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Thyroid Hormone Receptor Repression Linked to Type I Pneumocyte Associated Respiratory Distress Syndrome

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Abstract

Although the lung is a defining feature of air-breathing animals, the pathway controlling the formation of the type I pneumocyte, the cell that mediates gas exchange, is poorly understood. In contrast, glucocorticoids and their cognate receptor (GR) have long been known to promote type II pneumocyte maturation; prenatal administration of glucocorticoids is commonly used to attenuate the severity of infant respiratory distress syndrome (RDS). Here we show that knock-in mutations of the nuclear corepressor SMRT in C57Bl6 mice ($SMRT^{mRID}$) produces a novel respiratory distress syndrome due to prematurity of the type I pneumocyte. Though unresponsive to glucocorticoids, treatment with anti-thyroid hormone drugs (propylthiouracil or methimazole) completely rescues the SMRT-induced RDS, suggesting an unrecognized and essential role for the thyroid hormone receptor (TR) in lung development. We show that TR and SMRT control type I pneumocyte differentiation through Klf2, which in turn appears to directly activate the type I pneumocyte gene program. Conversely, mice without lung Klf2 lack mature type I pneumocytes and die shortly after birth, closely recapitulating the $SMRT^{mRID}$ phenotype. These results identify

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Author Contributions

L.P. led the project, designed and performed most of the experiments. M.L. is a pathologist who evaluated all the anatomy, histology and staining results. A.A. analyzed the blood T4/rT3 levels in newborn pups using mass spectrometry. R.N. generated the SMRT knock-in mice. R.T.Y analyzed the microarray data. H-R. L. provided expertise in EM studies. G.B., J.W., D.G., M.H., K.K., M.D., H.P. and J.B.L. provided intellectual input and technical expertise. J.B.L. provided the $Klf2^{-/-}$ ES cells. R.M.E. supervised the project. L.P. and R.M.E. wrote the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

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a second nuclear receptor, the TR, in type I pneumocyte differentiation and suggest a new type of therapeutic option in the treatment of glucocorticoid non-responsive RDS.

At birth the neonate undergoes a profound metabolic transition and exhibits a dramatically enhanced dependence on postnatal oxidative metabolism. While this metabolic switch is a hallmark of the fetal to newborn transition, little is known about the molecular genetics that direct this process. Physiologically, it is the first breath that expands the lung, enabling enhanced oxygenation of the blood. Failure of the lung to expand, such as in infant RDS, is one of the most common causes of neonatal mortality. The program of lung morphogenesis begins with the embryonic lung buds originating from foregut endoderm to form airways that branch into millions of alveoli required for air exchange immediately after birth[1,2]. These alveoli are lined by two types of epithelial cells: gas permeable type I pneumocytes which are responsible for air exchange, and type II pneumocytes which produce surfactant to reduce surface tension[3–5].

Nuclear receptors (NRs) are ligand-activated transcription factors that play important roles in normal physiology and pathological conditions[6]. Their activities are not only determined by the availability of their respective ligands, but also by their association with certain coactivators and corepressors. SMRT (Silencing Mediator of Retinoid and Thyroid hormone receptors) was initially identified as a transcriptional corepressor that maintains the ‘off state’ non-liganded NRs[7,8]. Its interaction with NRs is mediated by two C-terminal L-X-X-X-I-X-X-X-I/L motifs termed Receptor Interacting Domains (RIDs)[9,10]. SMRT facilitates transcriptional repression by serving as a scaffold protein to recruit histone deacetylase complexes and chromatin remodeling factors[11–13].

We have generated SMRT knock-in mice ($SMRT^{mRID}$) harboring point mutations within both RID1 and RID2 domains that specifically disrupt the interaction between SMRT and NRs[14]. Here we show that these mice on a pure C57BL6/J background die shortly after birth due to acute RDS resulting from an abnormal terminal differentiation of type I pneumocytes. Their lungs possess normal type II pneumocyte morphologies, including surfactant production, that display an unaltered gene expression program. Thus, these $SMRT^{mRID}$ mice provide evidence for a unique NR dependent pathway in type I pneumocyte differentiation. Unexpectedly, two anti-thyroid hormone drugs (but no other NR antagonists) restored functional type I pneumocytes, fully rescuing lung development and viability. Furthermore, we provide evidence that Klf2 is downstream of TR/SMRT signaling and is both necessary and sufficient for type I pneumocyte differentiation. Prenatal administration of glucocorticoids has long been known to promote type II pneumocyte maturation and is clinically used to mitigate the severity of infant RDS. This study identifies a second crucial NR pathway controlled by the TR, SMRT and Klf2 that, when disabled, blocks type I pneumocyte differentiation resulting in catastrophic lung collapse even in the presence of surfactant. The unexpected ability to fully rescue the syndrome with a commonly used drug suggests a potential new avenue in the management of lung prematurity and infant respiratory distress.

RESULTS

SMRT^{mRID} mice die due to acute respiratory failure

We have previously described SMRT^{mRID} mice harboring point mutations that specifically disrupt SMRT interactions with NRs[14]. Although these mice are viable in a C57BL6/Sv129 mixed background, only 14% (from a total of 361 pups) survive weaning (Supplementary Fig. 1). Viability drops to less than 1% when backcrossed to a pure C57BL6/J background (>99.5% BL6/J) (4 out of 494) (Supplementary Fig. 1). This was not due to early embryonic lethality, as all genotypes were represented at close to a Mendelian ratio at mid-gestation (E13.5, Supplementary Fig. 1).

The cause of this postnatal lethality appears to be a form of acute respiratory distress marked by severe dyspnea, cyanosis (Fig. 1a), deoxygenated blood (Fig. 1b) and diffuse lung atelectasis, as visualized by lungs that sink readily in saline (Fig. 1c). Gross evaluations showed that the lungs possessed normal orientation and lobation; there were no obvious upper respiratory airway obstructions or diaphragmatic defects. Analyses of whole body sections at different planes also revealed no histological abnormalities except in the lung (data not shown).

Microscopically, both WT and SMRT^{mRID} lungs from E18.5 to P0 mice were in the saccular stage (Fig. 1d and Supplementary Fig. 2). SMRT^{mRID} lungs appeared partially collapsed with irregular and narrower airspaces. Alveoli were about 8 times smaller and alveolar walls were thickened, hypercellular and lined by cuboidal cells. Other pulmonary structures appeared histologically normal. No prenatal gross or histological differences were observed between SMRT^{mRID} and WT mice at the early (E13.5) and late (E16.5) pseudoglandular stage, pointing to a late stage maturation problem (Supplementary Fig. 2 and data not shown).

SMRT^{mRID} lungs retain premature type I pneumocytes

To ascertain the cause of postnatal death in SMRT^{mRID} mice, we next examined their lungs under higher magnification using both light (Fig. 2a) and electron (Fig. 2b) microscopy (EM). Although type II pneumocytes exhibiting normal cuboidal morphology were readily observed, few cells with the distinctive flattened morphology typical of type I pneumocytes were found in SMRT^{mRID} alveoli (Fig 2a). Similar observations were made at E18.5 (not shown). Under EM analysis, the SMRT^{mRID} alveoli contained normal surfactant, lamellar bodies and tubular myelin. Alveolar walls were lined predominantly by type II pneumocytes of normal morphology and very few type I pneumocytes with the thin, gas permeable cytoplasmic extensions covering capillaries (Fig. 2b).

While immunohistochemical examination of markers specific for both type I and II cells revealed no changes in surfactant protein levels (E18.5, Supplementary Fig. 3), dramatic reduction of typical type I pneumocyte markers (T1α and Cav1) was observed in SMRT^{mRID} lungs (Fig. 2c). Judged by the marker staining, type I pneumocyte cytoplasmic extensions cover only 10~20% of alveolar surface in SMRT^{mRID} mice compared to greater than 90% coverage in WT littermates. Though less dramatic, the mRNA levels of type I cell markers (*T1α, Aqp5 and Cav1*) were also significantly lower at both E17.5 and E18.5 (Fig.

2d and Supplementary Fig. 4), suggesting that transcription of the type I pneumocyte differentiation program is compromised at these stages. Consistent with the immunohistochemistry results, mRNA levels of type II cell marker surfactant proteins were unaffected. The mRNA levels of transcription factors *NKX2.1* (also known as *TTF1* or *T/ebp*), *HNF3β* and *GATA6*, previously implicated in lung morphogenesis[15–17], were similar between WT and SMRT^{mRID} mice (Fig. 2d and Supplementary Fig. 4). Since glycogen is used in type II cells to generate surfactants, we performed PAS staining which revealed similar glycogen levels in WT and SMRT^{mRID} lungs, further confirming that type II cells were functionally intact (Supplementary Fig. 5). Finally, as pneumocyte and alveolar development depend on normal blood flow and vascular architecture, we analyzed the terminal vascular differentiation marker PECAM-1. This revealed normal alveolar vasculogenesis within the developing alveolar structure (data not shown) which in combination with the above data suggests that type II pneumocytes, alveolar structure and vasculogenesis are normal in SMRT^{mRID} mice.

Anti-thyroid drugs restore type I pneumocytes and rescue SMRT^{mRID} mice

The above observations lead to the hypothesis that SMRT dependent repression of one or more NRs is required to establish the type I pneumocyte lineage and prevent acute respiratory failure. As SMRT^{mRID} cells exhibit higher basal receptor activity as well as enhanced response to hormones such as PPARγ agonists[14] and T3 (Supplementary Fig. 6), we conjectured that inhibiting NR activity might rescue the SMRT^{mRID} phenotype.

Accordingly, several NR antagonists were tested by delivery at different doses to pregnant females at E16.5, just prior to the gestational age when the differences in gene expression and morphology were observed. Among those, antagonists of RARα (RO 41-5253), ERs (tamoxifen), and GR/PR (RU486) did not affect the survival rate of SMRT^{mRID} pups (Supplementary Table 1). Although glucocorticoids are used clinically in infant RDS to promote surfactant production from type II pneumocytes, dexamethasone was not able to rescue SMRT^{mRID} pups, consistent with a type I pneumocyte defect. In striking contrast, two different clinically approved anti-thyroid drugs which block iodine incorporation into thyroid hormone (TH) were observed to rescue the SMRT^{mRID} pups (Supplementary Table 1). Both oral propylthiouracil (PTU, which also inhibits T4 to T3 conversion) and methimazole (MMI) administered through i.p. injection were able to achieve efficient rescue.

Not only are PTU/MMI-treated SMRT^{mRID} mice born alive, but notably they show no evidence of respiratory distress, and survived to adulthood. Grossly, lungs were pink, inflated and floated in saline (Fig. 3a and 3c, compared to Fig. 1a and 1c). Their blood was found to be properly oxygenated (Fig. 3b, compared to Fig. 1b). Microscopically, both WT and SMRT^{mRID} lungs from PTU-treated mothers were normal and in the saccular stage at E18.5 and P0 (Fig. 3d). Electron microscopy revealed terminal sacculi that were composed of normal septae with continuous cytoplasmic extension coverage from flattened type I pneumocytes as well as cuboidal type II cells containing lamellar bodies (Fig. 3e). Consistent with the morphological rescue, transcriptional activation of type I pneumocyte markers was restored and there were no significant differences in type I or II cell markers

nor lung transcription factor expression between PTU-treated WT and SMRT^{mRID} mice (Supplementary Fig. 4).

To confirm that PTU decreases serum TH levels in treated litters, we developed a very sensitive mass spectrometry assay to measure the concentrations of thyroid hormones T4, T3 and their metabolite reverse T3 (rT3) from small blood volumes (10~15 μl). The results showed that the serum T4 levels in both genotypes were indeed reduced after a few days of PTU treatment; serum T3 was not detectable (Supplementary Fig. 7 and Supplementary Table 2). It is notable that serum T4 levels in SMRT^{mRID} mice were lower than in WT littermates. This is likely due to higher TR activity (as observed in SMRT^{mRID} MEFs, Supplementary Fig. 6) in their hypothalamic-pituitary-thyroid axes, resulting in suppressed T4 production. We also note that the major embryonic form of thyroid hormone is rT3, a T4 metabolite that does not activate TR. In contrast and although thyroid hormone crosses the placenta, T4 is the dominant thyroid hormone form in pregnant females, suggesting that at this stage of embryonic development there is an active mechanism to degrade T4 to rT3 to lower TR signaling in pups (ref[18] and data not shown). TR is encoded by two genes (*TRα* and *TRβ*) with distinct expression patterns; knockout animal studies have pointed to important yet different physiological functions[19–23]. QPCR analysis of E17.5 to P0 lungs revealed that both TRα and TRβ are expressed at these stages (Supplementary Fig. 4 and data not shown), with TRβ likely to be the more abundant form (QPCR Ct values: ~25 for *TRβ* and ~28 for *TRα*).

We also tested the effect of the timing of PTU treatment on the survival rate. Notably, PTU rescued the SMRT^{mRID} mice only when started at E16.5 and withdrawn right after birth, coinciding with dramatic lung differentiation and branching from pseudoglandular stage to saccular/alveolar stage (data not shown). Shorter periods failed to effect rescue and longer periods (starting PTU before E16.5 or withdrawing after P0) resulted in the death of all pups regardless of their genotype, presumably due to cretinism[24].

SMRT regulates *Klf2* and type I pneumocyte gene program *in vitro* and *in vivo*

The above results strongly indicated that TR and SMRT signaling regulate type I pneumocyte formation without affecting other cell types in developing lungs. As little is known about this cell lineage[5,15], our SMRT^{mRID} mouse provides a unique model to probe the molecular mechanisms of type I pneumocyte differentiation. As TR and SMRT do not directly bind to the promoter regions of *T1α*, *Aqp5* and *Cav1* (data not shown), we hypothesized that one or more “mediator” factors downstream of TR and SMRT must determine type I pneumocyte differentiation.

To identify such mediator proteins, we next performed genome-wide gene expression analysis of pooled WT and SMRT^{mRID} lungs at E18.5 with or without PTU by microarray. As type I pneumocytes comprise only a small percentage of total lung cells, unsurprisingly only 21 robustly expressed genes were observed to be significantly changed between WT and SMRT^{mRID} mice, while remaining statistically unaffected by PTU rescue at E18.5 (Supplementary Table 3). We compared expression levels by real-time PCR of these 21 candidate genes in individual littermate-controlled WT and SMRT^{mRID} embryos at both E17.5 and E18.5 when type I markers were reduced, and found that only one gene, *Klf2*, also

known as lung Kruppel-like factor (*Lklf*), was significantly different at both time points while remaining statistically unchanged with PTU treatment (Fig. 4a and data not shown). More importantly, Klf2 protein levels were almost undetectable in $SMRT^{mRID}$ lungs by immunohistochemistry, while strong nuclear staining of Klf2 could be clearly found in WT branching saccular epithelial cells (Fig. 4b).

We next investigated whether TR and SMRT could repress *Klf2* transcription through direct binding to its promoter or enhancers. Use of chromatin IP followed by deep sequencing (ChIP-Seq) to map genome-wide SMRT binding sites in macrophages (ref[25] and data unpublished) reveals that SMRT binds strongly to a conserved enhancer region downstream of the mouse *Klf2* locus (Supplementary Fig. 8). Conventional ChIP confirmed that both TR and SMRT also bind to this conserved *Klf2* enhancer region (Supplementary Fig. 9) in MLE12 cells.

To determine if this enhancer contains a positive or negative TRE, we cloned it into the 3' region of a pGL4 luciferase reporter vector. Transient transfection assays with a classical DR4 (TRE) shows, as expected, that T3 increases the DR4-Luc activity in the presence of TRβ/RXRα (Supplementary Fig. 10). However, the Klf2 enhancer is repressed by T3, identifying it as a negative or nTRE. nTREs, such as in the *TSH* promoter, have the property of being repressed by T3 and activated in its absence, similar to what is seen in the Klf2 gene.

Klf2 is expressed predominantly in the lung throughout embryonic and adult stages[26]. We next asked whether Klf2 is sufficient to induce a type I pneumocyte gene program. To this end, we overexpressed Klf2 in a mouse lung carcinoma cell line, MLE12, using adenoviral vectors. The dose of adenovirus used resulted in approximately 30~50% cell infection but did not affect cell survival or growth. Expression of type I markers, including *T1α* and *Aqp5*, was induced by ectopic Klf2 in a dose-dependent manner (Fig. 4c). Typical type II pneumocyte markers remained either unchanged (*Sp-a* and *Sp-b*) or slightly increased (*Sp-c*) by Klf2 overexpression (data not shown), suggesting that Klf2 specifically drove the type I cell differentiation program.

As Klf2 is a transcription factor, we next tested whether Klf2 could directly bind and activate type I cell marker gene promoters. We noticed that proximal promoters of multiple type I cell markers contain conserved Klf2 binding sites. ChIP assays demonstrated that Klf2 directly binds to the promoter regions containing these sites (Fig. 4d). Transient transfection assays showed that Klf2 directly activated the promoters of these type I markers including *T1α*, *Aqp5* and *Cav1* (Fig. 4e), while well-established type II pneumocyte transcription factor Nkx2.1 had little effect. These results suggest that Klf2 is sufficient to directly activate a type I pneumocyte gene program *in vitro*.

Klf2 is essential for type I pneumocyte differentiation and normal lung development *in vivo*

We next asked whether Klf2 is required for type I pneumocyte differentiation and normal lung development *in vivo*. Unfortunately *Klf2* knockout mice die between E11.5 and E14.5[27,28], precluding the analysis of their lungs at later stages of development (E16.5~P0). To circumvent this early embryonic lethality, we generated chimeric mice using $Klf2^{-/-}$ ES

cells to test whether Klf2 is required for normal lung development. Among 38 chimeras that were generated from 8 litters, we found two pups which showed signs of respiratory distress and died soon after delivery by C-section at E18.5. These two pups (#19 and #33) exhibited cyanosis with uninflated lungs (Fig. 5a), closely phenocopying the RDS of the SMRT^{mRID} pups. Viable pups had no or low chimerism while the two distressed pups had highly chimeric *Klf2*$^{-/-}$ lungs (data not shown), resulting in low *Klf2* mRNA and protein levels compared to their littermates (Fig. 5b and 5c). These two mice were found to have normal expression levels of *Klf2* in other tissues examined (tail, liver and muscle, data not shown), suggesting that the phenotype is due to the high chimerism in the lungs but not other tissues. Other important lung transcription factors including *Nkx2.1*, *Gata6* and *Hnf3β* were essentially unchanged (Supplementary Fig. 11).

Histological examination revealed that the two highly chimeric pups had lung atelectasis (Fig. 5d), with few flattened cells typical of type I pneumocytes, while there were abundant numbers of cuboidal type II-like cells. All type I markers were expressed at much lower levels in these two pups (Fig. 5b). Immunohistochemistry revealed a marked reduction of type I marker proteins in their lungs (Fig. 5c). The type II pneumocytes and endothelial cells were not affected as judged from SP-B, SP-C and PECAM-1 staining (Supplementary Fig. 12 and data not shown). These results demonstrate that Klf2 is essential for type I pneumocyte differentiation and normal lung development *in vivo*.

DISCUSSION

Embryonic lung development is orchestrated by a precisely regulated cascade of morphogens, cellular signaling molecules, hormones and transcription factors[15,16,29–32]. Specific cell lineage determination factors have been characterized for many specialized cells of the lung[17,33–37]. However, the factors that direct the terminal differentiation of type I pneumocytes, the cells responsible for gas exchange in the lung, have yet to be identified[5,15]. In this study, we delineate a novel signaling pathway in which a TR-SMRT complex is required for type I pneumocyte terminal differentiation, and provide evidence that the transcription factor Klf2 is downstream of TR signaling in normal lung morphogenesis. We show that Klf2 activates a genetic program consistent with type I—but not type II—pneumocyte development through direct interactions with type I marker promoters. Importantly, mice without lung Klf2 lack mature type I pneumocytes and die postnatally, closely recapitulating the phenotype of SMRT^{mRID} animals. These findings identify a critical developmental role for thyroid hormone in the maturation of type I pneumocytes, and suggest that Klf2 is an essential mediator of thyroid hormone signaling in the lung.

Nuclear receptors and their coregulators play important roles in many physiological and pathological conditions. In the developing lung, the glucocorticoid receptor is well recognized for its role in promoting surfactant production. The retinoid acid receptors are critical for early lung morphogenesis and tracheal/esophageal separation[38]. However, the role of TR is less clear. Though some earlier reports have demonstrated that TH enhanced surfactant phospholipids synthesis from type II pneumocytes, most studies revealed that TH does not enhance surfactant protein transcription and synthesis (summarized in ref[39]). In

supporting this, large scale human clinical trials show that administration of antenatal thyrotropin-releasing hormone in addition to surfactant supplement treatment does not provide further benefit to the neonatal outcome of preterm infants[40]. We show here that anti-thyroid hormone drugs could rescue the isolated type I pneumocyte defects in SMRT^{mRID} mice. We also demonstrate that TR achieves this at least partially through direct regulation of *Klf2* transcription. These results point to a critical and previously unappreciated role of the TR in type I pneumocyte development. However, it remains possible that SMRT mutation may alter the type I pneumocyte differentiation program partially through another orphan nuclear receptor or an as-yet unknown transcriptional partner, or the effects of anti-thyroid hormone drugs could be mediated though a TR-independent mechanism. It is also likely that in addition to directly regulating *Klf2*, the TR/SMRT complex can determine type I cell fate by affecting the proliferation and/or apoptosis of its precursors. Future work using genetic animals that have altered TR signaling in the developing lung would be instrumental to prove the essential role of TR in type I pneumocyte development.

The GR plays a critical role in lung development by acting on the immature type II pneumocyte to coordinate its maturation and the production of surfactant. It is this feature that has allowed the successful application of antenatal glucocorticoid therapy to accelerate lung development in premature infants and to ameliorate infant RDS[41–43]. It is noticeable that approximately 20% to 30% of infants with RDS do not respond to surfactant replacement therapy and babies with lung hypoplasia and who are extremely premature (less than 24 weeks of gestation in human) do not respond well to exogenous surfactant replacement because of structural immaturity[44], suggesting that overcoming lung immaturities in premature infants requires the full function of both types of pneumocytes. The rescue of type I pneumocyte immaturity in SMRT^{mRID} mice by maternal anti-thyroid hormone drug treatment (PTU or MMI) identifies a key and unexpected role of the TR in controlling this critical transition of the fetus associated with the first breath.

In summary, we show that SMRT has a non-redundant role to NCoR and that its association with TR is critical for the terminal differentiation of the type I pneumocyte. While the GR has long been known to promote type II pneumocyte development, our findings reveal a novel TR dependent pathway for type I cell maturation and lung development (Fig 5e).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Metzger RJ, Klein OD, Martin GR, Krasnow MA. The branching programme of mouse lung development. Nature. 2008; 453:745–750. [PubMed: 18463632]
2. Stripp BR. Hierarchical organization of lung progenitor cells: is there an adult lung tissue stem cell? Proc Am Thorac Soc. 2008; 5:695–698. [PubMed: 18684719]
3. Williams MC. Alveolar type I cells: molecular phenotype and development. Annu Rev Physiol. 2003; 65:669–695. [PubMed: 12428023]
4. Dobbs LG, Johnson MD, Vanderbilt J, Allen L, Gonzalez R. The great big alveolar TI cell: evolving concepts and paradigms. Cell Physiol Biochem. 25:55–62. [PubMed: 20054144]
5. Herzog EL, Brody AR, Colby TV, Mason R, Williams MC. Knowns and unknowns of the alveolus. Proc Am Thorac Soc. 2008; 5:778–782. [PubMed: 18757317]
6. Evans R. A transcriptional basis for physiology. Nat Med. 2004; 10:1022–1026. [PubMed: 15459694]
7. Chen JD, Evans RM. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature. 1995; 377:454–457. [PubMed: 7566127]
8. Bookout AL, et al. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell. 2006; 126:789–799. [PubMed: 16923397]
9. Privalsky ML. The role of corepressors in transcriptional regulation by nuclear hormone receptors. Annu Rev Physiol. 2004; 66:315–360. [PubMed: 14977406]
10. Hu X, Lazar MA. The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature. 1999; 402:93–96. [PubMed: 10573424]
11. Jepsen K, Rosenfeld MG. Biological roles and mechanistic actions of co-repressor complexes. J Cell Sci. 2002; 115:689–698. [PubMed: 11865025]
12. Perissi V, Jepsen K, Glass CK, Rosenfeld MG. Deconstructing repression: evolving models of co-repressor action. Nat Rev Genet. 11:109–123. [PubMed: 20084085]
13. Jones PL, Shi YB. N-CoR-HDAC corepressor complexes: roles in transcriptional regulation by nuclear hormone receptors. Curr Top Microbiol Immunol. 2003; 274:237–268. [PubMed: 12596910]
14. Nofsinger RR, et al. SMRT repression of nuclear receptors controls the adipogenic set point and metabolic homeostasis. Proc Natl Acad Sci U S A. 2008; 105:20021–20026. [PubMed: 19066220]
15. Morrisey EE, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell. 2010; 18:8–23. [PubMed: 20152174]
16. Mendelson CR. Role of transcription factors in fetal lung development and surfactant protein gene expression. Annu Rev Physiol. 2000; 62:875–915. [PubMed: 10845115]
17. Zhang Y, et al. GATA and Nkx factors synergistically regulate tissue-specific gene expression and development in vivo. Development. 2007; 134:189–198. [PubMed: 17164424]
18. Burrow GN, Fisher DA, Larsen PR. Maternal and fetal thyroid function. N Engl J Med. 1994; 331:1072–1078. [PubMed: 8090169]
19. Fraichard A, et al. The T3R alpha gene encoding a thyroid hormone receptor is essential for post-natal development and thyroid hormone production. Embo J. 1997; 16:4412–4420. [PubMed: 9250685]
20. Wikstrom L, et al. Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor alpha 1. Embo J. 1998; 17:455–461. [PubMed: 9430637]
21. Salto C, et al. Ablation of TRalpha2 and a concomitant overexpression of alpha1 yields a mixed hypo- and hyperthyroid phenotype in mice. Mol Endocrinol. 2001; 15:2115–2128. [PubMed: 11731613]
22. Gauthier K, et al. Genetic analysis reveals different functions for the products of the thyroid hormone receptor alpha locus. Mol Cell Biol. 2001; 21:4748–4760. [PubMed: 11416150]
23. Forrest D, et al. Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor beta: evidence for tissue-specific modulation of receptor function. Embo J. 1996; 15:3006–3015. [PubMed: 8670802]

24. Hetzel BS, Mano MT. A review of experimental studies of iodine deficiency during fetal development. J Nutr. 1989; 119:145–151. [PubMed: 2493080]
25. Barish GD, et al. Bcl-6 and NF-kappaB cistromes mediate opposing regulation of the innate immune response. Genes Dev. 2010; 24:2760–2765. [PubMed: 21106671]
26. Wani MA, Wert SE, Lingrel JB. Lung Kruppel-like factor, a zinc finger transcription factor, is essential for normal lung development. J Biol Chem. 1999; 274:21180–21185. [PubMed: 10409672]
27. Wani MA, Means RT Jr, Lingrel JB. Loss of LKLF function results in embryonic lethality in mice. Transgenic Res. 1998; 7:229–238. [PubMed: 9859212]
28. Kuo CT, et al. The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis. Genes Dev. 1997; 11:2996–3006. [PubMed: 9367982]
29. Warburton D, et al. The molecular basis of lung morphogenesis. Mech Dev. 2000; 92:55–81. [PubMed: 10704888]
30. Whitsett JA, Wert SE, Trapnell BC. Genetic disorders influencing lung formation and function at birth. Hum Mol Genet. 2004; 13(Spec No 2):R207–R215. [PubMed: 15358727]
31. Domyan ET, Sun X. Patterning and plasticity in development of the respiratory lineage. Dev Dyn. 2011; 240:477–485. [PubMed: 21337460]
32. Sheppard D. Functions of pulmonary epithelial integrins: from development to disease. Physiol Rev. 2003; 83:673–686. [PubMed: 12843406]
33. Minoo P, Su G, Drum H, Bringas P, Kimura S. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(−/−) mouse embryos. Dev Biol. 1999; 209:60–71. [PubMed: 10208743]
34. Chen G, et al. SPDEF is required for mouse pulmonary goblet cell differentiation and regulates a network of genes associated with mucus production. J Clin Invest. 2009
35. Tsao PN, et al. Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. Development. 2009; 136:2297–2307. [PubMed: 19502490]
36. Borges M, et al. An achaete-scute homologue essential for neuroendocrine differentiation in the lung. Nature. 1997; 386:852–855. [PubMed: 9126746]
37. Morimoto M, et al. Canonical Notch signaling in the developing lung is required for determination of arterial smooth muscle cells and selection of Clara versus ciliated cell fate. J Cell Sci. 2010; 123:213–224. [PubMed: 20048339]
38. Mendelsohn C, et al. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development. 1994; 120:2749–2771. [PubMed: 7607068]
39. Gross I. Regulation of fetal lung maturation. Am J Physiol. 1990; 259:L337–L344. [PubMed: 2260669]
40. Merrill JD, Ballard RA. Antenatal hormone therapy for fetal lung maturation. Clin Perinatol. 1998; 25:983–997. [PubMed: 9891625]
41. Effect of corticosteroids for fetal maturation on perinatal outcomes. NIH Consensus Development Panel on the Effect of Corticosteroids for Fetal Maturation on Perinatal Outcomes. JAMA. 1995; 273:413–418. [PubMed: 7823388]
42. Bizzarro MJ, Gross I. Effects of hormones on fetal lung development. Obstet Gynecol Clin North Am. 2004; 31:949–961. xii. [PubMed: 15550344]
43. Purdy IB, Wiley DJ. Perinatal corticosteroids: A review of research. Part I: Antenatal administration. Neonatal Netw. 2004; 23:15–30. [PubMed: 15077857]
44. Ho NK. Factors affecting responses of infants with respiratory distress syndrome to exogenous surfactant therapy. Singapore Med J. 1993; 34:74–77. [PubMed: 8266136]
45. Lim BK, et al. Coxsackievirus and adenovirus receptor (CAR) mediates atrioventricular-node function and connexin 45 localization in the murine heart. J Clin Invest. 2008; 118:2758–2770. [PubMed: 18636119]
46. Zhang Y, Conrad AH, Conrad GW. Detection and quantification of 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine by electrospray ionization tandem mass spectrometry. J Am Soc Mass Spectrom. 2005; 16:1781–1786. [PubMed: 16182556]

47. Yue B, et al. Free thyroid hormones in serum by direct equilibrium dialysis and online solid-phase extraction--liquid chromatography/tandem mass spectrometry. Clin Chem. 2008; 54:642–651. [PubMed: 18258669]

48. Hsiao A, Ideker T, Olefsky JM, Subramaniam S. VAMPIRE microarray suite: a web-based platform for the interpretation of gene expression data. Nucleic Acids Res. 2005; 33:W627–W632. [PubMed: 15980550]

49. Pei L, et al. NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism. Nat Med. 2006; 12:1048–1055. [PubMed: 16906154]

50. Nelson JD, Denisenko O, Bomsztyk K. Protocol for the fast chromatin immunoprecipitation (ChIP) method. Nat Protoc. 2006; 1:179–185. [PubMed: 17406230]

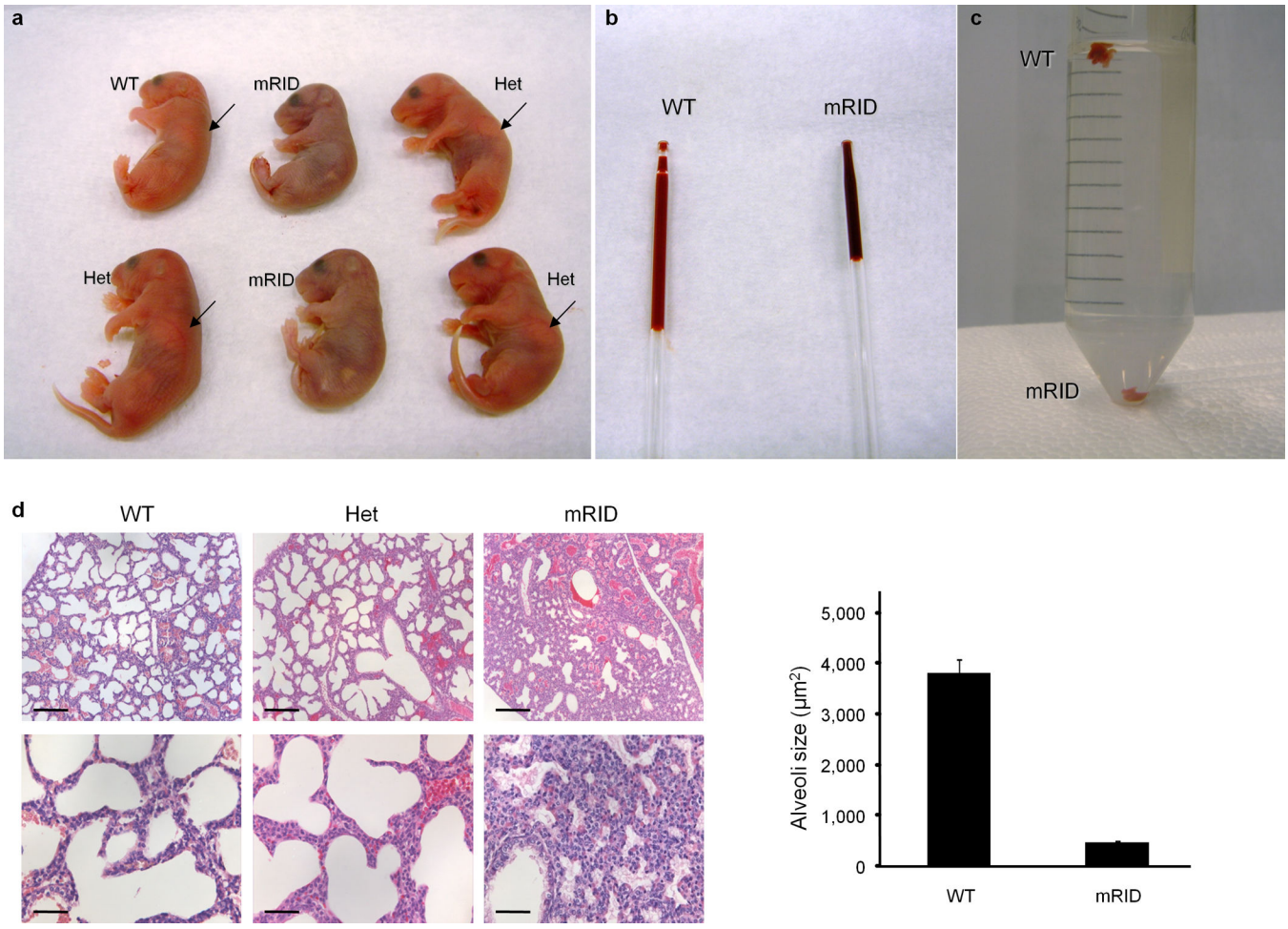

Figure 1. SMRT^{mRID} mice die postnatally due to acute respiratory failure

a) A representative newborn SMRT litter, with two SMRT^{mRID} pups in the middle. Arrows indicate the visible lungs.

b) A representative picture of blood from newborn WT and SMRT^{mRID} littermates.

c) Comparison of the lungs from newborn WT and SMRT^{mRID} littermates in PBS.

d) Microscopic images of H&E stained lung tissue from newborn SMRT littermates and the quantification of alveoli sizes. Scale bars, upper panel, 200 μm. Scale bars, lower panel, 50 μm.

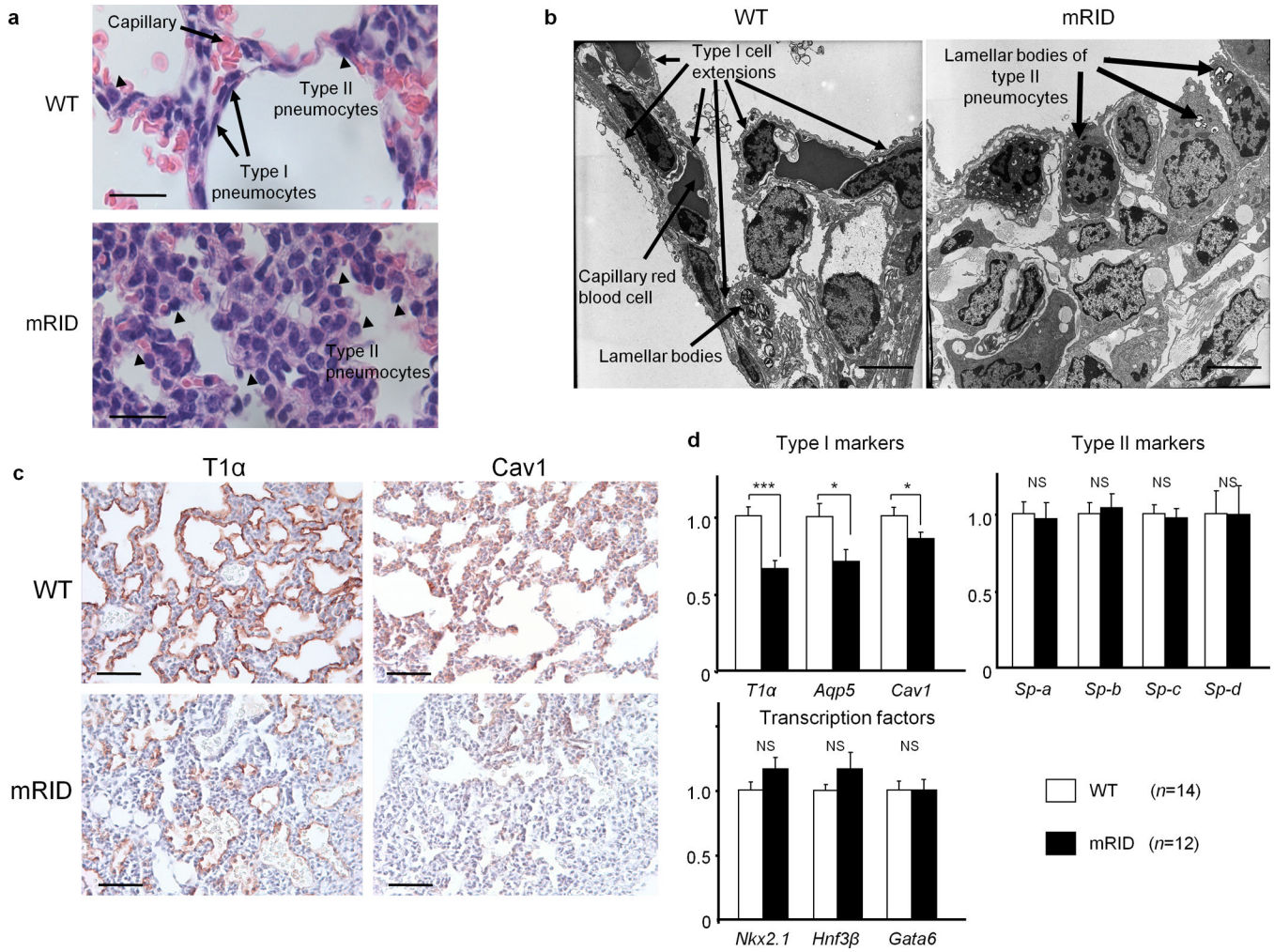

Figure 2. Lungs of SMRT^{mRID} mice lack mature type I pneumocytes

a) High magnification light microscopic images of H&E stained lung tissue from newborn WT and SMRT^{mRID} littermates. Arrowheads indicate some of the type II pneumocyte-like cells. Scale bars, 20 μm.

b) Electron microscopic images of lung tissue from newborn WT and SMRT^{mRID} littermates. Scale bars, 5 μm.

c) Immunostaining of type I pneumocyte markers (T1α and Cav1) in E18.5 WT and SMRT^{mRID} littermate lungs. Scale bars, 50 μm.

d) Expression of type I and II pneumocyte markers in E17.5 SMRT littermate lungs. Gene expression in each individual embryo was analyzed. * p<0.05; *** p<0.001.

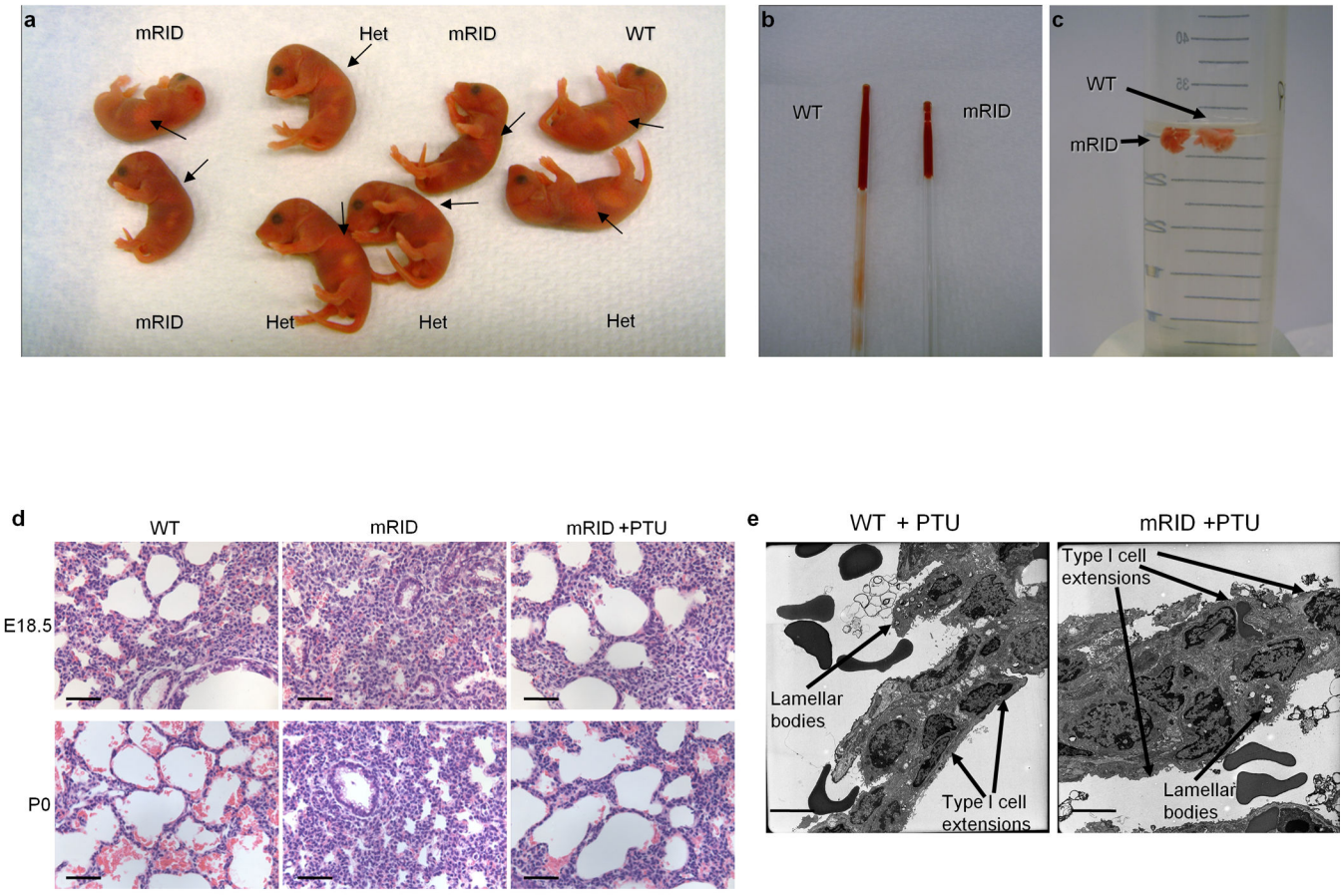

Figure 3. Anti-thyroid drugs rescue SMRT^{mRID} animals and restore type I pneumocyte development

a) A representative PTU treated newborn SMRT litter. Arrows indicate the visible lungs.

b) A representative picture of the blood from PTU treated newborn WT and SMRT^{mRID} littermates.

c) Comparison of the lungs from PTU treated newborn WT and SMRT^{mRID} littermates in PBS.

d) Microscopic images of H&E stained lung tissue from SMRT littermates (E18.5 and P0). Scale bars, 50 μm.

e) Electron microscopic images of lung tissue from PTU treated newborn WT and SMRT^{mRID} littermates. Scale bars, 5 μm.

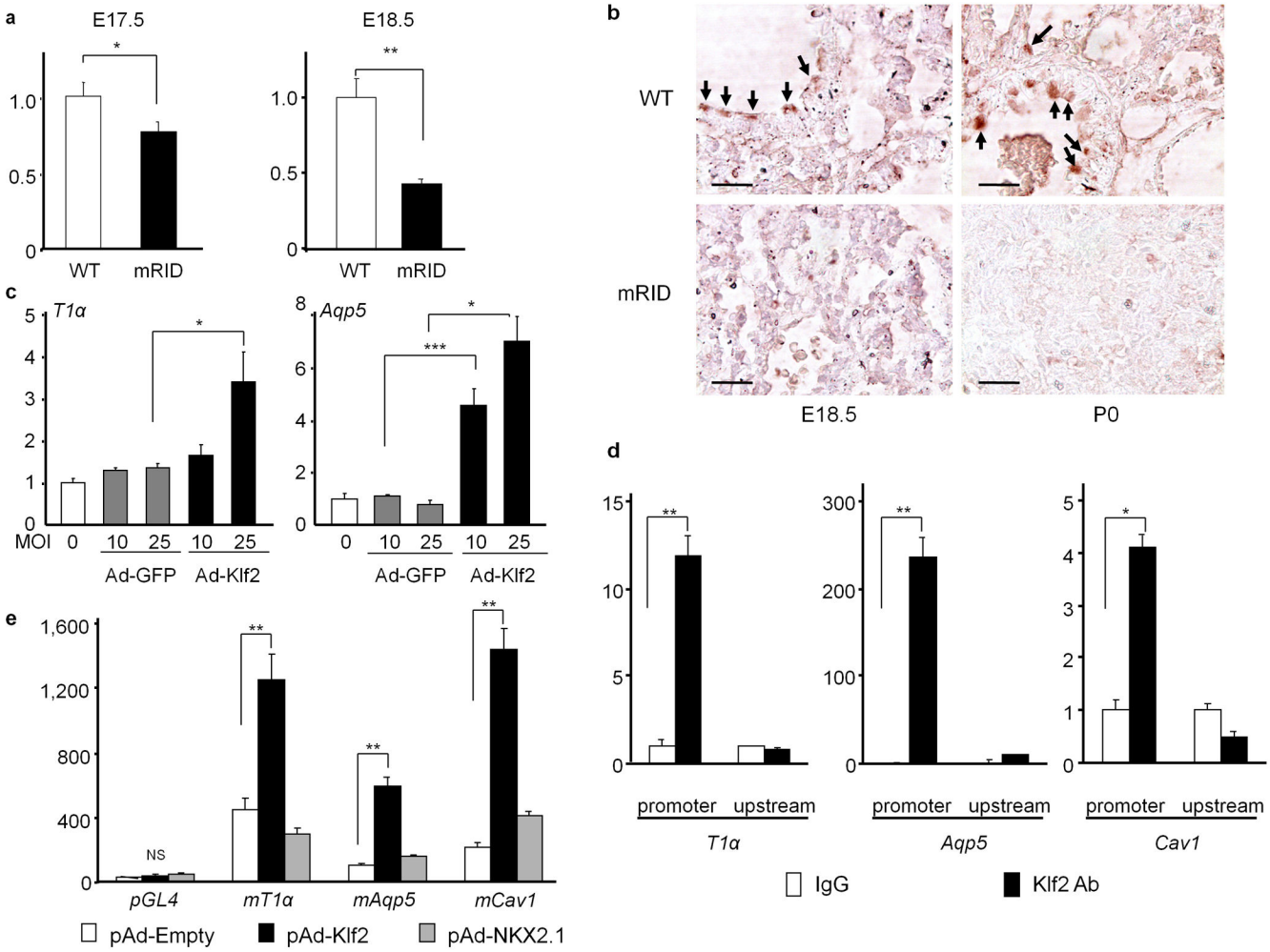

Figure 4. Klf2 is reduced in SMRT^{mRID} lungs and activates type I pneumocyte gene program

a) Expression of *Klf2* in E17.5 and E18.5 SMRT littermate lungs. Gene expression in each individual embryo was analyzed. * $p<0.05$; ** $p<0.01$.

b) Immunostaining of Klf2 in E18.5 and P0 WT and SMRT^{mRID} littermate lungs. Scale bars, 50 μm.

c) Adenoviral-mediated Klf2 expression induces type I pneumocyte marker expression in MLE12 cells. The MOI of adenovirus used is indicated. * $p<0.05$; *** $p<0.001$.

d) Klf2 binds to the promoter region of type I pneumocyte marker genes in a ChIP assay. * $p<0.05$; ** $p<0.01$.

e) Klf2, but not NKX2.1, directly activates *T1α, Aqp5* and *Cav1* promoters. Transient transfections were done in triplicate wells in CV-1 cells and repeated at least 3 times. Luciferase values normalized to β-gal were shown. ** $p<0.01$.

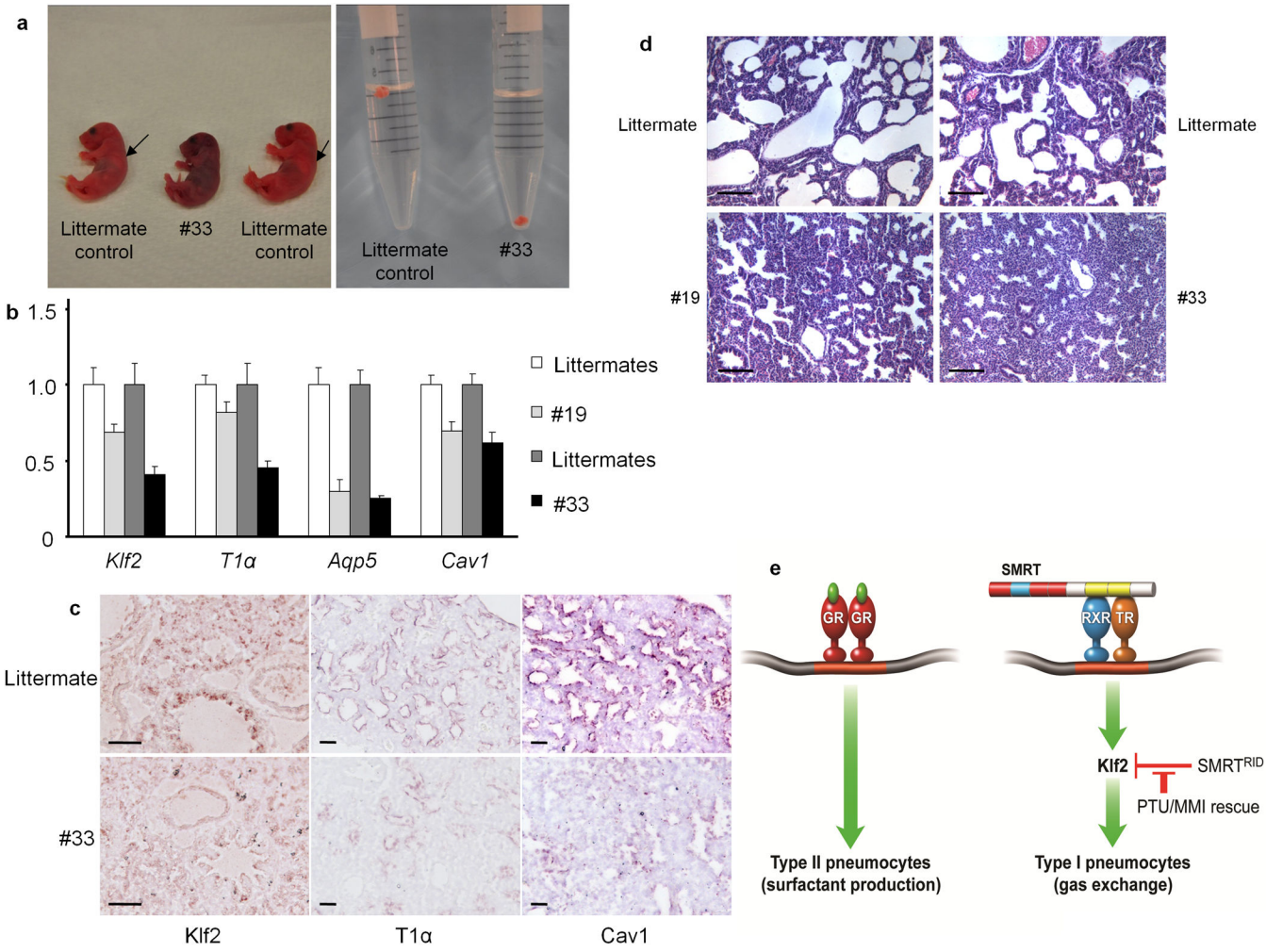

Figure 5. Klf2 is essential for type I pneumocyte and normal lung development *in vivo*

a) A representative picture showing a highly *Klf2* chimeric mouse (#33, in the middle) and its sinking lung in PBS compared to its littermates. The lungs of its littermates are clearly visible and indicated by arrows.

b) Expression of *Klf2* and type I pneumocyte markers in highly *Klf2* chimeric mice and their respective littermates.

c) Immunostaining of Klf2 and type I pneumocyte markers in highly *Klf2* chimeric mice and their respective littermates. Scale bars, 50 μm.

d) H&E staining of lungs from highly *Klf2* chimeric mice and their respective littermates. Scale bars, 100 μm.

e) A working model proposing that GR and TR regulate distinct pathways of pneumocyte development.