shorter than that of the Mig6+/− mice. A similar trend was observed in EGFRDL L11 (line 11) mice, although the EGFRDL-driven tumors appeared later than EGFRLSRR-driven tumors. Thus, the survival of mice with EGFRDL-induced tumors in a Mig6−/− background appears more curtailed than in a Mig6+/− background due to the longer latency of tumor induction by transgenic EGFRDL. We euthanized littermates with various genotypes 9 days after doxycycline induction to demonstrate the possible early appearance of tumors in Mig6−/− mice. Although there was mild hyperplasia of type II cells in Mig6+/− mice (Fig. 2A), Mig6−/− mice showed intermediate histopathology with increased type II hyperplasia, adenomas, and adenocarcinomas (Fig. 2B; Supplementary Fig. S2A; A–D), and Mig6−/− littermates exhibited dramatic effacement of lung alveoli with diffuse adenocarcinoma.
MIG6 Loss Potentiates Lung Tumorigenesis by Mutant EGFR

Figure 1. Accelerated initiation and progression of tumorigenesis and decreased overall survival in Mig6−/− mice. A, CC10mut/EGFRmut/Mig6+/+ and CC10mut/EGFRmut/Mig6−/− mice were treated with doxycycline (dox) to induce expression of the EGFRmut transgene, and serial MRI imaging was performed to monitor tumor initiation and progression. Representative images at 35 and 28 days of doxycycline induction show that there is more tumor burden at earlier time periods in CC10mut/EGFRmut/Mig6−/− mice. B, hematoxylin and eosin staining of lung tissue sections from CC10mut/EGFRmut/Mig6−/− mice treated with doxycycline for 107 and 22 days at survival endpoint. C–F, Kaplan–Meier survival curves of doxycycline-treated CC10mut/EGFRmut/Mig6−/−, CC10mut/EGFRmut/Mig6+/+, and CC10mut/EGFRmut/Mig6+/+ mice from line 57 (L57) (C) and line 56 (L56) (D), and CC10mut/EGFRmut/Mig6−/−, CC10mut/EGFRmut/Mig6+/+, and CC10mut/EGFRmut/Mig6+/+ mice from line 11 (L11) (E) and line 9 (L9) (F) show reduced survival of Mig6-deficient mice. The number (n) of mice used in each group for survival analysis is indicated in each graph. P value was calculated between two groups (double arrow) separately and shown in each panel; P < 0.05 indicates a significant difference in survival.

(33) We performed immunohistochemistry on lung tissue sections from Mig6−/−, Mig6+/+, and Mig6+/− mice 9 days following doxycycline induction, using antibodies against TTF1 (a type II epithelial cell marker and hence also a marker for lung cancer cells), EGFRmut, the proliferation marker Ki67, and phosphorylated ERK (pERK). There was an increase in TTF1, EGFRmut, Ki67, and pERK immunoreactive cells in both Mig6+/− and Mig6−/− mice, compared with the lungs of Mig6+/+ mice (Fig. 2D–O), confirming significantly increased lung tumor burden 9 days following doxycycline induction of mutant EGFRs in the Mig6-deficient background.

Loss of Mig6 cooperates with loss of Pten for endometrial cancer initiation and progression in a mouse model (37). This acceleration of tumorigenesis was shown to be due to the prevention of apoptosis. Normal mammary gland development in Mig6-null mice demonstrated that MIG6 promotes apoptosis in terminal end buds (38). Mig6 has also been shown to be an inducer of replicative or oncogene-induced senescence in fibroblasts (39–41). To further investigate whether mutant EGFR-driven tumor cells in Mig6−/− mice escape apoptosis and/or senescence, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and senescence-specific p19ARF staining (42, 43) of lung tissue sections from Mig6−/−, Mig6+/−, and Mig6+/+ mice 9 days after doxycycline induction. However, we did not observe any significant apoptosis or senescence in mutant EGFR–induced mouse lung tumorigenesis (Supplementary Fig. S2B, A–H), further confirming that the rapid progression of mutant...
EGFR-driven tumorigenesis is likely a result of increased proliferation as a result of increased ERK/MAPK pathway activation.

Because we observed that median survival of EGFR\textsuperscript{L858R} /Mig6\textsuperscript{−/−} mice is about 13 days, we performed hematoxylin and eosin (H&E) staining and immunohistochemistry on lung tissue sections from Mig6\textsuperscript{+/+}, Mig6\textsuperscript{+/−}, and Mig6\textsuperscript{−/−} mice 7 days following doxycycline induction. Staining with hematoxylin and eosin (H&E; A–C), TTF1 (D–F), EGFR\textsuperscript{L858R} (G–I), Ki67 (J–L), and pERK (M–O) shows significantly increased tumor burden in Mig6\textsuperscript{+/−} and Mig6\textsuperscript{−/−} mice. Scale bars, 100 μm.

Figure 2. Rapid progression of tumorigenesis at 9 days after doxycycline induction of mutant EGFR in Mig6\textsuperscript{+/−} and Mig6\textsuperscript{−/−} mice. Immunohistochemistry of lung tissue sections from CC10\textsuperscript{rtTA}/EGFR\textsuperscript{L858R}/Mig6\textsuperscript{+/+}, CC10\textsuperscript{rtTA}/EGFR\textsuperscript{L858R}/Mig6\textsuperscript{+/−}, and CC10\textsuperscript{rtTA}/EGFR\textsuperscript{L858R}/Mig6\textsuperscript{−/−} littermates after 9 days of doxycycline induction. Staining with hematoxylin and eosin (H&E; A–C), TTF1 (D–F), EGFR\textsuperscript{L858R} (G–I), Ki67 (J–L), and pERK (M–O) shows significantly increased tumor burden in Mig6\textsuperscript{+/−} and Mig6\textsuperscript{−/−} mice. Scale bars, 100 μm.
early, arguing against LOH (Supplementary Fig. S4). Hence, Mig6 is a haploinsufficient tumor suppressor for initiation and progression of tumorigenesis, at least in the context of expression of transgenic EGFR<sup>L858R</sup> in mice.

**Increased EGFR–MAPK Signaling in Mig6–Deficient Tumors**

To examine whether accelerated tumor growth in Mig6<sup>−/−</sup> mice is correlated with increased phosphorylation and activity of transgenic EGFR mutants, we measured the levels and phosphorylation status of mutant EGFR proteins in mouse lung tissue lysates and lung tissue sections by immunoblotting and immunohistochemistry. Early after doxycycline induction, cells reactive with TFF1 and EGFR<sup>L858R</sup> antisera were significantly more abundant in lungs from Mig6<sup>−/−</sup> and Mig6<sup>+/−</sup> as opposed to Mig6<sup>+/+</sup> mice (Fig. 2D–I), indicating early tumor initiation due to Mig6 deficiency. This was also associated with increased immunoreactivity to Ki67 and pERK, suggesting increased proliferation due to activation of the MAPK pathway (Fig. 2J–O). However, we found both by immunohistochemistry and Western blotting that EGFR<sup>L858R</sup> levels were paradoxically reduced in tumors collected at survival endpoint from Mig6–deficient animals (Fig. 3A and B).

When adjacent tumor sections were stained with anti-TFF1 and anti-EGFR antibodies, levels of EGFR<sup>L858R</sup> were significantly lower in TFF1<sup>+</sup> cells from EGFR<sup>L858R/Mig6<sup>−/−</sup></sup> mice than in those from EGFR<sup>L858R/Mig6<sup>+/−</sup></sup> mice (Fig. 3B).

To confirm this unexpected result, we immunoprecipitated EGFR from mouse tumor lysates and examined the immunoprecipitated EGFR with anti-pERK (Y1068), anti-EGFR<sup>L858R</sup>, and anti-EGFR antibodies (Fig. 3C and D). Although the levels of EGFR<sup>L858R</sup> were reduced in established tumors, the levels of pERK remained high; the ratio of pERK to total EGFR was higher in tumors from Mig6<sup>−/−</sup> mice than in tumors from Mig6<sup>+/−</sup> or Mig6<sup>+/+</sup> mice (Fig. 3D). We also observed decreased EGFR<sup>L858R</sup> levels in tumors from EGFR<sup>L858R/Mig6<sup>−/−</sup></sup> mice compared with protein levels in tumors from EGFR<sup>L858R/Mig6<sup>+/−</sup></sup> or EGFR<sup>Del</sup>/Mig6<sup>−/−</sup> mice at the survival endpoint (Fig. 3E). Likewise, as with Mig6<sup>−/−</sup> mice expressing the EGFR<sup>L858R</sup> transgene, the pERK/EGFR ratio was higher in the EGFR<sup>Del</sup>/Mig6<sup>−/−</sup> mice (Fig. 3F). Thus, the higher proportion of pERK appears to be sufficient for tumor maintenance in Mig6<sup>−/−</sup> mice.

We further measured components of the MAPK and PI3K signaling pathways in established tumors. Immunoblots of whole lung extracts from Mig6<sup>−/−</sup>, Mig6<sup>+/−</sup>, and Mig6<sup>+/+</sup> mice expressing either EGFR<sup>L858R</sup> or EGFR<sup>Del</sup> showed highly variable levels of pERK and pMEK. The level of pERK was higher in whole lung lysates from Mig6<sup>−/−</sup> mice than from Mig6<sup>+/−</sup> or Mig6<sup>+/+</sup> mice, particularly those with the transgene encoding EGFR<sup>L858R</sup> (Fig. 3G and H). Furthermore, immunohistochemistry demonstrated increased pERK reactivity in distinct, comparable areas of lung tumors in Mig6<sup>−/−</sup> mice compared with those from Mig6<sup>+/−</sup> mice (Fig. 3I–L). Interestingly, we observed increased levels of both p4EBP and 4EBP protein synthesis factor in Mig6<sup>−/−</sup> mice (Fig. 3G and H).

The reduced levels of mutant EGFR in established lung tumors at survival endpoint was surprising. We next determined whether this reduction in protein levels could be due to reduced levels of EGFR RNA. We examined whole-lung lysates for levels of Mig6, transgenic human EGFR, mouse Egfr, rtTA, and mouse Sfpcc RNAs in tumors obtained at the survival endpoint (Supplementary Fig. S5). We confirmed complete loss of Mig6 transcripts in Mig6<sup>−/−</sup> mice (Supplementary Fig. S5A). Although the transcript level of endogenous mouse Egfr was the same regardless of Mig6 status, at this late time-point, the levels of the human EGFR transgene (EGFR<sup>L858R</sup> or EGFR<sup>Del</sup>) RNAs were slightly lower in tumors from Mig6<sup>−/−</sup> mice (Supplementary Fig. S5B and S5C). This was accompanied by a slight decrease in transgenic rtTA RNA levels (Supplementary Fig. S5D) but no significant change in levels of Sfpcc RNA (Supplementary Fig. S5E). We also examined transcript levels in lung lysates obtained early (9 or 14 days) after doxycycline induction of mutant EGFRs and found no significant differences in EGFR<sup>L858R</sup> or rtTA expression at these early time points among mice with different Mig6 genotypes (Supplementary Fig. S6A–S6E).
EGFR:TTF1 ratio. The EGFR:TTF1 ratio was significantly lower in the band intensities for pY1068-EGFR and EGFR from the above experiment were quantified and plotted as pEGFR:EGFRD.

Immunohistochemical analysis of pERK expression performed on tumor tissue sections from I–L, against signaling components downstream of EGFR. Expression analyses of lung epithelial cell–specific prosurfactant C (Pro-SPC) and Rho-GDI (load-

Figure 3. Expression of mutant EGFR and downstream signaling components in tumors of mice at survival endpoint. A, immunoblot analysis of protein lysates from the lungs of Mig6+/+, Mig6−/−, and Mig6−/− mice expressing transgenic doxycycline (dox)-induced EGFRL858R. Lysates from mice designated as M1–M10 (Supplementary Table 1) were probed with M166, EGFRL858R, and Rho-GDI (control)–specific antibodies. B, immunohistochemical staining of tumor-bearing sections from the lungs of CC10+/+EGFRL858R/Mig6+/+ and CC10−/−EGFRL858R/Mig6−/− mice with EGFRL858R and TTF1–specific antibodies shows reduced expression of mutant EGFR in EGFRL858R/Mig6−/− mice. The intensities of LS58R and TTF1 were quantified and shown in the graph as EGFR.TTF1 ratio. The EGFR.TTF1 ratio was significantly lower in Mig6−/− mice than in Mig6+/+ mice. C, immunoprecipitation of tumor-bearing mouse lung lysates using EGFR antibody followed by immunoblotting with pY1068-EGFR, EGFRL858R (clone 18D1), and EGFR to detect the expression of phospho-EGFR and mutant EGFR. D, the band intensities for pY1068-EGFR and EGFR from the above experiment were quantified and plotted as pEGFR:EGFR ratios. The graph represents average value ± SE from 4 experiments. E, immunoblot analysis of tumor-bearing mouse lung lysates from Mig6+/+, Mig6−/−, and Mig6−/− mice expressing EGFRL858R using specific antibodies against Mig6, pY1068-EGFR, and EGFR. F, the band intensities for pEGFR and EGFR in the above experiment were quantified and plotted as pEGFR:EGFR ratio. The graph represents average value ± SE from 4 experiments. G and H, immunoblot analyses of lung extracts from Mig6+/+, Mig6−/−, and Mig6−/− mice expressing EGFRL858R (G) or EGFRDel (H) using both phospho-specific and total antibodies against signaling components downstream of EGFR. Expression analyses of lung epithelial cell–specific prosurfactant C (Pro-SPC) and Rho-GDI (loading control) were also performed. I–L, immunohistochemical analysis of pERK expression performed on tumor tissue sections from CC10+/+EGFRL858R/Mig6+/+ and CC10−/−EGFRL858R/Mig6−/− mice (J and K) and from CC10−/−EGFRL858R/Mig6+/+ and CC10−/−EGFRDel/Mig6−/− mice (J and L) showed increases in pERK immunoreactivity in Mig6−/− tumors. Scale bars, 100 μm.
MIG6 Loss Potentiates Lung Tumorigenesis by Mutant EGFR

**A** Lung adenocarcinoma cell lines

- Light
- Medium
- Heavy

Serum starved → EGF stimulated → TKI inhibited

Trypsin digestion followed by pTyr IP → LC/MS-MS pTyr-peptide quantification

**B** MIG6 peptide: VSSTHYLPERPPYLDKYEK (Y394/Y395)

- M/L: 1.2
- H/M: 0.2

Relative abundance

**C** MIG6 peptide: VSSTHYLPERPPYLDKYEK (Y394/Y395)

- H1975 (L858R/T790M EGFR)

Relative abundance

**D** MIG6 peptide: VSSTHYLPERPPYLDKYEK (Y394/Y395)

EGFR*E850K* mouse tumor lysate

Relative abundance

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**Figure 4.** Identification of Y394/Y395 phosphorylation in human lung adenocarcinoma cells and mutant EGFR-driven mouse lung tumors in vivo by mass spectrometry. **A**, B, SILAC-based quantitative phosphoproteomics reveals constitutive phosphorylation of MIG6 at Y394/Y395 and significant reduction of phosphorylation upon erlotinib treatment in TKI-sensitive lung adenocarcinoma cells, but not in TKI-resistant cells. **A**, schematic of experimental design for SILAC-based quantitative phosphoproteome analysis of lung adenocarcinoma cells. **B**, a representative MS spectrum of a MIG6 peptide from PC9 cells containing tyrosine 394/395 residues indicates that phosphorylation of MIG6 (Y394) is not altered in the presence of EGF, but in vivo phosphorylation of MIG6 at Y394.

We have demonstrated that MIG6 residues Y394 and Y395 are constitutively phosphorylated in mutant EGFR–expressing lung adenocarcinoma cells. Using *in vitro* kinase assays with purified proteins, others have shown that EGFR can directly phosphorylate MIG6 on tyrosines (44). A 77-amino acid region of MIG6 segment 1 (aa336–412) has been shown to be necessary for EGFR inhibition (31). The structural determinants of MIG6 required for binding to EGFR have been previously mapped to an EGFR-binding region (EBR), spanning residues 323 to 411 at the C-terminus of MIG6 protein (31, 45). The binding domain contains six tyrosine residues at positions 341, 358, 394, 395, 403, and 407, all of which have been phosphorylated in PC9 cells, whereas both Y394 and Y395 were phosphorylated in H1975 cells (Supplementary Fig. S7). To validate whether Y394/Y395 is the major site of MIG6 phosphorylation *in vivo*, we carried out phosphoproteomic analysis of pTyr peptides from tumor lysates of *EGFR*E850K transgenic mice and identified the MIG6 phosphopeptide with phosphorylation at the Y394 residue (Fig. 4D). Using purified proteins for *in vitro* kinase assays, it has been shown that EGFR can phosphorylate MIG6 directly (44). Our results in this study and in human bronchial epithelial cells (HBEC) expressing mutant EGFRs (24) provide *in vivo* evidence consistent with three conclusions: that MIG6 is a direct target of mutant EGFR; that Y394 and Y395 are sites constitutively phosphorylated by mutant EGFRs; and that erlotinib inhibits such phosphorylation in TKI-sensitive cells, but not TKI-resistant cells.

Phosphorylation of MIG6 at Y394 and Y395 Residues Promotes the Interaction of MIG6 and EGFR

We have demonstrated that MIG6 residues Y394 and Y395 are constitutively phosphorylated in mutant EGFR–expressing lung adenocarcinoma cells. Using *in vitro* kinase assays with purified proteins, others have shown that EGFR can directly phosphorylate MIG6 on tyrosines (44). A 77-amino acid region of MIG6 segment 1 (aa336–412) has been shown to be necessary for EGFR inhibition (31). The structural determinants of MIG6 required for binding to EGFR have been previously mapped to an EGFR-binding region (EBR), spanning residues 323 to 411 at the C-terminus of MIG6 protein (31, 45). The binding domain contains six tyrosine residues at positions 341, 358, 394, 395, 403, and 407, all of which have...
Tyrosine phosphorylation of MIG6 Y394/Y395 is critical for its interaction with EGFR. A, HEK293 cells were transiently cotransfected with wild-type (WT) or L858R EGFR, together with either WT or Y394/Y395F MIG6-expressing plasmids. Cells were serum starved for 18 hours and then treated with 100 ng/mL EGF for 10 minutes. Cell lysates were immunoprecipitated with anti-EGFR antibody and analyzed on Western immunoblots for total and phosphorylated (4G10) EGFR and MIG6 levels. B and C, H322M cells, stably expressing WT or mutant MIG6, were transiently transfected with either WT or L858R EGFR. B, cell lysates were immunoprecipitated with EGFR and analyzed on Western immunoblots for EGFR and MIG6. C, cell lysates were immunoprecipitated with FLAG antibody (MIG6-FLAG) and analyzed on Western immunoblots for total (FLAG) and phosphorylated (4G10) MIG6. Input lysates were immunoblotted with EGFR, MIG6, or Rho-GDI.

been shown to be phosphorylated in mass spectrometry-based experiments (data from Phosphosite Plus; refs. 24, 46). The crystal structure of the EGFR kinase domain bound to part of segment 1 of MIG6 indicates that Y358 resides in the binding interface, and mutation of this residue to alanine (Y358A) disrupts binding (31). Frosi and colleagues (32) also showed that, unlike wild-type (WT) MIG6, the Y358A mutant failed to promote endocytosis of EGFR, indicating that the Y358 residue is important for MIG6 function. However, Y358 is not a major site of phosphorylation in vivo. Moreover, the effect of phosphorylation of the major sites, Y394/Y395, on MIG6 function has not been studied in detail. Recently, using purified proteins and in vitro kinase assays, it has been shown that phosphorylation of MIG6Y394 reduces the inhibitory function of MIG6 on the EGFR kinase (47). We postulate that phosphorylation of Y394/Y395 and other tyrosine sites within the EBR domain of MIG6 affects the binding of EGFR and MIG6 and regulation of EGFR kinase activity by MIG6.

To determine whether phosphorylation of MIG6 at Y394 and Y395 is important for the interaction of MIG6 with EGFR, we replaced these tyrosines with phenylalanine to mimic unphosphorylated tyrosine. Expression vectors containing WT or mutant MIG6 cDNAs were cotransfected into HEK293 cells with vectors containing WT EGRF, EGRF<sup>L858R</sup>, or an empty vector as a control. After serum starvation for 18 hours, some cultures were stimulated with EGF for 10 minutes. Cell extracts were examined for interacting proteins by immunoprecipitation with EGFR-specific antibodies, followed by Western blotting with second antibodies against MIG6 and other proteins (Fig. 5A). These studies indicated that MIG6 interacts with both WT and mutant EGFR. EGFR stimulation increased the interaction of MIG6 with WT EGFR. Furthermore, MIG6 interacted more efficiently with EGFR<sup>L858R</sup> than with WT EGFR in unstimulated cells, and the increase was associated with increased Tyr phosphorylation of MIG6. More importantly, mutation of residues Y394/Y395 to phenylalanine, abolishing phosphorylation of these sites, impaired the ability of MIG6 protein to bind both WT and mutant EGFR proteins. However, it is possible that Y-F mutants may affect hydrogen-bonding interactions due to the loss of a hydroxyl in the phenylalanine, and hence influence interactions beyond just the loss of phosphorylation.

Because HEK293 cells express endogenous MIG6, we also performed coimmunoprecipitation studies in H322M cells, a lung adenocarcinoma cell line that does not express detectable levels of endogenous MIG6 because of a homozygous MIG6 nonsense mutation, E83Stop (30). H322M cells stably producing WT or Y-F MIG6 mutants were transiently transfected with either WT EGFR or EGFR<sup>L858R</sup> expression vectors and analyzed by EGFR coimmunoprecipitation assays (Fig. 5B). These experiments demonstrated a reduced interaction of MIG6 Y-F mutants with both WT EGFR and EGFR<sup>L858R</sup>, except for the MIG6<sup>Y394/F</sup> mutant. To examine the overall tyrosine phosphorylation of MIG6, we immunoprecipitated MIG6 from lysates of H322M cells transfected with WT EGFR or EGFR<sup>L858R</sup> and probed the blots with pTyr and
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Figure 6. Delayed degradation of EGFR<sub>L858R</sub> upon EGF stimulation in the presence or absence of wild-type (WT) or Y394/Y395F-mutant MIG6. A, HBECs with endogenous WT EGFR or stably transduced L858R EGFR expression were grown in serum-free medium for 18 hours followed by cycloheximide (100 μmol/L) treatment for 1 hour to inhibit new protein synthesis, and then treated with 100 ng/mL of EGF for indicated time points. RIPA cell lysates were immunoblotted with EGFR (clone 13), EGFR<sub>L858R</sub>-, and EGFR<sub>L858</sub>-specific antibodies to detect total, mutant, and corresponding WT EGFR. Rho-GDI-specific antibody was used as a loading control. B, HEK293 cells stably expressing WT or L858R EGFR alone or together with WT or Y394/Y395F MIG6 were serum starved for 18 hours and treated with 100 μmol/L of cycloheximide for 1 hour followed by 100 ng/mL of EGF for indicated time points to induce receptor degradation. Lysates from treated cells were immunoblotted and probed with specific antibodies against EGFR and MIG6. Alpha-tubulin-specific antibody was used to probe cell lysates for loading control. C, the band intensities for EGFR in the above experiment were quantified and plotted as a percentage of EGFR retained following EGF treatment. The graph represents average value ± SD from 3 experiments. D and E, stably transduced HEK293 cells, as described above, were serum starved for 18 hours and treated with cycloheximide (100 μmol/L). After 45 minutes, chloroquine (100 μmol/L) was added, and 15 minutes later, EGF (100 ng/mL) was added for the indicated time points. Lysates from stably transduced WT EGFR-expressing cells were immunoblotted with EGFR<sub>L858R</sub>-specific antibodies (D), and those expressing EGFR<sub>L858R</sub> were immunoblotted with EGFR<sub>L858</sub>-specific antibodies (E). All lysates were also probed with EGFR, MIG6, and Rho-GDI-specific antibodies. F, H322M cells stably expressing WT or Y394/Y395F MIG6 and endogenous WT EGFR were serum starved for 18 hours and treated with 100 μmol/L of cycloheximide for 1 hour followed by 100 ng/mL of EGF for indicated time points to induce receptor degradation. Lysates from treated cells were immunoblotted with EGFR and MIG6 antibodies, and Rho-GDI-specific antibody was used as a loading control.

FLAG (MIG6) antibodies. There was increased tyrosine phosphorylation of MIG6 in EGFR<sup>L858R</sup>-expressing cells. Y394/Y395 were again demonstrated to be the predominant sites of tyrosine phosphorylation in MIG6 (Fig. 5C).

MIG6 Does Not Promote Degradation of Mutant EGFR, and May Also Stabilize Activated WT EGFR

Previous studies have shown that EGF promotes activation-dependent endocytosis and degradation of EGFR, potentially regulating the duration of downstream signaling (48, 49). However, mutant EGFRs are ineffectively internalized (50, 51) and exhibit diminished downregulation following ligand activation (52, 53). To assess differences in the degradation of WT EGFR and mutant EGFR, we first used isogenic HBECs. Lysates from HBECs treated with EGF and cycloheximide for 0 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours were immunoblotted with antibodies that specifically recognize pan-EGFR (both WT and L858R EGFR), EGFR<sup>L858R</sup>, and EGFR<sup>L858</sup> (epitope surrounding the L858 residue in WT EGFR; Supplementary Fig. S8). Substantial degradation of WT EGFR occurred within 2 hours of EGF-induced activation of EGFR. In contrast, degradation of EGFR<sup>L858R</sup> was significantly reduced in HBECs expressing the mutant EGFR (Fig. 6A), indicating that EGFR<sup>L858R</sup> is more resistant to degradation than WT EGFR upon EGF stimulation.

MIG6 inhibits EGFR kinase activity and promotes WT EGFR trafficking to the degradation pathway (29, 32). Kinetic modeling based on EGFR endocytosis experiments...
performed on lung adenocarcinoma cells expressing WT and mutant (Del 746–750) EGFRs has suggested that MIG6 promotes WT EGFR, but not mutant EGFR, internalization (54, 55). To investigate the effect of MIG6 on EGFR-induced degradation of EGFR, we performed an EGFR degradation assay with cycloheximide on HEK293 cells stably transfected with WT or Y394/Y395F MIG6, together with WT EGFR or EGFRL858R (Fig. 6B) and quantified retained EGFRs (Fig. 6C). As predicted, WT EGFR was degraded upon EGF stimulation. However, there was little effect of EGFR on mutant EGFR degradation. Furthermore, expression of MIG6 had no effect on the degradation of mutant EGFR, and it slowed the degradation of activated WT EGFR. To ascertain whether WT EGFR degradation utilizes the lysosomes, we performed these degradation experiments in the presence of chloroquine, a lysosomotropic inhibitor. We observed that chloroquine significantly delayed degradation of WT EGFR (Fig. 6D). However, there was no additional effect of chloroquine on the retention of EGFRL858R, suggesting that the lysosomal pathway is utilized for WT EGFR, but probably not for mutant EGFR degradation (Fig. 6E). We further performed these degradation assays in WT or Y394/Y395F MIG6-transduced H322M lung adenocarcinoma cells that contain WT EGFR and no endogenous MIG6. WT EGFR was efficiently degraded upon EGF stimulation in the absence or the presence of Y394/Y395F MIG6; however, WT EGFR appeared more stable in the presence of MIG6, findings similar to those observed in HEK293 cells (Fig. 6F).

**DISCUSSION**

MIG6 is known to inhibit EGFR kinase activity and promote the degradation of WT EGFR, so it is considered a suppressor of tumors with active WT EGFR signaling. However, the role of MIG6 in regulating lung cancer–specific mutant EGFRs has not been studied in detail. Here, we show for the first time that Mig6 deficiency, even haploinsufficiency, accelerates the initiation and progression of tumorigenesis and lethality driven by mutant EGFR in mouse models. We have previously shown in isogenic HBECs that mutant EGFR enhances tyrosine phosphorylation of MIG6 more efficiently than WT EGFR (24). Here, we demonstrate constitutive phosphorylation of MIG6 at Y394/Y395 in lung adenocarcinoma cells harboring EGFR mutations. Phosphorylation at these sites is inhibited by erlotinib in TKI-sensitive, but not TKI-resistant, cells, suggesting that mutant EGFRs directly phosphorylate and possibly regulate MIG6. This conclusion is supported by published evidence that purified EGFR protein can directly phosphorylate MIG6 in vitro (44). We have further examined the functional consequences of tyrosine phosphorylation of Y394/Y395 on MIG6 and how this affects its tumor suppressor function. We show that phosphorylation increases binding of MIG6 to mutant EGFRs; but, in contrast to WT EGFR, the increased interaction does not direct mutant EGFR to the degradation pathway. Our observation is consistent with another study in which MIG6 was shown to be a poor inhibitor of the kinase activity of nearly full-length mutant EGFR in vitro (44). A phosphorylated Y394-containing fragment of MIG6 was also shown to be a poor kinase inhibitor of WT EGFR compared with its unphosphorylated counterpart using kinase assays with purified proteins in vitro (47). Taken together, our findings of constitutive phosphorylation of MIG6 Y394/Y395 in lung adenocarcinoma cell lines and the increased binding of MIG6 with mutant EGFRs (possibly leading to stability of mutant EGFR), along with published in vitro studies of inadequate EGFR kinase inhibition by phosphorylated MIG6, provide strong evidence that mutant EGFRs can partially circumvent inhibition by MIG6 in lung adenocarcinoma cells through tyrosine phosphorylation of MIG6 on key residues. However, most importantly, we show that the residual inhibitory activity of MIG6 is still tumor suppressive in mutant EGFR-driven lung tumor models, because MIG6 deficiency reduces survival of mice due to accelerated tumorigenesis. This was not expected based on the results of enzmologic studies published to date (44, 47). It is also possible that MIG6 is capable of inhibiting the formation of heterodimers of mouse WT EGFR and human mutant EGFR, a likely scenario in the early stages of doxycycline induction in our mouse model, especially when levels of transgenic mutant EGFR are low. A schematic of our model of inhibitory activity of MIG6 against WT and mutant EGFRs is depicted in Fig. 7A and B.

The accelerated initiation and progression of mutant EGFR–driven tumorigenesis in Mig6-deficient background was quite striking. The manifestation of early tumorigenesis was dramatic in the early time periods of doxycycline induction of mutant EGFRs. There was almost complete effacement of normal alveoli by type II cells 7 to 9 days after doxycycline induction in Mig6−/− mice, the earliest times at which tissue was analyzed in this study, at a time when only focal type II cellular hyperplasia was observed in Mig6+/+ littermates. Increased proliferation was associated with increased pERK immunoreactivity in the lungs of Mig6−/− mice. Interestingly, our experiments also demonstrate a statistically significant difference in the survival of Mig6−/− compared with Mig6+/+ mice in the presence of an EGFRL858R transgene, at least in line 9, with a strong trend toward decreased survival in EGFRL858R line 11 mice. In two EGFRL858R mouse lines, we saw a statistically insignificant trend toward decreased survival for Mig6−/− mice. The EGFRL858R mice develop tumors much later than EGFRL858R mice, which may allow for the difference in survival between the Mig6−/− and Mig6+/+ mice to manifest. We did not observe Mig6 LOH in a select group of Mig6−/− mice we studied. However, it is still possible that there could be LOH in individual mice that we did not analyze. The fact that there is strong evidence of Mig6 being a haploinsufficient tumor suppressor in our studies has implications in mutant EGFR-driven human lung adenocarcinoma biology. A recent study evaluated the relative expression level of Mig6 and EGFR in a small cohort of patients with lung cancer treated prospectively with gefitinib. This study concluded that a lower Mig6:EGFR ratio is associated with sensitivity to TKIs, whereas a higher Mig6:EGFR ratio is a predictor of TKI resistance (56). In another study, the ratio of Mig6 and miR200c RNA levels correlated with EMT and resistance to erlotinib (57). However, these studies were performed primarily with patients harboring WT EGFR. Further prospective clinical studies are warranted to ascertain whether absolute Mig6 levels can influence the initiation, progression, and EGFR-TKI response in mutant EGFR-driven human lung adenocarcinoma.
MIG6 Loss Potentiates Lung Tumorigenesis by Mutant EGFR

We saw comparable induction of steady-state levels of transgenic mutant EGFR transcripts in the early time periods of doxycycline induction, regardless of MIG6 status. However, there were more TTF1 and EGFRL858R-immunostained cells at these time periods, consistent with enhanced stimulation of growth by mutant EGFR and early tumorigenesis in Mig6−/− transgenic mice. We demonstrated that the low level of transgenic EGFR protein was still hyperphosphorylated in these late-stage tumors, and the pEGFR:EGFR ratio was higher in tumors from Mig6−/− mice than in tumors from Mig6+/− mice. We demonstrated that the low level of transgenic EGFR protein was still hyperphosphorylated in these late-stage tumors, and the pEGFR:EGFR ratio was higher in tumors from Mig6−/− mice than in tumors from Mig6+/− mice, suggesting that the residual mutant EGFR activity was sufficient to maintain these aggressive tumors. We did not find any difference in the mutant EGFR and rTA transcript levels at earlier time periods. However, there was a slight decrease of rTA and mutant EGFR transcript expression in the late-stage tumors. This could be a result of decreased CCSP promoter activity in the lungs of older Mig6−/− mice, reducing mutant EGFR transcripts. This is corroborated by the fact that MIG6 is essential for normal lung development (34). However, the modest decrease in rTA or mutant EGFR mRNA does not explain the reduced levels of mutant EGFR protein. We speculate that during the progression of tumorigenesis, there is selection for lower transgenic EGFR-expressing cells. However, the residual mutant EGFR signal strength is still enough to maintain these aggressive tumors. We also speculate that MIG6 inhibits heterodimers of mutant and WT EGFR and promotes their degradation in the early stages of doxycycline induction of mutant EGFRs in these models, thus explaining the dramatic tumor-suppressive role. At later stages, because of increased transgenic mutant EGFR levels, the mutant EGFRs exist predominantly as homodimers. MIG6 is unable to traffic these homodimers to degradation pathways because of increased feedback tyrosine phosphorylation; instead, MIG6 binds more strongly and stabilizes mutant EGFR homodimers at this stage. Hence, MIG6 deficiency results in lower levels of transgenic mutant EGFRs in end-stage tumors.

We postulate a two-pronged mechanism by which mutant EGFRs dampen inhibition by MIG6; one acts to regulate the levels and the other modulates the function of MIG6. The first is by a downregulation of MIG6 protein levels similar to the regulation of a classic tumor suppressor. In a recent study, lung cancer–specific EGFR mutations correlated with loss of MIG6 protein; 12 of 16 EGFR-mutant tumors lacked MIG6 protein (58). At least one lung adenocarcinoma cell line, H322M, harbors a homozygous nonsense mutation in MIG6, with undetectable MIG6 protein in the context of WT EGFR expression (30). Around 50% of primary glioblastoma multiforme (GBM) tumor samples and cell lines have reduced MIG6 RNA and protein expression (29). MIG6 levels may also be regulated by epigenetic mechanisms. MIG6 promoter methylation was observed in 79% of papillary thyroid...
The second mechanism of reduced MIG6 inhibitory function is the increased tyrosine phosphorylation of Y394/Y395 by mutant EGFRs, leading to decreased kinase inhibition (44, 47) and increased constitutive binding of MIG6 to mutant EGFRs, possibly stabilizing mutant EGFRs presented in this study. Recently, Ying and colleagues (29) also observed that the EGFRvIII mutant does not undergo MIG6-mediated endocytosis and degradation in lysosomes of GBM cell lines, unlike WT EGFR. A recent study showed a modest decrease in EGFR internalization upon MIG6 knockdown in PC9 cells, a lung adenocarcinoma cell line harboring the EGFRvIII mutant (54). The study by Walsh and colleagues does not distinguish between mutant EGFR and WT EGFR in PC9 cells, which are heterozygous for the EGFRvIII mutant. Furthermore, we also noticed that mutant EGFRs could be effectively internalized into early endosomes in HBECs (Supplementary Fig. S9A–B). Interestingly, we found strong colocalization of mutant or WT EGFR and MIG6 in discrete vesicles upon EGF stimulation of HBECs. However, there was less colocalization of mutant EGFR compared with WT EGFR and LAMP1, a lysosomal marker even after 2 hours of EGF stimulation, suggesting that mutant EGFR may not traffic through the lysosomal degradation pathway (Supplementary Fig. S9C–D). Our data suggest that mutant EGFR degradation is inhibited in spite of the increased interaction of MIG6 and mutant EGFRs (Fig. 6 and Supplementary Fig. S9).

Prospective biomarker-validation studies are warranted to establish the role of MIG6 expression or phosphorylation in the overall prognosis of patients harboring WT EGFR or mutant EGFRs. Such clinical studies are needed to ascertain whether absolute MIG6 levels can influence the initiation, progression, and EGFR-TKI response in mutant EGFR-driven lung adenocarcinoma.

**METHODS**

Additional methods are described in the Supplementary Materials and Methods section.

**Reagents and Antibodies**

RPMI and DMEM tissue culture media and FBS were obtained from Invitrogen. Defined FBS for H3255 adenocarcinoma cell culture was obtained from HyClone. All chemicals were obtained from Sigma-Aldrich, unless stated otherwise. Egg-crateGENE 9 DNA transfection reagent, complete mitatib protease inhibitor, and PhosStop phosphatase inhibitor were obtained from Roche Applied Science. Nitrocellulose Western transfer sandwich was obtained from Invitrogen, and nitrocellulose membrane was obtained from GE Healthcare Life Sciences. EGF was obtained from Millipore and Peprotech. The tyrosine kinase inhibitor erlotinib was obtained from Sigma-Aldrich and p190Rho-specific antibodies were obtained from Abcam. TUNEL staining was performed using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore).

**Cell Lines**

H1975 and HEK293 cell lines were purchased from the ATCC, the PC9 cell line was obtained from the Varmus Laboratory, and the H322M cells were obtained from the Division of Cancer Treatment and Diagnosis (DCTD) Tumor Cell Line Repository (NCI, Frederick, MD). All human lung adenocarcinoma cells were maintained in RPMI supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. The human embryonic kidney cell line HEK293 was cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. The HBECs were a kind gift from Dr. John D. Minna (University of Texas Southwestern, Dallas, TX), and were maintained in keratinocyte serum-free medium (Invitrogen) supplemented with bovine pituitary extract (BPE) and EGF. Cells were authenticated by short tandem repeat (STR) profiling using the AmpFISTR Identifiler kit at the Protein Expression Laboratory (NCI, Frederick, MD) in February 2015.

**Plasmids**

Site-directed mutagenesis of human EGFR and MIG6 and subcloning of wild-type and mutant constructs for lentivirus production were performed at the Protein Expression Laboratory, a Frederick National Laboratory for Cancer Research (FNLCR) core facility.

**Cell Extract and Mouse Tissue Extract Preparation, Immunoprecipitation, and Immunoblot Analysis**

Tissue culture or mouse tissue lysates used for immunoblot were prepared in RIPA lysis buffer (150 mmol/L NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/L Tris, pH 8.0). For immunoprecipitation, cell extracts were prepared in NP-40 lysis buffer. Mouse tissue extracts were prepared in RIPA buffer using a tissue lyser (Qiagen) following the manufacturer’s protocol. For phosphoproteomic analysis, mouse tissue extracts were prepared in urea lysis buffer (20 mmol/L Hepes pH 8.0, 9 mol/L urea, 1 mmol/L sodium orthovanadate, 2.5 mmol/L sodium pyrophosphate, and 1 mmol/L β-glycerophosphate). All lysis buffers contained protease and phosphatase inhibitor cocktails from Roche, and 1 mmol/L sodium orthovanadate to inhibit protease and phosphatase activities. Protein concentrations were quantified using a modified Lowry method (BioRad). For immunoprecipitation, 800 to 1,000 µg of lysate was incubated overnight at 4°C with 2 to 5 µg of mouse anti-EGFR (MAB108) or mouse anti-Flag (MIG6) monoclonal antibody. The antigen–antibody complex was then captured by incubating the mixture with protein G beads for an additional 1 hour. The immunocomplexes were washed with NP-40 lysis buffer twice and once with PBS buffer containing 1 mmol/L sodium orthovanadate. The bound proteins were then extracted with 2× SDS loading buffer [6.8 mmol/L Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol] by incubating at 95°C for 5 minutes and fractionated by SDS-PAGE (4%–15%). The proteins were transferred to nitrocellulose membrane using either the semidyry or wet transfer method, and probed with the specified antibody.
EGFR Degradation Assays

Cells were serum starved for 18 hours and treated with 100 μmol/L cycloheximide (Sigma-Aldrich) for 1 hour to inhibit new protein synthesis. Cells were either mock stimulated or stimulated with EGF (100 ng/mL) for different time points at 37°C. Following EGF treatment, the cells were quickly chilled and washed with cold PBS, and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors, as previously described. For the degradation assay in the presence of chloroquine, cells were serum starved and treated with cycloheximide as described above. Fifteen minutes before EGF addition, cells were treated with or without 100 μmol/L of chloroquine. Extracts for Western blot analysis were prepared at various time points after EGF stimulation.

Mouse Strains

The doxycycline-inducible EGFRloxPlox and EGFRloxΔlox transgenic models have been described previously (35). All mice were maintained in a pathogen-free facility approved by the NCI and Memorial Sloan Kettering Cancer Center (MSKCC) Animal Care and Use Committees (ACUC). Animal studies were carried out with the approval of research protocols by the ACUC. We bred Mig6+/− mice with CCSPrtTA mice, and Mig6+/− mice with TetO-EGFRloxΔlox mice. Resulting Mig6lox+/−/CCSPrtTA and Mig6lox+/−/TetO-EGFRloxΔlox offspring were then crossed to generate mutant EGFR-expressing mice in a Mig6 wild-type (Mig6+/+), Mig6 heterozygous (Mig6+/−), or Mig6-null (Mig6−/−) background.

Tumor Monitoring

Transgenic mice were fed with doxycycline-impregnated food pellets (625 ppm; Harlan-Teklad) to induce mutant forms of human EGFR from a doxycycline-regulated promoter. Mice were monitored for EGFR-driven tumor development by MRI of the lungs. MRI was carried out with respiratory gating at the MRI Core Facility of NCI or MSKCC. Serial MRI analyses were performed, and the tumor burden was quantified by ImageJ software. Regions of interest tool (ROI) was used to outline the lung and tumor within the lung. ROI measurements provided the area and mean intensity of lung and tumor. For each MRI time point, the percentage of tumor burden was calculated from the total lung and tumor measurements obtained. Mice were selected to be euthanized for survival analysis primarily using clinical criteria such as hunched posture, trachypnea, weight loss, and decreased movement. Lungs were perfused with PBS, and a representative portion of the tumor tissue was frozen in liquid nitrogen for further analysis. Another representative lung tumor tissue was processed by perfusion with phosphate-buffered 4% paraformaldehyde for histopathology evaluation.

Statistical Analysis

For replicate experiments, SD or SE was calculated to indicate the variation between experiments, and values given represent the mean ± SD. Statistical analyses of the results to assess the significance of differences were performed using an unpaired Student t test. A threshold of P ≤ 0.05 was used for significance. Kaplan-Meier survival analyses were performed on tumor-bearing mice using Graph-Pad Prism.

Disclosure of Potential Conflicts of Interest

R.M. Simpson is president-elect at the American College of Veterinary Pathologists. K. Politi reports receiving a commercial research grant from AstraZeneca, has ownership interest (including patents) in Molecular MD/MSKCC, and is a consultant/advisory board member for Takeda. No potential conflicts of interest were disclosed by the other authors.

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