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Transgenic Mice for Cre-Inducible Overexpression of the *Cul4A* Gene

Tong Li[1,2], **Ming-Szu Hung**[1,3], **Yucheng Wang**[4], **Jian-Hua Mao**[5], **Jia-Li Tan**[6,7], **Kenneth Jahan**[1], **Hannah Roos**[1], **Zhidong Xu**[1], **David M. Jablons**[1,*], and **Liang You**[1,*]

[1]Thoracic Oncology Laboratory, Department of Surgery, Comprehensive Cancer Center, University of California, San Francisco, California

[2]Thoracic Surgery Department, Beijing Chao-Yang Hospital, Capital University of Medical Science, Beijing, People's Republic of China

[3]Division of Pulmonary and Critical Care Medicine, Chang Gung Memorial Hospital, Chiayi, Taiwan, Republic of China

[4]Department of Surgery, University of California, San Francisco, California

[5]Life Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, California

[6]Department of Orthodontics, School of Stomatology, The Fourth Military Medical University, Xi'an, Shaanxi Province, People's Republic of China

[7]Department of Orofacial Science, University of California, San Francisco, California

Summary

The *Cullin 4A* (*Cul4A*) gene is important in cell survival, development, growth, and cell cycle control and is amplified in breast and hepatocellular cancers. Recently, we reported that *Cul4A* plays an oncogenic role in the pathogenesis of mesothelioma. An important strategy for studying *Cul4A* in different tissues is targeted overexpression of this gene *in vivo*. Studies of *Cul4A* in mice have been restricted to the loss-of-function studies using *Cul4A* knockout mice; gain-of-function studies of *Cul4A* using transgenic mice have not been reported. We, therefore, generated a gain-of-function transgenic mouse model that over-expresses *Cul4A* in a Cre-dependent manner. Before Cre recombination, these mice express *LacZ* during development in most adult tissues. After Cre-mediated excision of the *LacZ* reporter, the transfected *Cul4A* gene is expressed along with a C-terminal *Myc-tag* in different tissues. In this study, Cre-excision was induced in mouse lungs by inhalation of an adenovirus vector encoding Cre recombinase. This mouse model provides a valuable resource for investigating the significance of *Cul4A* overexpression in various tissues.

Keywords

Cul4A; Cre-mediated expression; mouse model

Cullin 4A (Cul4A) belongs to the family of evolutionarily conserved cullin proteins, including seven related ones (Cul1, 2, 3, 4A, 4B, 5, and 7) in humans. Cul4A forms a part of

*Correspondence to: David M. Jablons or Liang You, Thoracic Oncology Laboratory, Department of Surgery, Comprehensive Cancer Center, University of California, San Francisco, CA 94143. Liang.You@ucsfmedctr.org.
The first two authors contributed equally to this work.

the multifunctional ubiquitin-protein ligase E3 complex by interacting with ring finger protein (ROC1) and damaged DNA binding protein (DDB1). Through ubiquitin-mediated proteolysis, Cul4A regulates many critical processes in cells. *Cul4A* is a critical gene for hematopoietic cell survival and development. Overexpression of *Cul4A* reportedly increases cell growth and causes the disruption of the G2–M cell cycle in irradiated mammary epithelial cells. Previous studies (Kopanja *et al*., 2009) showed that *Cul4A* is amplified in breast and liver cancers, is implicated in the ubiquitination and proteolysis of tumor suppressors such as p53, and may contribute to tumorigenesis and cancer development through ubiquitination and then proteolysis of tumor suppressors.

The *Cul4A* gene restricts cellular repair capacity (Angers *et al*., 2006; Li *et al*., 2010). It does so by orchestrating the concerted actions of nucleotide excision repair (NER) and the DNA damage-responsive G1/S checkpoint through selective degradation of the DDB2 and XPC DNA damage sensors and the p21/CIP1/WAF1 checkpoint effector (Liu *et al*., 2009). We previously found that *Cul4A* is an oncogene for mesothelioma (Hung *et al*., 2009). However, the relationship between Cul4A and the occurrence and metastasis of other tumors such as mesothelioma and lung cancer is seldom reported, and the potential oncogenic role of Cul4A has been little studied.

Mouse models have been instrumental in developing our knowledge of signaling pathways *in vivo* and of the aberrations in these pathways that are causally associated with disease. Studies of the *Cul4A* gene in mice have been restricted to the loss-of-function studies using *Cul4A* knockout mice (Liu *et al*., 2009). For instance, it has been shown that *Cul4A* knockout mice are resistant to ultraviolet (UV)-induced skin carcinogenesis and that Cul4A restricted cellular repair capacity through selective degradation of p21, DDB2, and XPC (Liu *et al*., 2009). However, gain-of-function studies of *Cul4A* using transgenic mice have not been reported.

The relationship between *Cul4A* and cancer is not well understood. Because a transgenic mouse model is needed to explore the potential oncogenic role of *Cul4A*, we sought to generate one with lineage-tracing that conditionally overexpresses Cul4A protein on Cre-mediated recombination. In this mouse model, before Cre-mediated recombination, *LacZ* was expressed in the majority of embryonic and adult tissues that were successfully transfected with the gene vector. On Cre-excision in selected organs, *LacZ* expression was replaced by expression of *Cul4A* and a *Myc-tag*. In our model, Cre-excision was induced in mouse lungs by inhalation of an adenovirus vector encoding Cre recombinase (Ade-Cre) (DuPage *et al*., 2009). To generate this mouse model, we used the pCALL2 expression vector, which has been previously reported (Ding *et al*., 2002; Elghazi *et al*., 2008; Lobe *et al*., 1999; Novak *et al*., 2000). In our study, a Myc-tagged full-length *Cul4A* cDNA was subcloned downstream of the loxP-flanked *LacZ/neoR*. Between the loxP sites, the *LacZ* sequence is followed by a triple repeat of the SV40 polyadenylation signal (Zinyk *et al*., 1998) to ensure a transcriptional stop.

Before the Cre-mediated excision, the promoter regulated expression of the loxP-flanked *LacZ/neoR*. The Cre recombinase of the P1 bacteriophage catalyzes recombination between two specific 34-bp consensus sequences (loxP sites) with high specificity and efficiency (Sternberg and Hamilton, 1981). The loxP sites are palindromic, except for an 8-bp asymmetric core sequence that provides each loxP site with an orientation (Hoess *et al*., 1986). Introduction of Cre enzyme results in recombination between the loxP sites and excision of the intervening DNA sequence. Upon Cre-mediated excision, *LacZ* expression is switched to the expression of the *Cul4A* and a *Myc-tag* as a monitor of expression (Fig. 1a). The expression of Cul4A can be manipulated in an organ of interest by Cre-excision in that organ.

The pCALL2 vector constructed with the *Cul4A* gene (We hereafter refer to the constructed vector as pCALL2-*Cul4A*.) was used to generate transgenic mice using embryonic stem cell (ES)-based transgenesis. By standard methods (Nagy, 1997), the pCALL2-*Cul4A* was transfected into ES cells. After clone selection, the chosen clones of ES cells with pCALL2-*Cul4A* were used to generate chimeric mice. Transgenic mice were mated with FVB/N mice to produce transgenic offspring. The pCALL2-*Cul4A* transgene was genotyped by polymerase chain reaction (PCR) using genomic DNA isolated from the tail. We successfully obtained three chimeric mouse lines (lines 1, 6, and 23) from 24 lines (Fig. 1c). Whole mount X-gal staining was positive in the tails (Fig. 1d–g) and the lungs (Fig. 1h) of these three mouse lines, but varied in strength. Line 6 exhibited weak *LacZ* expression in the staining of tail and lung, whereas Lines 1 and 23 showed similar strong *LacZ* expression.

Transgene expression was assessed by X-gal staining in the brain, heart, lung, stomach, gut, liver, pancreas, spleen, kidney, and muscle of transgenic mice from the chimeric mouse lines 1 and 23. These lines showed similar stronger *LacZ* (X-gal) expression in most organs when compared with negative controls (The X-gal staining data for lung, brain, stomach, gut, muscle, pancreas, and kidney are shown in Fig. 2. Data for heart, liver, and spleen are not shown.). However, *LacZ* expression varied in different organs and tissues. In the brain, *LacZ* expression was higher in the white matter than in the gray matter. In the lung, the bronchi and alveoli showed the same level of *LacZ* expression. Overall, *LacZ* expression was strongest in the gastrointestinal organs, especially in mucous membrane, although it was weak in the chorion and smooth muscle layers and the glands of mucous membrane in the gastrointestinal organs (Fig. 2f,h). In the kidney, expression was higher in the medulla than in the cortex (Fig. 2n). In the pancreas, *LacZ* expression was apparent, especially in the pancreas islets (Fig. 2l). In the spleen, expression of *LacZ* was homogeneous (data not shown). Lower levels of *LacZ* expression were observed in the muscle (Fig. 2j) and heart (data not shown), and none was observed in the liver (data not shown). The fact that high levels of the transgene were expressed in the stomach, gut, lung, brain, spleen, and pancreas suggests that, in this mouse model, studies of *Cul4A* should be feasible in these organs. The lower levels of *LacZ* expression in the muscle and heart and the lack of expression in the liver suggest that studies of *Cul4A* would not be feasible in those organs.

We also did immunofluorescence staining for anti-LacZ (X-gal) in mammary glands. The data showed strong *LacZ* expression in the chimeric mouse lines 1 and 23 and a remarkable difference between control and pCALL2-*Cul4A* mouse lines 1 (data not shown) and 23 (Fig. 2o–t). This result suggested that the pCALL2 vector highly expressed in mammary glands of the chimeric mouse lines and that studies of *Cul4A* should be feasible in mammary glands in this mouse model.

To assess *Cul4A* expression, Cre excision was induced in the lungs of transgenic mice. Half of the transgenic mice inhaled Ade-Cre. Simultaneously, the other half inhaled Ade-GFP without Cre. Six weeks later, lungs from these mice were dissected. To determine the expression of *Cul4A* and *Myc-tag* with or without Cre-excision, lung sections from pCALL2-*Cul4A* mouse lines 1, 6, and 23 with Ade-GFP or Ade-Cre were immunostained with anti-Cul4A and anti-Myc-tag antibodies, respectively. The lungs of mice with Ade-Cre were positive for both anti-Cul4A and anti-Myc-tag, whereas the lungs of mice without Cre-excision were negative for both. Interestingly, we noticed some potential hyperplasia regions in the mouse lungs after Ade-Cre inhalation (Fig. 3, the potential hyperplasia regions were marked by arrows.). Possibly, this is due to the oncogenic functions of the Cul4A protein. Further studies with longer induction periods will be needed to study these effects. Among the three lines, immunostaining was weakest in Line 6 (Fig. 3b,f). This result was similar to that of the whole mount X-gal staining (Fig. 1d–h). The lung sections from pCALL2-*Cul4A* mouse lines 1, 6, and 23 with Ade-GFP and Ade-Cre were also immunofluorescence stained

for anti-Cul4A, anti-Myc-tag, and anti-LacZ. The results for *Cul4A* and *Myc-tag* were the same as those for immunostaining—the lungs of mice with Ade-Cre were positive, and the lungs of mice with Ade-GFP were negative. Immunofluorescence staining for LacZ was negative in lungs with Cre-excision and positive in lungs without Cre-excision (Fig. 4a–c for mouse line 23; data not shown for mouse lines 1 and 6). Taken together, all of these results confirmed that Cre-excision was successful. In mice that inhaled Ade-Cre, the *LacZ* gene was excised; therefore, these lungs stained positive for anti-Cul4A and anti-Myc-tag antibody and negative for anti-LacZ antibody. We used the same virus particle of Ade-Cre but with GFP (instead of Cre) as control. In mice that inhaled Ade-GFP, the pCALL2-*Cul4A* gene could not be expressed because the *LacZ* and the triple repeat of the SV40 sequences were not excised. Consequently, *LacZ* continued to be expressed instead of *Cul4A* and *Myc-tag*.

In conclusion, we have produced a gain-of-function transgenic mouse model that overexpresses *Cul4A* in a Cre-dependent manner. We believe that these transgenic mice are valuable for further characterization of the *Cul4A* gene in different organs and at different developmental stages. The lineage tracing capabilities of our model are powerful tools to elucidate the importance of *Cul4A* in cell fate during development, as well as in the plasticity of mature cells. This mouse strain provides a new model for studying the *Cul4A* complex *in vivo*.

METHODS

Construction of the Vector

The study was approved by the University of California at San Francisco (UCSF) Institutional Animal Care and Use Committee. To generate a mouse model that would conditionally overexpress Cul4A protein, we used the pCALL2 vector (Lobe *et al*., 1999; Novak *et al*., 2000). This construct contains a chicken *β-actin* promoter with upstream *cytomegalovirus (CMV)* enhancer (pCAGGS) (Niwa *et al*., 1991). This promoter is followed by a *loxP*-flanked *LacZ/neoR* fusion with three SV40 polyadenylation (PA) signals and the full-length *Cul4A* cDNA with a joint but independent sequence of Myc-tag. After the *Myc-tag* sequence, the construct contained a rabbit *β-globin* polyadenylation sequence (Fig. 1a). This allowed us to detect cells that expressed *Cul4A* and to perform lineage tracing experiments. We refer to the expression vector as pCALL2-*Cul4A*.

ES Culture

We used ES-based transgenesis to generate chimeric mice. The pCALL2-Cul4A vector was transfected by standard methods into ES cells (Nagy, 1997). After cells were cultured, ES cell clones that carried the transgene were selected through neomycin resistance. After selection, colonies were picked and replica-plated in 96-well formats. DNA was extracted from the plates for confirmatory analysis. One clone of cells was chosen, expanded, and frozen. Three clones containing the transgene were selected for generation of chimeric mice by ES cell/embryo aggregation.

Generation of Transgenic Mice Expressing Cul4A

The chosen clones with a single copy of the transgene were used for microinjection to generate transgenic mice by conventional techniques (Nagy, 1997). Transgenic mice were mated with FVB/N mice to produce transgenic offspring. The pCALL2-*Cul4A* transgene was genotyped by PCR using genomic DNA isolated from the tail. We used the following *Cul4A* primers (Fig. 1b shows the design and positions of the primers): forward: 5′-GCACTGGAGCGAGTACATCA-3′, and reverse: 5′-CACATGCTTTGCGATCAGTT-3′.

A group of mice from the PCR-positive lines were fed for up to 6 months and used to generate offspring.

Tissue Preparation and LacZ Staining

Organs, including brain, lung, heart, stomach, pancreas, liver, gut, kidney, spleen, tail, and muscle, from 4-week-old mice from three different lines and a negative control group were obtained after the mice were killed using cervical dislocation. The organs were removed from the mice and fixed for 6 h in LacZ fixing solution (0.2% glutaraldehyde, 5 mM ethylene glycol tetraacetic acid (EGTA), pH 7.3, 0.1 M $MgCl_2$ in phosphate buffered saline (PBS), pH 7.3) as described previously (Elghazi *et al*., 2008). Organs were washed in PBS and cryoprotected in 30% sucrose overnight. Tissues were then embedded in optimal cutting temperature (OCT) medium over dry ice. Ten-micrometer frozen sections were cut, collected onto polylysine-coated slides, and stored at −20°C. Slides were fixed in PBS containing 0.2% glutaraldehyde for 10 min, washed in LacZ wash buffer (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 in PBS, pH 7.3) and then stained in LacZ wash buffer containing 1 mg/mL X-gal (Roche Diagnostics, Indianapolis, IN), 5 mM potassium ferrocyanide, and 5 mM ferricyanide at 37°C. After staining, slides were rinsed in LacZ wash buffer and then PBS.

Cre-Excision

The intranasal administration of the adenoviruses was performed as previously described (DuPage *et al*., 2009). The Ade-Cre (Ad5CMVCre) and Ade-GFP (Ad5CMVeGFP) were purchased from the Gene Transfer Vector Core of the University of Iowa, and these adenoviruses were used in several studies (Roh *et al*., 2006; DuPage *et al*., 2009). Transgenic mice were anesthetized with 2.5% Avertin via intraperitoneal injection, after which half of the mice inhaled approximately 10^6 or 10^7 particles of Ade-Cre introduced directly into the lungs. Simultaneously, the other half inhaled Ade-GFP without Cre. Six weeks later, the mice were killed, and the lungs were dissected.

Assessment of *Cul4A* and *Myc-Tag* Expression in Transgenic Cell Lines

Lung sections from pCALL2-*Cul4A* mice with Ade-GFP or Ade-Cre were immunostained with anti-Cul4A and anti-Myc-tag and immunofluorescence-stained for anti-Cul4A, anti-Myc-tag, and anti-LacZ antibodies, respectively. The sections were washed with PBST (0.1% Triton X-100 in PBS) for 15 min and blocked with 2% horse serum in PBST for 2 h. The sections were then incubated with primary antibody diluted in PBST with 2% horse serum overnight at 4°C. The primary antibodies used in this study were rabbit polyclonal anti-Cul4A (1:400, ab34897, Abcam, Cambridge, MA), rabbit polyclonal anti-Myc-tag (1:20; sc-789; Santa Cruz Biotechnology, Santa Cruz, CA), and goat polyclonal anti-LacZ (1:2,500; 4600-1409; Biogenesis, Poole, UK). The sections were washed four times for 5 min each in PBST. Appropriate secondary antibody was diluted in PBST with 2% horse serum and used in the sections. For histostaining, biotinylated secondary goat anti-rabbit antibody (1:200; BA1000; Vector Laboratories, Burlingame, CA) was used for both anti-Cul4A and anti-Myc-tag primary antibodies. The tissue stainings were visualized using the Vectastain ABC-HRP kit (PK6100; Vector Laboratories). Finally, the slides were counterstained with hematoxylin. For immunofluorescence staining, fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit antibody (1:200; 711-097-003; Jackson Immuno Research Laboratories, West Grove, PA) was used for both anti-Cul4A and anti-Myc-tag primary antibodies; FITC-conjugated donkey anti-goat antibody (1:200; 705-095-003; Jackson ImmunoResearch Laboratories) was used for the anti-LacZ primary antibody. The sections were placed in dark. Two hours later, the sections were washed three times with PBS for 5 min each, counterstained with Vectashield, covered with cover-slips,

and viewed using a fluorescent microscope. All sections were photographed using a Zeiss AxioCam camera with Zeiss AxioVision software.

Mammary gland sections from pCALL2-*Cul4A* mice and control mice were immunofluorescence-stained for anti-LacZ antibody, respectively, using the same procedure that was used for lungs.

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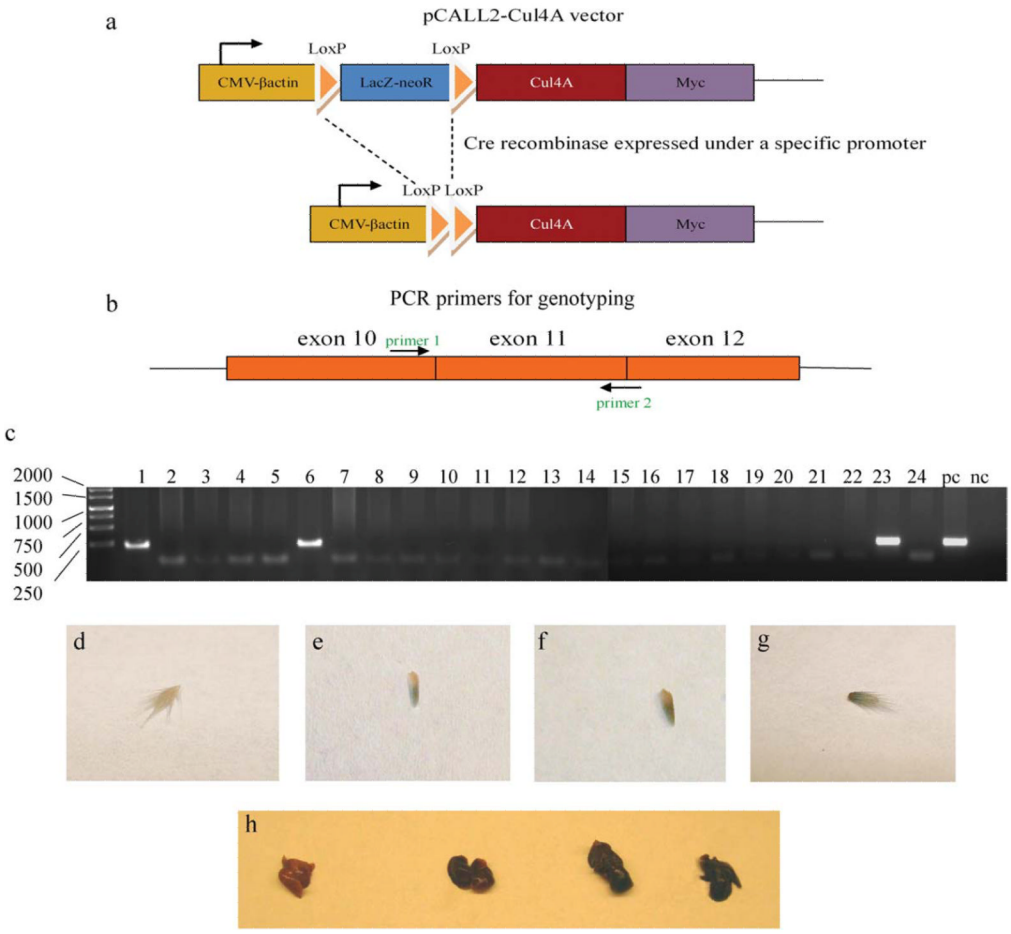

FIG. 1.

pCALL2-*Cul4A* and the three positive lines. (**a**) Diagram of the pCALL2-*Cul4A* transgene construct before and after Cre-mediated excision of the floxed *LacZ-neoR* sequence. (**b**) Design of the PCR primers for genotyping. (**c**) PCR data (pc, positive control; nc, negative control). The transferred gene could be amplified from lines 1, 6, and 23. (**d**–**g**) The stained tails of the transgenic mouse lines (from left to right: (d) negative control, (e) Line 6, (f) Line 1, and (g) Line 23). (**h**) The stained lungs of the transgenic mouse lines (from left to right: negative control, Line 6, Line 1, and Line 23).

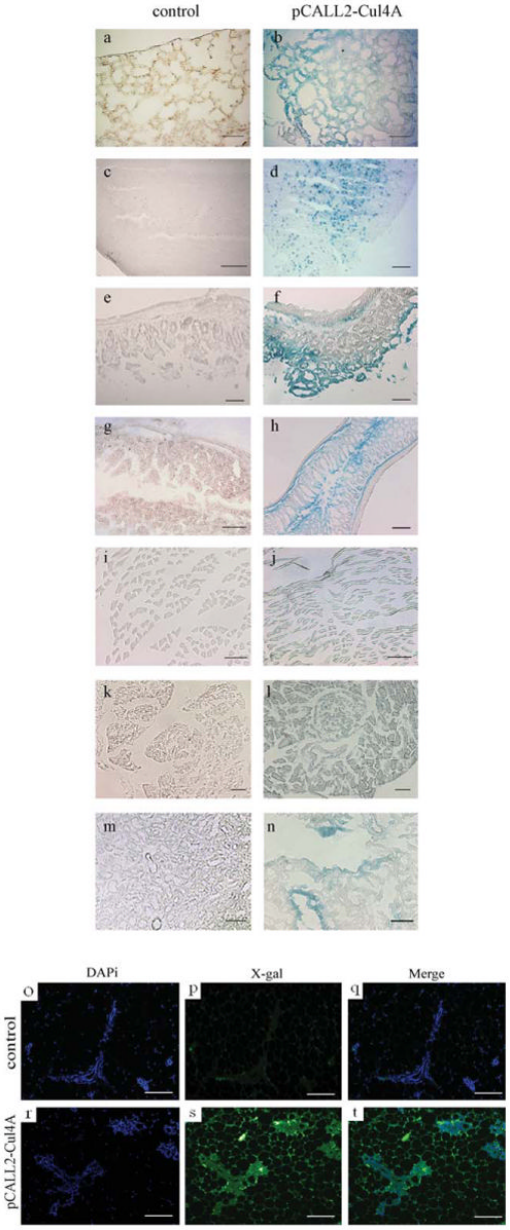

FIG. 2.

Transgene expression assessed by X-gal staining in several organs of transgenic mice from lines 1 and 23 (**a**–**n**), and immunofluorescence staining for anti-LacZ (X-gal) in mammary glands from Line 23 (**o**–**t**). In (a–n), the transgene line (pCALL2-*Cul4A*) is shown on the right, and the negative control is shown on the left. (a,b) Lung; (c,d) Brain; (e,f) Stomach; (g,h) Gut; (i,j) Muscle; (k,l) Pancreas; and (m,n) Kidney. (o–t) Immunofluorescence staining for anti-LacZ in mammary gland sections from control mice (top panels) and pCALL2-*Cul4A* mice (bottom panels). Scale bar, 100 μm for (a–h,m–t) and 50 μm for (i–l).

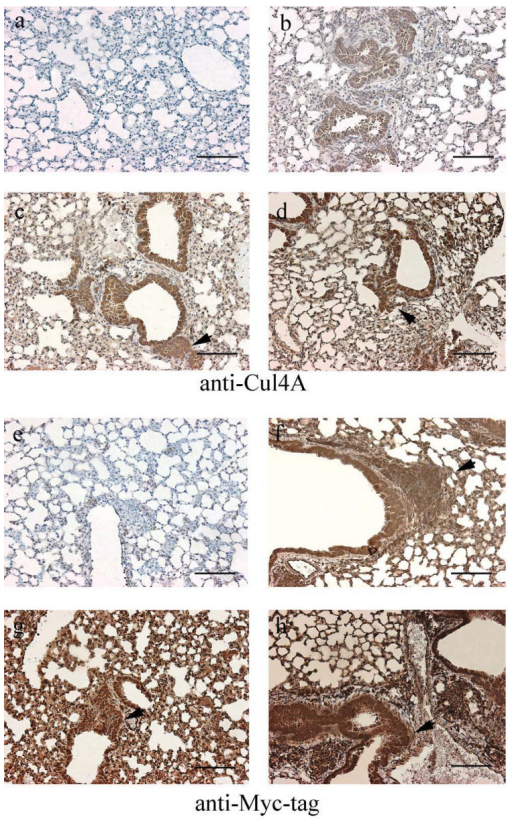

FIG. 3.

Immunostaining in lung sections from pCALL2-*Cul4A* mice with Ade-GFP (**a**,**e**) or Ade-Cre (**b**–**d**, **f**–**h**). (a–d) Anti-Cul4A; (e–h) Anti-Myc-tag; (b,f) Line 6; (c,g) Line 1; and (d,h) Line 23. The potential hyperplasia regions were marked by arrows in c, d, f, g, and h. Scale bar, 100 μm.

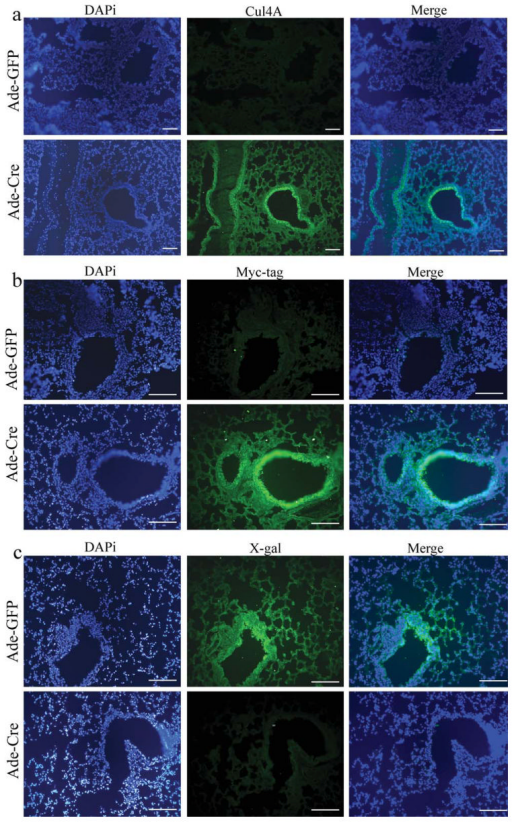

FIG. 4.

Immunofluorescence staining in lung sections from pCALL2-*Cul4A* mice with Ade-GFP (top panels) or Ade-Cre (bottom panels). (**a**) Anti-Cul4A. (**b**) Anti-Myc-tag. (**c**) Anti-LacZ. Scale bar, 100 μm.