Transcription factor Etv5 is essential for the maintenance of alveolar type II cells

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Alveolar type II (AT2) cell dysfunction contributes to a number of significant human pathologies including respiratory distress syndrome, lung adenocarcinoma, and debilitating fibrotic diseases, but the critical transcription factors that maintain AT2 cell identity are unknown. Here we show that the E26 transformation-specific (ETS) family transcription factor Etv5 is essential to maintain AT2 cell identity. Deletion of *Etv5* from AT2 cells produced gene and protein signatures characteristic of differentiated alveolar type I (AT1) cells. Consistent with a defect in the AT2 stem cell population, *Etv5* deficiency markedly reduced recovery following bleomycin-induced lung injury. Lung tumorigenesis driven by mutant KrasG12D was also compromised by Etv5 deficiency. ERK activation downstream of Ras was found to stabilize Etv5 through inactivation of the cullin-RING ubiquitin ligase $CRL4^{COP1/DET1}$ that targets Etv5 for proteasomal degradation. These findings identify Etv5 as a critical output of Ras signaling in AT2 cells, contributing to both lung homeostasis and tumor initiation.

lung cancer | Ras | Etv5 | Cop1

Alveolar type II (AT2) cells are a stem cell population that self renews and differentiates into alveolar type I (AT1) cells during lung homeostasis or in response to injury (1, 2). They also give rise to lung adenocarcinoma induced by oncogenic Kras (1, 3–5). AT2 cells are derived from bipotent progenitor cells at the sacculation stage (1). In the adult lung, mature AT2 cells also secrete surfactant phospholipids to maintain normal alveolar function. Sustained Kras activity stimulates the self-renewal of AT2 cells, which eventually leads to abnormal tissue growth (1). An AT2 gene-expression signature has been reported (6, 7), but it is unclear how the identity of AT2 cells is specified and maintained.

Given that Ras/MAPK signaling is essential for the self-renewal of AT2 cells and for the initiation of lung adenocarcinoma, identification of the transcription factors that are activated by Ras in AT2 cells is a step toward defining the transcriptional programs underlying AT2 stemness. The PEA3 subgroup of the ETS family of transcription factors, comprised of ETS transcription variants 1, 4, and 5 (Etv1, Etv4, and Etv5) (8), is known to be engaged by Ras/MAPK signaling. *Etv4* and *Etv5* are expressed in distal lung epithelium during development and are involved in branching morphogenesis and epithelial cell differentiation (9–11). Alveolar epithelial cells in the adult lung also express *Etv5* (7, 12), but a critical role for Etv5 in this setting remains elusive.

PEA3 transcription factors are also implicated in tumorigenesis. Overexpression of ETV1 or ETV4 has been linked to prostate cancer (13, 14), and stabilization of ETV1 by mutant, active tyrosine kinase receptor KIT is thought to drive an oncogenic program in gastrointestinal stromal tumors (GIST) (15). ETV1, ETV4, and ETV5 are labile proteins because of their regulation by the ubiquitin–proteasome system. In GIST, ETV1 protein is stabilized by activation of the Ras/MAPK pathway through an unknown mechanism. In prostate cancers, most ETV1 mutants lack the region required for interaction with COP1 (also called "RFWD2"), which is the substrate adapter of the E3 ubiquitin ligase $CRL4^{COP1/DET1}$. Consequently, these ETV1 mutants escape COP1-mediated ubiquitination and proteasomal degradation (14). These findings suggest that stabilization of PEA3 transcription factors is critical for their oncogenicity.

In addition to ETV1, ETV4, and ETV5, $CRL4^{COP1/DET1}$ substrates include c-Jun (16), C/EBPα (17), and ETS1/2 (18). COP1 binds to DET1, which in turn engages the DDB1–CUL4A–RBX1 core complex that associates with an E2 ubiquitin-conjugating enzyme (16). Conserved in plants, COP1 represses photomorphogenesis until exposure to light causes COP1 to be excluded from the nucleus and apart from its nuclear substrates (19). In mammals, COP1 controls lung-branching morphogenesis (20) and functions as a tumor suppressor by targeting c-JUN or ETV1 for degradation (14, 21). How vertebrate $CRL4^{COP1/DET1}$ is regulated is less clear.

Here we show that Etv5 is expressed specifically in AT2 cells and is stabilized when $CRL4^{COP1/DET1}$ is inhibited by ERK signaling. Genetic deletion of *Etv5* in AT2 cells revealed that Etv5 is essential for maintaining AT2 cell identity. As a consequence, Etv5 loss impairs lung recovery from bleomycin-induced

Significance

Alveolar type II (AT2) cells are a stem cell population in the lung contributing to the repair of alveolar damage and the formation of Ras-induced lung adenocarcinoma. Here we show that a critical output of Ras signaling in AT2 cells is inactivation of the ubiquitin ligase COP1, resulting in stabilization of the transcription factor ETV5. Etv5 deficiency markedly reduced mouse lung hyperplasia driven by mutant KrasG12D or lung repair following bleomycin-induced lung injury, indicating that Etv5 contributes to both tumor initiation and lung homeostasis. Deletion of *Etv5* from AT2 cells expressing KrasG12D produced a gene and protein signature characteristic of differentiated AT1 cells, suggesting that ETV5 is critical for the maintenance of AT2 cell identity.

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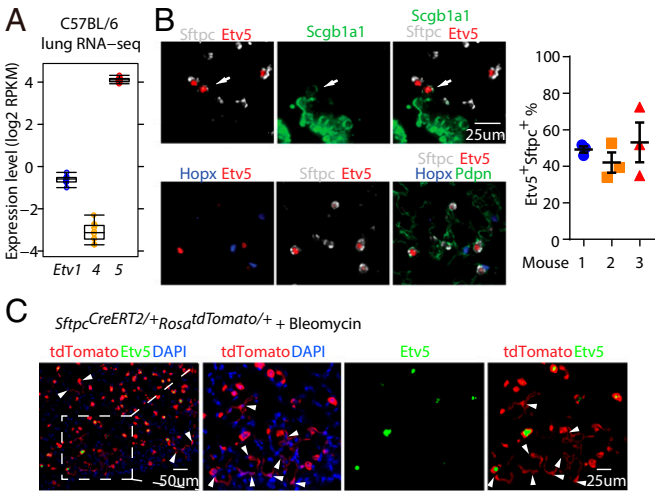

Fig. 1. Expression of Etv5 in AT2 cells in adult lung. (*A*) *Etv1*, *Etv4*, and *Etv5* gene expression in C57BL/6 mouse lung ($n = 10$). (*B*, *Left*) Immunostaining of wild-type mouse lung. The arrow indicates a Sftpc$^+$Scgb1a1$^+$Etv5$^+$ cell. (*Right*) Percentage of Sftpc$^+$Etv5$^+$cells in total Sftpc$^+$ cells counted in three wild-type mice. Data are shown as mean ± SEM. (*C*) *Sftpc*$^{CreERT2/+}$*Rosa*$^{tdTomato/+}$ lung after dosing with tamoxifen for 5 d and then s.c. dosing with bleomycin for 1 wk. Arrowheads point to the nuclei of AT2-derived AT1 cells.

damage and lung tumor initiation by oncogenic Kras. Therefore, Etv5 stabilization is a critical output of Ras signaling in AT2 cells and contributes to both lung homeostasis and tumor initiation.

Results

Etv5 Protein Is Expressed in AT2 Cells in Adult Lung. *Etv5* mRNA is more abundant than *Etv1* or *Etv4* mRNA in normal mouse lung (Fig. 1*A*). *Etv5* mRNA has been reported in AT2 cells (1, 7), but mRNA expression does not always correlate with the abundance of labile proteins such as Etv5 (22). By immunostaining, we detected Etv5 protein in a subset of AT2 cells expressing surfactant protein c (Sftpc) and in cells expressing both Sftpc and the secretoglobin Scgb1a1 (Fig. 1*B*). In contrast, Etv5 was not detected in bronchiole or AT1 cells expressing Hopx1 or Pdpn (Fig. 1*B*).

The differentiation of AT2 cells into the AT1 cells that mediate pulmonary gas exchange is critical for replacing damaged AT1 cells after lung injury. To evaluate AT2 cell differentiation following bleomycin-induced lung injury, we performed lineage tracing using *Sftpc*^{CreERT2}*Rosa*^{tdTomato} mice that exclusively express tdTomato in their Sftpc$^+$ AT2 cells (23, 24). We identified tdTomato$^+$ AT1 cells that were derived from AT2 cells based on their elongated, flattened cell morphology, but they did not express Etv5 (Fig. 1*C*). Therefore, Etv5 expression is a defining feature of AT2 cell identity in both lung homeostasis and repair.

Etv5 Is Required to Maintain AT2 Cell Identity. Signaling by Kras regulates the self-renewal of AT2 cells (1), so we studied the effect of Etv5 deficiency on the growth of cultured AT2 cell colonies. Lineage$^-$ CD24$^-$ Epcam$^+$ cells (25) were isolated from adult lungs bearing tamoxifen-inducible (*Rosa*^{CreERT2}) (26) conditional alleles of mutant *Kras* (Kras.LSL.G12D) (27) and *Etv5* (28). More than 99% of sorted cells expressed the AT2 cell marker Sftpc (Fig. S1*A*). The primary AT2 cells were cultured on Matrigel for 10–12 d total, with 4-hydroxytamoxifen (4-OHT) added on the first 4 d to induce expression of Kras^{d12} and to delete *Etv5*. *Etv5*$^{+/+}$ cells formed compact, spherical colonies in the presence of exogenous growth factors [hepatocyte growth factor (HGF), FGF10, and keratinocyte growth factor (KGF)] or after Kras^{d12} induction (Fig. S1*B*). Colony numbers in both contexts were reduced significantly by Etv5 deficiency (Fig. 2*A*).

To determine the molecular consequence of Etv5 deletion, we performed RNA-sequencing (RNA-seq) on *Etv5*$^{+/+}$*Kras*$^{LSL/+}$ *Rosa*$^{CreERT2/+}$ and *Etv5*$^{fl/fl}$*Kras*$^{LSL/+}$*Rosa*$^{CreERT2/+}$ cells after 4 d of culture in 4-OHT, when there was no detectable difference in their growth. Etv5 deficiency had a dramatic effect on the expression of AT1 and AT2 marker genes (7); AT2 marker genes were down-regulated significantly, and AT1 marker genes were

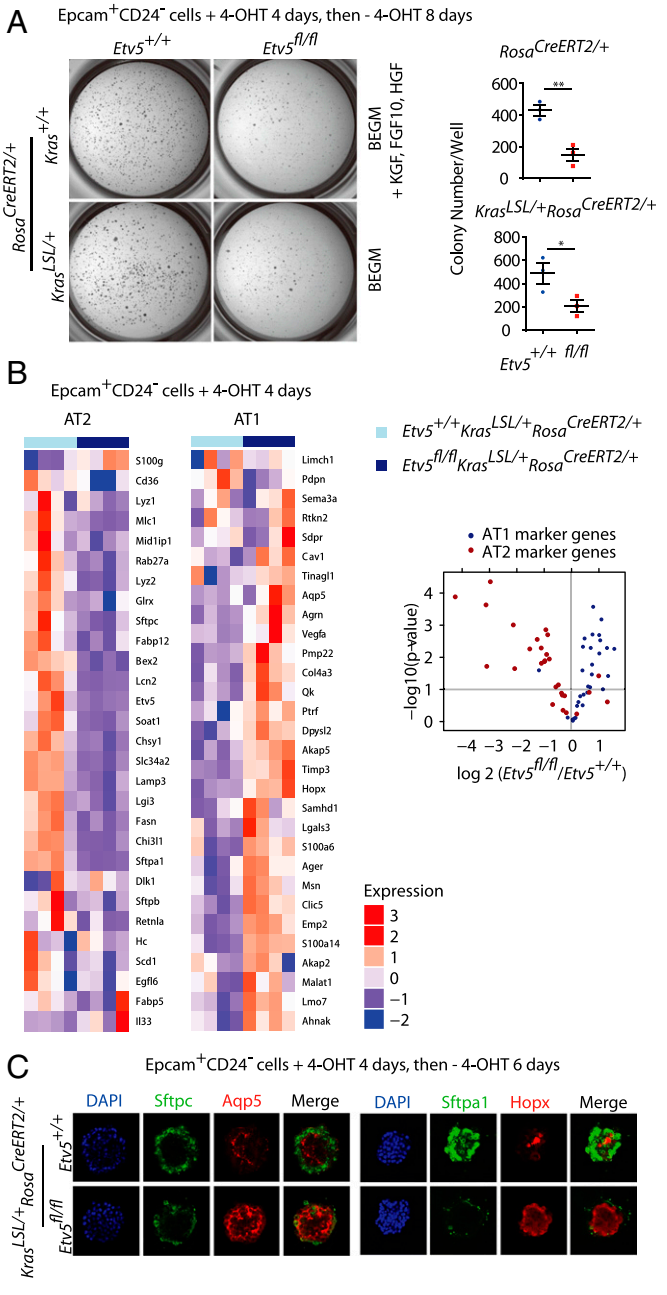

Fig. 2. Etv5 is required to maintain AT2 cell fate in vitro. (*A*, *Left*) Colonies formed by AT2 cells after culture on Matrigel with or without growth factors. (*Right*) Each symbol indicates cells from one mouse. Data are shown as mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$ (Student's *t* test). (*B*, *Left*) Heatmap shows expression of AT1 and AT2 marker genes (7) in cells sorted and then cultured with 4-OHT. RNA-seq values are scaled and centered, normalized counts to account for library size and gene length ($n = 4$ mice per genotype). (*Right*) The volcano plot indicates the effect of Etv5 deficiency on AT1 (blue) and AT2 (red) marker genes. (*C*) Immunostaining of colonies formed by AT2 cells after induction of Kras^{d12}.

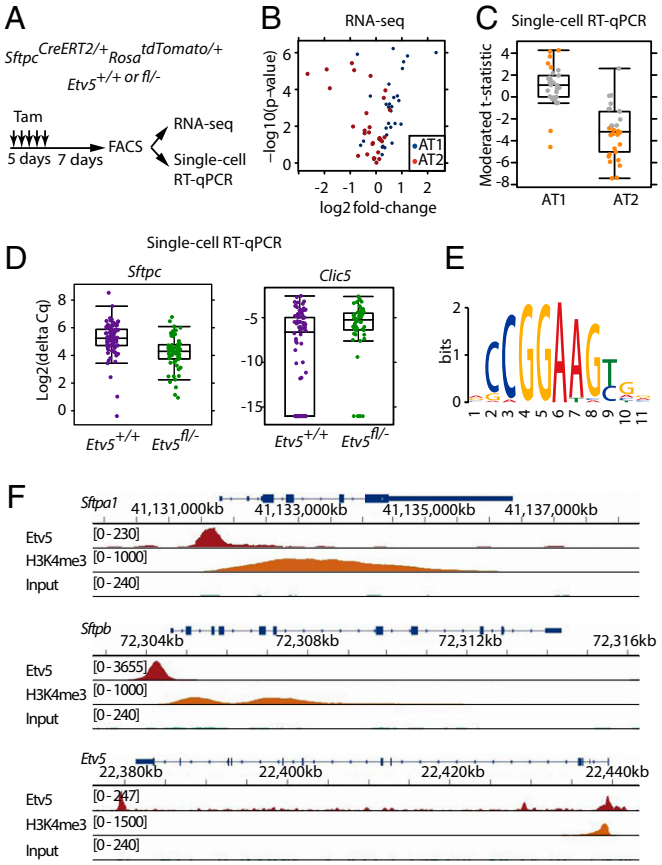

Fig. 3. Etv5 regulates AT2 cell identity in vivo. (*A*) Scheme for deleting Etv5 from AT2 cells in the lungs. Tam, tamoxifen. (*B*) Volcano plot indicating the effect of Etv5 deficiency on AT1 (blue) and AT2 (red) marker gene expression in tdTomato$^+$ cells isolated as in *A*. (*C*) Comparison of AT1 and AT2 marker gene expression in *Etv5*$^{+/+}$ versus ΔEtv5 tdTomato$^+$ Epcam$^+$ CD24$^-$ lineage$^-$ cells by single-cell qRT-PCR. The *t* statistic for each gene is plotted. Orange dots indicate genes significantly changed by *Etv5* deficiency (Benjamini–Hochberg adjusted $P < 0.05$, moderated *t* test). Genes that are up-regulated in ΔEtv5 tdTomato$^+$ cells show larger positive *t* statistics, whereas those down-regulated show larger negative *t* statistics. (*D*) Box-and-whisker plots showing the expression of the most differentiated AT2 and AT1 genes, *Sftpc* and *Clic5*, in tdTomato$^+$ cells by single-cell RT-PCR. (*E*) Consensus sequence motif identified by the MEME motif-finding software in the Etv5-binding sites found in C57BL/6 AT2 cells. (*F*) ChIP-Seq normalized coverage of representative AT2 marker genes.

up-regulated significantly (Fig. 2*B*). Immunostaining confirmed that Etv5-deficient colonies expressed more of the AT1 markers Aqp5 and Hopx and less of the AT2 markers Sftpa1 and Sftpc than control colonies (Fig. 2*C*). These data suggest that Etv5 preserves the AT2 cell phenotype and that, in the absence of Etv5, cells adopt a more AT1-like identity.

To verify that data from cultured AT2 cells reflect AT2 cell characteristics in vivo, we also examined gene expression after deleting *Etv5* from AT2 cells in mice. tdTomato$^+$ cells were isolated from *Sftpc*$^{CreERT2/+}$*Rosa*$^{tdTomato/+}$ lungs after tamoxifen treatment to delete *Etv5* (Fig. 3*A* and Fig. S2 *A*–*C*). Whole lungs were analyzed also. Reassuringly, *Etv5* deletion from AT2 cells in vivo increased the expression of AT1 marker genes and decreased the expression of AT2 marker genes, as observed in vitro (Fig. 3*B*). There were 185 genes that were both enriched in wild-type AT2 cells (compared with whole lung) and suppressed by *Etv5* deficiency (Fig. S2*D*). This overlap in genes was significantly larger than expected by chance ($P < 2.2e\text{-}16$, χ^2 test). Single-cell quantitative RT-PCR (qRT-PCR) on *Etv5*$^{+/+}$ tdTomato$^+$ cells and Δ*Etv5* tdTomato$^+$ cells confirmed that *Etv5* deficiency reduced the expression of AT2 marker genes and increased the expression of AT1 marker genes (Fig. 3 *C* and *D*). Similar changes in gene expression occurred when the tdTomato$^+$ cells expressed Kras^{d12} (Fig. S2*E*) or when lineage$^-$ CD24$^-$ Epcam$^+$ cells from tamoxifen-treated *Etv5*$^{+/+}$*Rosa*$^{CreERT2/+}$ and *Etv5*$^{fl/-}$*Rosa*$^{CreERT2/+}$ mice were compared (Fig. S2*F*). Collectively, these results indicate that an Etv5-dependent transcriptional program is critical for the maintenance of AT2 cell identity.

We searched for direct transcriptional targets of Etv5 in AT2 cells by performing ChIP-sequencing (ChIP-seq). We identified 10,545 Etv5-binding sites that were close to transcription start sites (TSS) in primary AT2 cells from C57BL/6 mice. Of these sites, 9,610 contained the GGAA ETS core motif (Fig. 3*E*) and overlapped with H3K4Me3, an active promoter mark (Fig. 3*F* and Fig. S2*G*). Interestingly, Etv5 peaks were located within the promoters of both AT1 and AT2 marker genes (Fig. S2*H*), and thus the regulation of gene expression by Etv5 is likely complex and influenced by the presence of other factors.

Etv5 Is Required for the Proliferation of AT2 Cells Following Lung Damage. The altered gene-expression profile of Etv5-deficient AT2 cells suggested they might be more prone to differentiate into AT1 cells. When challenged with s.c. bleomycin, tamoxifen-treated *Etv5*$^{fl/-}$*Sftpc*$^{CreERT2/+}$*Rosa*$^{tdTomato/+}$ mice tended to develop more extensive and severe lung lesions than control mice (Fig. 4 *A*–*D* and Fig. S3). Lesions in *Etv5* mutant lungs contained more tdTomato$^+$ AT1 cells, and fewer AT2 cells were labeled with BrdU, which marks cells that have proliferated (Fig. 4 *B*–*D*). These data indicate that Etv5 deficiency in AT2 cells compromises their ability to divide/self-renew and favors their differentiation into AT1 cells.

Etv5 Is Required for Lung Tumor Initiation by Oncogenic Kras. AT2 cells are the cells-of-origin in Kras-induced lung adenocarcinoma (1, 3–5). Therefore, we determined whether Etv5 deficiency in AT2 cells impairs Kras-driven tumor initiation. Adenovirus expressing Cre was delivered intranasally to induce *Kras*^{d12} expression in the lungs of mice with wild-type *Etv5* (*Etv5*$^{+/+}$*Kras*$^{LSL/+}$) or floxed *Etv5* alleles (*Etv5*$^{fl/fl}$*Kras*$^{LSL/+}$). Etv5 protein was detected in early *Kras*^{d12} lesions with little hyperplastic morphology and in more advanced tumors (Fig. 4*E*). *Etv5* deficiency, concurrent with Kras^{d12} expression, significantly reduced the proliferative lung lesion burden, including hyperplastic and adenomatous lesions (Fig. 4*F*). Seventeen adenomas that did develop in *Etv5*$^{fl/fl}$ *Kras*$^{LSL/+}$ mice were genotyped after laser-capture microdissection. All 17 tumors retained the floxed *Etv5* exons (showing 43–100% retention) (Fig. 4*G*), indicating that complete ablation of Etv5 prevents Kras-driven lung tumor initiation.

ERK Signaling Inhibits COP1-Mediated Degradation of Etv5. *Kras*^{d12} lung tumors overexpressed Etv5 protein compared with normal lung tissue (Figs. 4*E* and 5*A*), but they did not overexpress *Etv5* mRNA (Fig. 5*B*). ETV5 is targeted for proteasomal degradation by CRL4$^{COP1/DET1}$ (29). Therefore, we hypothesized that Ras signaling stabilized Etv5 by inactivating CRL4$^{COP1/DET1}$. Indeed, human HT55 colon cancer cells treated with TGFα for 30 min to activate Ras contained more ETV5 protein but not *ETV5* mRNA than cells receiving DMSO vehicle (Fig. 5*C*). The increase in ETV5 was similar to that induced by the proteasome inhibitor MG-132 and was prevented by ERK inhibitor 11e (Vertex). Robust expression of Etv5 in mouse embryonic fibroblasts (MEFs) transformed with E1A and HrasG12V was also suppressed by inhibiting mitogen-activated protein kinase kinase (MEK)/MAP2K7 or ERK, but not PI3K, for as little as 10 min. Importantly, the MEK and ERK inhibitors had little impact on *Etv5* mRNA in the MEFs (Fig. 5*D*). These data suggest that

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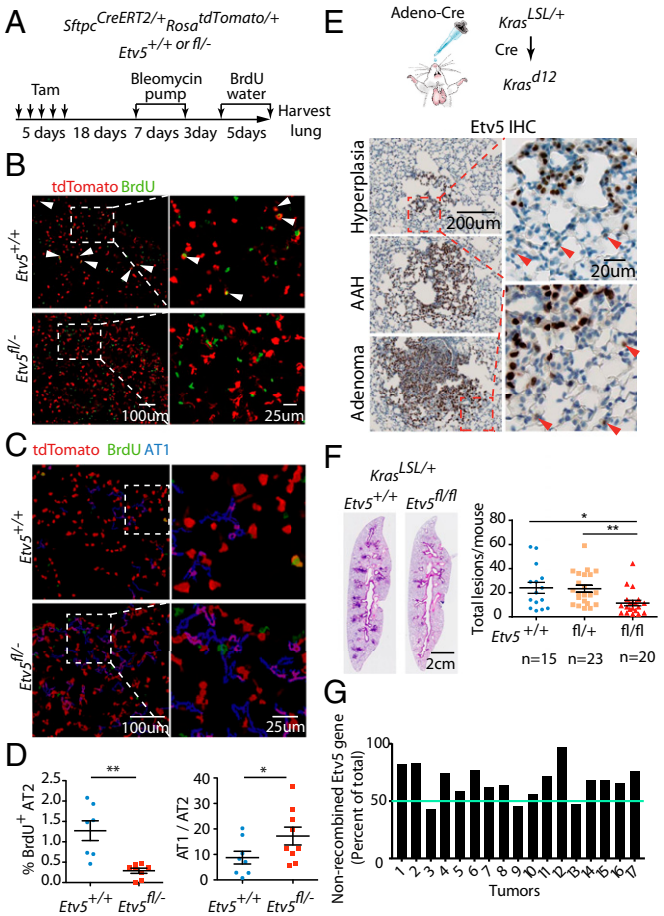

Fig. 4. Etv5 in AT2 cells is required for the repair of bleomycin-induced lung injury and for Kras-driven tumor initiation. (*A*) Scheme for the study of bleomycin-induced lung injury. Tam, tamoxifen. (*B*) Bleomycin-treated $Sftpc^{CreERT2/+}Rosa^{tdTomato/+}$ lung showing tdTomato expression (red) and BrdU incorporation (green). Arrowheads indicate tdTomato$^+$ BrdU$^+$ cells. (*C*) Lungs in *B* with tdTomato$^+$ AT1 cells pseudocolored blue after being differentiated from AT2 cells by their lower staining intensity and elongated shape. (*D*) Quantification of tdTomato$^+$ BrdU$^+$ cells and relative numbers of AT1 versus AT2 cells. Data are shown as mean ± SEM; $^{**}P < 0.01$, unpaired one-tailed Student's *t* test with Welch's correction; $^{*}P < 0.05$, unpaired one-tailed Student's *t* test. AT1 and AT2 cells are distinguished as in *C*. (*E*) Immunostaining of a $Kras^{LSL/+}$ lung lesion 20 wk after infection with adenovirus expressing Cre. Arrowheads indicate Etv5$^+$ cells adjacent to the lesions. AAH, atypical adenomatous hyperplasia. (*F*) Representative lung lesions at 20 wk after infection with adenovirus expressing Cre. The graph shows mean lesion numbers. Each dot represents one mouse. Bars indicate mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$ (Mann–Whitney test). (*G*) The graph indicates the extent to which the floxed *Etv5* allele was retained in microdissected tumors from $Kras^{LSL/+}$ $Etv5^{fl/fl}$ mice infected with adenovirus expressing Cre.

activation of ERK downstream of Ras stabilizes ETV5 in cells that express the *ETV5* gene.

Next we tested if ERK stabilizes ETV5 by inhibiting $CRL4^{COP1/DET1}$. COP1 is recruited to the DDB1–CUL4A–RBX1 core complex via the adaptor protein DET1 (Fig. 6*A*). Although inhibition of Ras/MAPK signaling reduced Etv5 abundance in wild-type transformed MEFs, it had no effect on Etv5 levels in similarly transformed $Cop1^{-/-}$ or $Det1^{-/-}$ MEFs (Fig. 6*B*). We also observed COP1-dependent degradation of ETV5 following ERK inhibition in human H460 and H1299 nonsmall cell lung cancer (NSCLC) cells and in mouse $Kras^{d12}$; $\Delta Trp53$ (KP) lung cancer cells (Fig. 6*C*). Cullin-based E3 ligases require neddylation for their activity, and MLN4924, an inhibitor of the Nedd8-activating enzyme (30), also prevented ETV5 degradation after ERK inhibition in human NSCLC lines (Fig. 6*D*). Therefore, $CRL4^{COP1/DET1}$ targets ETV5 for degradation when the Ras–Raf–MEK–ERK pathway is switched off.

Phosphorylation of DET1 by ERK Inhibits $CRL4^{COP1/DET1}$. We investigated what ERK phosphorylates to inactivate $CRL4^{COP1/DET1}$. ERK activity did not alter the abundance, subcellular localization, or known interactions of components of $CRL4^{COP1/DET1}$ or the activity of the CRL4 core complex (Fig. S4). Therefore, we explored whether ERK phosphorylated ETV5 rather than $CRL4^{COP1/DET1}$. HEK293 cells expressing KrasG12D and cRaf in response to doxycycline (hereafter called "293.Kras cells") were transfected with either wild-type *ETV5* or mutant *ETV5* having all putative ERK, mitogen- and stress-activated protein kinase 1 (MSK), ribosomal S6 kinase (RSK), and PKA phosphorylation sites mutated to alanine. Phosphorylation of mutant ETV5 was impacted because it did not migrate like wild-type ETV5 in a Phos-tag acrylamide gel. However, the mutant protein was stabilized following ERK activation (Fig. S5 *A* and *B*), suggesting that ERK-dependent phosphorylation of ETV5 does not shield it from COP1-dependent degradation. In addition, the COP1 substrate c-JUN also accumulated when ERK was active (Fig. 6 *B* and *D*).

Next, we determined whether COP1 and/or DET1 were the targets of ERK signaling. COP1 contains six putative ERK sites, three of which are conserved in vertebrates. Mutation of these six sites to either alanine or phospho-mimetic residues (aspartic acid or glutamic acid) did not prevent ectopic COP1 from targeting coexpressed ETV5 for degradation (Fig. S5*C*). Mutagenesis of putative ERK sites in DET1 proved more informative: Replacement of DET1 Ser66 or Ser458 with phospho-mimetic residues impaired the ability of $CRL4^{COP1/DET1}$ to reduce ETV5 protein abundance (Fig. 7*A*). We noted that an alanine substitution

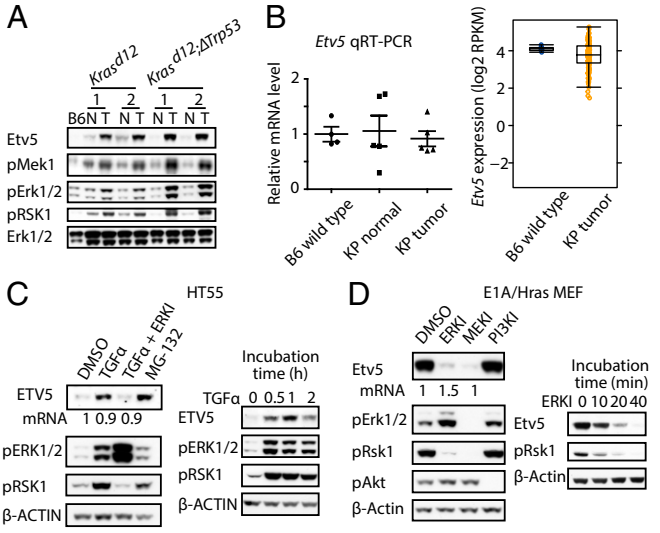

Fig. 5. ERK activation stabilizes ETV5. (*A*) Immunoblots of normal (N) and tumor (T) lung tissue from two $Kras^{LSL/+}$ and two $Kras^{LSL/+}Trp53^{fl/fl}$ mice after infection with adenovirus expressing Cre. B6, C57BL/6 wild-type. pRSK1 (phospho-Ribosomal protein S6 kinase alpha-1) is a readout of ERK activity. (*B*) *Etv5* gene expression in lung tissue from wild-type (B6) mice and in normal or tumor lung tissue from $Kras^{LSL/+}Trp53^{fl/fl}$ (KP) mice after infection with adenovirus expressing Cre. (*C*) Immunoblots of HT55 cells. Treatments shown on the left were for 1 h, with the exception of MG-132, which was added for 3 h. Numbers indicate relative *ETV5* mRNA expression by real-time RT-PCR. (*D*) Immunoblots of E1A/Hras-transformed MEFs. Inhibitor treatments shown on the left were for 1 h. Numbers indicate relative *Etv5* mRNA expression by real-time RT-PCR.

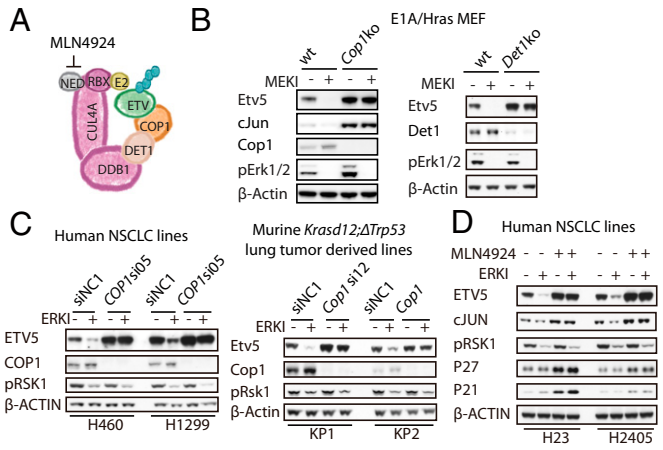

Fig. 6. Active ERK stabilizes ETV5 by inhibiting the COP1 ubiquitin ligase. (*A*) Organization of the $CRL4^{COP1/DET1}$ ubiquitin ligase. NED, Nedd8. (*B*) Immunoblots of E1A/Hras-transformed MEFs treated with MEK inhibitor (MEKI) for 1 h. (*C*) Immunoblots of H460, H1299, and $Kras^{d12}\Delta Trp53$ (KP1 and KP2) cells transfected with *Cop1* siRNAs and then treated with ERK inhibitor (ERKI) for 1 h. SiNC1, siRNA negative control 1. (*D*) Immunoblots of human NSCLC lines treated with MLN4924 for 1 h before the addition of ERK inhibitor.

at Ser66, but not at Ser458, in DET1 also compromised ETV5 degradation, suggesting that Ser66 is a structurally important residue.

MS using absolute quantification (AQUA) peptide standards confirmed that some Flag-tagged DET1 was phosphorylated on Ser66 or Ser458 after coexpression with active ERK2 in 293.Kras cells (Fig. S6). Approximately 12% of DET1 was phosphorylated at Ser458, but less than 2% of DET1 was phosphorylated at Ser66 (Fig. 7*B* and Table S1). Given the apparently weak interaction between COP1 and DET1 (Fig. S7), we investigated whether tethering COP1 to DET1 with an artificial linker would increase phosphorylation on DET1 Ser458. Indeed, DET1–COP1 fusion proteins with linkers of different lengths exhibited enhanced phosphorylation at DET1 Ser458 (Fig. 7*B* and Table S1). By contrast, phosphorylation at DET1 Ser66 was not increased in these fusion proteins (Fig. 7*B* and Table S1). Further evidence that DET1 is phosphorylated on Ser458 in an ERK-dependent manner was obtained with a DET1 Ser458 phospho-specific antibody. Thus, endogenous DET1 phosphorylated on Ser458 was detected in 293.Kras cells after doxycycline treatment to activate the Ras/MAPK pathway, and this phosphorylation could be prevented by either MEK or ERK inhibition (Fig. 7*C*). Collectively, these data support a model wherein ERK phosphorylates DET1 at Ser458 (Fig. 7*D*) and this phosphorylation inactivates $CRL4^{COP1/DET1}$ by an as yet unknown mechanism. ETV5 accumulates even when very little DET1 is phosphorylated, presumably because only DET1 encountering the COP1–ETV5 complex needs to be modified.

Discussion

We show that the transcription factor Etv5 is critical for maintaining AT2 cell identity. Although Etv4 and Etv5 are expressed in the distal lung epithelium of the embryo (10), adult lung expressed *Etv5* more than *Etv1* or *Etv4* (Fig. 1*A*), and Etv5 was largely restricted to AT2 cells (Fig. 1*B*). *Etv5* deletion caused a global reduction in the expression of genes associated with AT2 cells (GSE80102), including the AT2 marker genes identified by single-cell sequencing (7). In contrast, AT1 marker genes were up-regulated by Etv5 deficiency. The genes down-regulated in Etv5-deficient AT2 cells included not only those linked to AT2 cell physiology but also genes such as *Id2* that mark the distal progenitor population in the developing embryonic lung (31) and genes involved in cell-cycle regulation (Fig. S2 *B* and *C*). Therefore, Etv5 probably regulates both the specialized physiological and progenitor roles of AT2 cells.

Etv5 ChIP-seq indicated that Etv5 binds to the promoters of a wide range of genes in AT2 cells (Fig. 3*F*). This binding probably was functionally significant, because transcription of many of these genes was dysregulated in Etv5-deficient AT2 cells (Fig. 3*B* and Fig. S2 *D* and *E*). Therefore, it was somewhat surprising that deletion of *Etv5* from AT2 cells did not impact lung morphology or function over the next 3 mo. All the surfactant protein genes were still expressed in the mutant AT2 cells, although at a reduced level, and this reduced expression must have sustained lung function. The turnover rate of AT2 cells in normal mouse lung is also very slow (1), so defective AT2 cell replication would be difficult to detect in the absence of challenge. Bleomycin-induced lung injury is known to trigger AT2 cells to proliferate and differentiate into AT1 cells (24), and it was in the context of this damage that Etv5-deficient AT2 cells were found to differentiate into AT1 cells with less replication (Fig. 4 *A*–*D*). These data suggest that ETV5 plays a critical role in preserving the pool of AT2 cells needed to repair alveolar damage.

We also show that Etv5 is essential for lung tumor initiation by oncogenic Kras. Although it is well established that the Ras/MAPK pathway is a major driver of lung tumorigenesis, relatively little is known about the transcriptional programs that this pathway triggers. Ras signaling via ERK dramatically stabilized Etv5 in cells, whereas Etv5 was labile when ERK was inhibited (Fig. 5 *C* and *D*). Interestingly, not all $Sftpc^{+}$ AT2 cells in normal wild-type mouse lung were $Etv5^{+}$ by immunohistochemistry (Fig. 1*B*); it is possible that this observation reflects unique subsets of AT2 cells or that transient growth signals cause Etv5 abundance in AT2 cells to fluctuate. Regardless, constitutive activation of the Ras pathway appears to sustain high levels of Etv5 in the AT2 cells that give rise to tumors

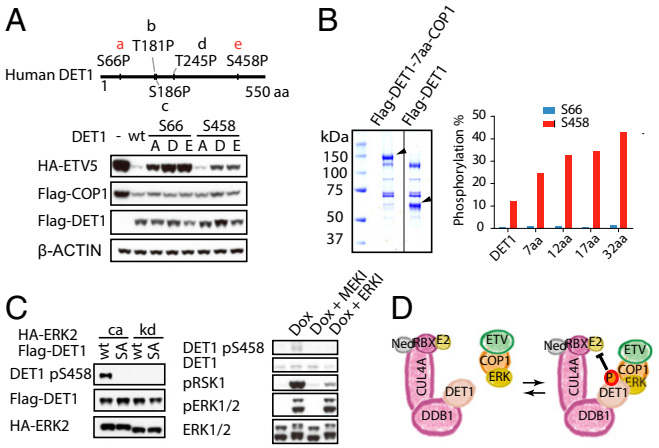

Fig. 7. Phosphorylation of DET1 by ERK. (*A*, *Lower*) Immunoblots of HEK293T cells cotransfected with ETV5, COP1, and either wild-type or mutant DET1. (*Upper*) The schematic of DET1 indicates the position of putative ERK sites. Residues conserved through evolution are shown in red. (*B*, *Left*) Coomassie blue-stained wild-type DET1 or DET1-COP1–tethered protein that was affinity purified from 293.Kras coexpressing constitutively active (CA) ERK2. Arrowheads indicate DET1-COP1 and DET1 bands. (*Right*) Percentage of DET1 phosphorylated at Ser66 or Ser458 estimated by MS. aa, the number of amino acids forming the linkers between DET1 and COP1. (*C*, *Left*) Immunoblots of 293.Kras cells coexpressing wild-type or S458A mutant DET1 with constitutively active (ca) or kinase-dead (kd) ERK2. (*Right*) Immunoblots of 293.Kras cells after treatment with doxycycline (Dox) and then with DMSO, 1 μM MEK inhibitor (MEKI), or 1 μM ERK inhibitor (ERKI) for 1 h before harvest. (*D*) Model of ERK regulation of COP1 complex activity. NED, Nedd8.

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(Fig. 4*E*). Stabilization of Etv5 appears to be an early event in cell transformation, because Etv5 was readily detected even in early hyperplastic lesions (Fig. 4*E*). Furthermore, Etv5 must be critical for oncogenesis, because we never have identified any Etv5-deficient KrasG12D adenomas (Fig. 4*G*). The findings that Etv5 is stabilized downstream of ERK and specifically regulates AT2 cell biology distinguish it from other transcription factors involved in lung tumor growth, such as Nkx2-1, Foxa1/2, or Gata2, which have broader expression in lung and are not directly regulated by Ras/MAPK signaling.

ERK inhibition of $CRL4^{COP1/DET1}$ enables Etv5 protein to accumulate rapidly in cells. Etv1, Etv4, and Etv5 are also phosphorylated by ERK, MSK, and RSK, and this phosphorylation boosts their transcriptional activity (32–36). It was surprising that Etv5 phosphorylation by ERK was dispensable for COP1-mediated degradation, because phosphorylation of the COP1 substrates ETS1 and ETS2 is reported to regulate their interaction with the ligase (18). Instead, DET1 was phosphorylated in an ERK-dependent manner, although how this phosphorylation inhibits the function of the ligase remains to be elucidated. Future studies will need to address whether other COP1 substrates are also subject to regulation by the Ras/MAPK pathway.

In summary, we demonstrate that Ras–Raf–MEK–ERK signaling inactivates the COP1 ubiquitin ligase complex, resulting in the accumulation of the transcription factor ETV5. This post-translational mechanism facilitates very rapid changes in transcriptional programs in response to external cues such as growth factors. In the lung, sustained Ras activation in AT2 stem cells stabilized ETV5, and this stabilization was an essential initiating event in the development of atypical adenomatous hyperplasia. The related PEA3 transcription factor, ETV1, has a similar role in GIST (15), so it is tempting to speculate that *Etv* genes are key factors in maintaining the populations that give rise to other Ras-driven cancers.

Materials and Methods

The Genentech Animal Care and Use Committee approved all experiments using mice. Mouse alleles *Cop1fh*, *Det13xf*, and *Det1fl* were generated by gene targeting (Fig. S8). An s.c.-implanted micro-osmotic pump was used to deliver bleomycin. $Kras^{LSL}$ mice intranasally infected with Adeno-Cre virus were killed at 20 wk postinfection to evaluate lung tumor burden. Primary AT2 cells were isolated by FACS from single-cell suspensions of mouse lung and were cultured on Matrigel. Detailed methods, reagents, and statistical analyses are provided in *SI Materials and Methods*.

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