Aberrant lung lipids cause respiratory impairment in a Mecp2-deficient mouse model of Rett syndrome

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Abstract

Severe respiratory impairment is a prominent feature of Rett syndrome, an X-linked disorder caused by mutations in methyl CpG-binding protein 2 (MECP2). Despite MECP2's ubiquitous expression, respiratory anomalies are attributed to neuronal dysfunction. Here, we show that neutral lipids accumulate in mouse Mecp2-mutant lungs, whereas surfactant phospholipids decrease. Conditional deletion of Mecp2 from lipid-producing alveolar epithelial 2 (AE2) cells causes aberrant lung lipids and respiratory symptoms, whereas deletion of Mecp2 from hindbrain neurons results in distinct respiratory abnormalities. Single-cell RNA sequencing of AE2 cells suggests lipid production and storage increase at the expense of phospholipid synthesis. Lipid production enzymes are confirmed as direct targets of MECP2-directed nuclear receptor co-repressor 1/2 transcriptional repression. Remarkably, lipid-lowering fluvastatin improves respiratory anomalies in Mecp2-mutant mice. These data implicate autonomous pulmonary loss of MECP2 in respiratory symptoms for the first time and have immediate impacts on patient care.

Introduction

Altered pulmonary lipid metabolism is a feature of many respiratory diseases. Pulmonary surfactant, a lipid-rich complex of phospholipids, proteins and neutral lipids, is essential for lowering surface tension in the lung and preventing alveolar collapse (1). The abundance and species composition of surfactant lipids requires tight control; phosphatidylcholines (PCs) represent 85% of the lipids in pulmonary surfactant, whereas phosphatidylglycerols make up 11%. Other components, including cholesterol and other neutral lipids are present, though the role of triglycerides (TGs) in the lung has not been well defined (2). Pulmonary surfactant is an absolute requirement for normal gas exchange. Preterm infants born before producing enough surfactant develop respiratory distress syndrome (3). Mutations in surfactant production genes, surfactant-protein B (SFTPB), C (SFTPC), or the lipid transporter ATP-binding cassette subfamily A member 3 (ABCA3) cause surfactant dysfunction; neonates born with these mutations generally experience respiratory failure due to low quantities of surfactant phospholipids and surfactant dysfunction (4–6). In addition, reduced activity of sterol...
regulatory element-binding protein (SREBP), a lipid-sensing transcriptional activator, increases lung cholesterol and TGs while decreasing phospholipids in mice (7).

Breathing abnormalities, including hyperventilation, apneas and breath holds, are a prominent feature of Rett syndrome (RTT), a severe neurological condition (8). RTT is caused by mutations in the X-linked gene methyl CpG-binding protein 2 (MECP2) (9). Affected patients reach normal developmental milestones until 6–18 months of age, when they experience a developmental regression involving loss of acquired verbal and motor skills, stereotypic hand wringing and seizures (10). Respiratory dysfunction causes up to 80% of RTT patient deaths (11), to breathing symptoms in male Mecp2−/− mice. Transmission electron microscopy (TEM) was performed on perfused, inflation-fixed lungs. Lipid accumulation was present in postnatal day (P) 56 Mecp2−/− mouse lungs, a time point after the onset of symptoms (symptomatic) (Fig. 1A). Lipid droplets were confirmed by histological staining (Fig. 1B, Supplementary Material, Fig. S1A and B) and were absent in age-matched +/Y mice. Although brain and liver lipid metabolism defects are present in Mecp2−/− mice (30,33), the observation of aberrant lung lipids was unexpected. Female Mecp2+/− mice, a more clinically relevant model, also exhibit drastic lung lipid accumulation at the peri- and post-symptomatic time points of 6 and 9 months of age, respectively, with a more dramatic progression likely due to their longer lifespan (Fig. 1C and D).

High-performance liquid chromatography (HPLC) was used to quantify lung lipids. In the lung, de novo lipid synthesis produces pulmonary surfactant, a biochemically heterogeneous complex of lipids and proteins that lines the epithelial layer to facilitate normal gas exchange. Consistent with the TEM findings, Mecp2−/−/Y mice have excess TGs in their lungs at the pre-symptomatic time point of P21 (5.31 ± 0.56 μg/g in +/Y; 9.46 ± 0.58 μg/g in Mecp2−/−/Y, P = 0.002) and the symptomatic time point of P56 (11.15 ± 1.18 μg/g in +/Y; 19.39 ± 2.15 in Mecp2−/−/Y, P = 0.015) (Fig. 1D). Mecp2−/− mice also have excess cholesterol in their lungs at P21 (2.39 ± 0.19 in +/Y; 3.00 ± 0.06 in Mecp2−/−/Y, P = 0.022) but not at P56 (Fig. 1F). Therefore, lung lipid accumulation precedes respiratory and behavioral symptoms, which typically appear at ~P28. To quantify lipids in the pulmonary surfactant, bronchoalveolar lavage (BAL) fluid was subjected to liquid chromatography-mass spectrometry (LC-MS/MS). Dipalmitoylphosphatidylcholine (DPPC) is the major constituent of pulmonary surfactant, making up ~40% of its normal composition. DPPC is the strongest surface-active molecule in the pulmonary surfactant mixture. Other PC molecules in lung surfactant regulate its fluidity. DPPC was markedly reduced in the Mecp2−/−/Y BAL fluid at both P21 (156.91 ± 12.58 μg/ml in +/Y; 64.80 ± 3.02 in Mecp2−/−/Y, P = 0.0024) and P56 (117.58 ± 3.96 μg/ml in +/Y; 86.78 ± 9.38 in Mecp2−/−/Y, P = 0.0116) (Fig. 1G). Other PC species were also detected at lower quantities in Mecp2−/−/Y BAL fluid (Supplementary Material, Fig. S1D and F), but total lung PCs were not changed (Supplementary Material, Fig. S1C and E). Finally, cholesterol was increased in Mecp2−/−/Y BAL at P21 (243.25 ± 283.52 μg/ml in +/Y; 8643.25 ± 381.76 in Mecp2−/−/Y, P = 0.0436), but was at normal levels by P56 (Fig. 1H). Altogether, these findings indicate an increase in cholesterol in neutral lipids in the lung tissue with a parallel decrease in PCs in the BAL fluid.

Female Mecp2+/− mice also have elevated lung TGs at 3 months of age (6.87 ± 0.35 μg/g in +/Y; 8.80 ± 0.41 in Mecp2+/−/+, P = 0.012), and this elevation remains over the course of disease progression up to 12 months (P < 0.05, Fig. 1I). In contrast, lung cholesterol transiently increases at 6 months in Mecp2−/−/+ lungs, during the peri-symptomatic period (4.48 ± 0.47 mg/g in +/Y; 6.38 ± 0.25 in Mecp2−/−/+, P = 0.012) (Fig. 1J). DPPC (PC 32:0) was markedly decreased in Mecp2−/−/+ BAL fluid at both 6 months (117.58 ± 7.9 μg/ml in +/Y; 88.14 ± 3.8 in Mecp2−/−/+, P = 0.016) and 9 months of age (115.02 ± 3.25 μg/ml in +/Y; 85.09 ± 11.9 in Mecp2−/−/+, P = 0.045) (Fig. 1K), and again, other PC species were altered (Supplementary Material, Fig. S1G–I). However, BAL cholesterol was unchanged at both time points (Fig. 1I).

Conditional deletion of Mecp2 from mouse lung is sufficient to cause lung lipid accumulation and respiratory symptoms

In the lung, alveolar epithelial 2 (AE2) cells are metabolically and functionally complex epithelial cells whose primary role is to synthesize, secrete and recycle pulmonary surfactant. AE2 cells produce all subclasses of surfactant lipids, including PCs and
Figure 1. Mecp2 deletion alters lipid composition of the mouse lung. (A) Electron micrographs of the alveolar area of the lung in male +/Y and Mecp2/Y mice at P56. Scale bars represent 2 μm. Asterisk indicates lipid droplets. (B) Oil Red O staining of neutral lipids (red) in alveoli of male +/Y and Mecp2/Y mice at P56. Scale bars represent 50 μm. Electron micrographs of female +/+ and Mecp2/+ lungs at (C) 6 months and (D) 9 months. Scale bars represent 2 μm. Asterisk indicates lipid droplets, n = 2 per age/genotype. Whole-lung (E) triglycerides and (F) cholesterol as measured by high-performance liquid chromatography in +/Y and Mecp2/Y mice, n = 4. Bronchoalveolar lavage (BAL) fluid (G) PC 32/0 (dipalmitoylphosphatidylcholine, DPPC) and (H) cholesterol as measured by LC–MS/MS in +/Y and Mecp2/Y mice, n = 4. Lung (I) triglycerides and (J) cholesterol and BAL fluid (K) PC 32/0 and (L) cholesterol in female +/+ and Mecp2/+ mice, n = 4. Data are expressed as mean ± SEM. Statistics were performed using Student’s t-test. *P < 0.05, **P < 0.01.

Neutral lipids. Mecp2<sup.tm3.1Bird</sup> mice, which have a green fluorescent protein (GFP) tag fused to the endogenous Mecp2 locus, were used to visualize expression. High MECP2 expression was present in lung AE2 cells (Fig. 2A), consistent with previous reports in mouse and human (32,34). Therefore, Mecp2 deficiency in lung AE2 cells alone could alter lung lipid metabolism, which may contribute to abnormal breathing in Mecp2-mutant mice. As respiratory symptoms of RTT are traditionally attributed to neuronal loss of Mecp2, the effect of AE2 cell-specific Mecp2 deficiency was compared with
Figure 2. Conditional deletion of MeCP2 in lung AE2 cells causes lipid accumulation and respiratory symptoms. (A) Lungs of MeCP2<sup>flx<sup>tm3.1Bird</sup> mice were used to assess MECP2’s localization. Blue: DAPI, green: GFP (MECP2), red: surfactant protein C (SPC, marker of alveolar epithelial 2 (AE2) cells). Scale bars represent 40 μm, arrows point to AE2 cells expressing MECP2. (B) Breeding scheme: B6.Mecp2<sup>flx<sup>tm1(Tmox)<sup>ERT2</sup>>tm1(Sftpctm1<sup>cre/ERT2</sup>)BLH mice, which express Cre under a tamoxifen (Tmx)-inducible a neuron-specific deletion in the hindbrain, the site of neuronal control of respiration (Fig. 2B). MeCP2 was removed from AE2 cells by breeding B6.Mecp2<sup>flx<sup>tm3.1Bird</sup> mice to B6.Sftpctm1<sup>cre/ERT2</sup>>tm1(Sftpctm1<sup>cre/ERT2</sup>)BLH mice, which express Cre under a tamoxifen (Tmx)-inducible
promoter of the surfactant protein C (Sftpc) gene, resulting in Sftpc-CreERT2, Mecp2-flx conditional knockout (AE2-cKO) mice. Sftpc is expressed exclusively in AE2 cells. Mecp2 was deleted from hindbrain neurons using Cg-Tg(Atoh1-cre)1Bri mice, which express Cre under the promoter of atonal basic helix loop helix (BHLH) Transcription Factor 1 (Atoh1). The resulting Atoh1-Cre; Mecp2-flx (‘hindbrain-cKO’) mice lack Mecp2 in the medulla oblongata and pons, the respiratory centers of the brain responsible for maintaining respiratory rhythm. As a final control, B6.Mecp2-null (Mecp2Δ/Y) mice were generated by treating B6.Mecp2flxO/Eym embryos with a ubiquitously expressed Cre recombinase. Male C57BL/6 mice were used in this experiment, allowing the assessment of lung lipids on a second genetic background as previous experiments were carried out on the 129Sv/Ev genetic background. Tissue-specific deletion and reduced Mecp2 expression was confirmed by polymerase chain reaction (PCR) (Supplementary Material, Fig. S2A).

As Mecp2 deficiency causes neurological disease, neurobehavioral phenotypes were assessed through subjective health scores and deficits in motor coordination (Fig. 2C, Supplementary Material, Fig. S2B–F). As expected, Mecp2Δ/Y mice develop neurobehavioral phenotypes, whereas AE2-cKO do not. Hindbrain-cKO have increased subjective health scores and deficits in motor coordination (Fig. 2C, Supplementary Material, Fig. S2B–F). Whole lung, BAL fluid and serum lipids were also measured. At P70, AE2-cKO mice show a significant increase in lung TGs (Fig. 2D, 6.86 ± 0.57 in +/Y; 14.76 ± 3.59 mg/g in AE2-cKO, P = 0.043), along with a decrease in BAL fluid PC 32/0 (Fig. 2F, 124.59 ± 8.51 in +/Y; 80.14 ± 6.72 in AE2-cKO, P = 0.0087) and BAL fluid cholesterol (Fig. 2G, 38.99 ± 3.33 in +/Y; 27.14 ± 2.39 in AE2-cKO, P = 0.030). However, they do not have altered serum TGs or cholesterol (Supplementary Material, Fig. S2G–H, P > 0.05). Mecp2Δ/Y mice have the same altered lung lipid profile as AE2-cKO mice; however, they also have increased lung cholesterol (Fig. 3F, 5.61 ± 0.55 in +/Y; 8.49 ± 0.84 in Mecp2Δ/Y, P = 0.015), as well as increased serum TGs and cholesterol (Supplementary Material, Fig. S2G–H). In contrast, hindbrain-cKO mice do not show any changes in lung, BAL fluid or serum lipids (P > 0.05). No significant changes were found in any parameters across littermate controls of AE2-cKO and hindbrain-cKO mice (Supplementary Material, Figs S3 and S4).

To assess respiratory symptoms, we used whole-body plethysmography (WBP), a non-invasive, quantitative method in which minute pressure changes in unrestrained mice are monitored and converted into respiratory waveforms. Compared with +/Y mice, both AE2-cKO and Mecp2Δ/Y mice have an elevated baseline breathing frequency (378.93 ± 3.6 breaths/min in +/Y, 442.93 ± 17.4 in Mecp2Δ/Y, P = 0.008, and 452.77 ± 17.4 in AE2-cKO, P = 0.002) (Fig. 2H). However, only hindbrain-cKO mice have an increase in tidal volume (0.24 ± 0.006 ml in +/Y; 0.27 ± 0.007 in hindbrain-cKO, P = 0.02) (Fig. 2I). Thus, Mecp2 deficiency in the lung and hindbrain may impart distinct respiratory symptoms. Apneas are the most characteristic respiratory symptom of RTT patients. Both AE2-cKO and hindbrain-cKO show a significant increase in the number of apneas produced (1.71 ± 0.24 apneas/min, P = 0.0002 and 1.25 ± 0.15, P = 0.004, respectively), though apneas in Mecp2Δ/Y mice are far greater (0.08 ± 0.03 apneas/min in +/Y; 4.82 ± 1.34 in Mecp2Δ/Y, P < 0.0001) (Fig. 2J). Thus, as loss of Mecp2 in either AE2 cells or the hindbrain is sufficient to cause apneas, MECF2 may act in lung- and hindbrain-autonomous processes to regulate breathing; the combined loss of MECF2 from both centers likely imparts the full spectrum of respiratory symptoms in RTT.

Loss of Mecp2 from lung alveolar cells alters lipogenic gene expression

The data presented thus far support the idea that altered lipids in AE2 cells contribute to respiratory symptoms in mouse models of Mecp2 deficiency. Altered lipids could be a direct result of the inability to regulate lipid production upon loss of MECF2. In the liver, loss of Mecp2 increases the transcription of lipogenesis genes through loss of NCOA1/2 binding (30). Thus, we hypothesized that MECF2 regulates lipid metabolism enzymes in lung AE2 cells. To identify genes that are misregulated in Mecp2 deficiency, AE2 cells were isolated from P18 +/Y and Mecp2/Y mouse lungs by flow cytometry for single-cell RNA sequencing analysis (Supplementary Material, Fig. S5A). A pre-symptomatic time point was chosen with the aim of identifying primary disease-causing transcriptional changes (Fig. 3A). A total of 436 significantly differentially expressed genes were identified in Mecp2/Y AE2 cells (Supplementary Material, Table S1). Of these, 113 had increased expression and 323 had decreased expression. The top 20 genes with altered expression are highlighted in Figure 3B.

Many lipid metabolism genes were misregulated in Mecp2/Y AE2 cells (Table 1) and the expression of a subset of genes was confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) (Supplementary Material, Fig. S5B). The expression of hydroxymethylglutaryl-CoA synthase (Hmgcs1) is increased in Mecp2/Y AE2 cells. HMGCS1 catalyzes an early step in cholesterol biosynthesis, converting acetocetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), suggesting cholesterol biosynthesis is increased, which is consistent with lipid quantification results in Mecp2/Y lungs. Acyl-CoA thioesterase 1 (Acot1) is the second most significantly increased gene in Mecp2/Y AE2 cells [log-fold change (FC): 1.13, P-value: 3.10E-123]. Consistently, its family member Acot2 is also expressed at high levels compared with +/Y cells. When required for energy or other metabolic processes, fatty acids are converted to acyl-CoAs by long-chain acyl-CoA synthetases (ACSs) and transported to the mitochondria for beta-oxidation. Acyl-CoA thioesterases (ACOTs) catalyze the reverse reaction, and as such, ACSs and ACOTs direct the metabolic fate of fatty acids by channeling substrates toward or away from beta-oxidation. These results suggest fatty acids in the Mecp2/Y lung are shuttled away from mitochondrial recycling and pushed toward lipid storage pathways. Consistent with previous literature on mitochondrial involvement in RTT (35–37), loss of Mecp2 impacted electron transport chain (ETC) components, with 11 of the 13 mitochondrialy expressed ETC components being expressed at lower levels in Mecp2/Y AE2 cells (Table 1).

Despite high levels of TGs in the Mecp2/Y lung, a global decrease in lipid biosynthesis gene expression was found. Fatty acid biosynthesis genes including fatty acid synthase (Fasn), stearoyl-CoA desaturase 1/2 (Scd1,2) and elongation of very long chain fatty acids (ELOVL) fatty acid elongase were unchanged in both AE2 cells and whole lung from Mecp2/Y mice, with the exception of lipoprotein lipase (Lpl), which was decreased in Mecp2/Y AE2s (Supplementary Material, Fig S5C). Therefore, we assessed SREBP, membrane-bound transcriptional regulators of lipid homeostasis (38,39). SREBPs are synthesized as inactive precursors bound to endoplasmic reticulum membranes. When lipid levels are low, SREBPs migrate to the nucleus and bind and activate the transcription of lipogenic genes containing sterol response elements (SREs) in their promoters (40). In Mecp2/Y AE2 cells, the expression of Srebf1, which
Figure 3. Metabolic genes are altered in Mecp2/Y AE2 cells due to loss of MECP2- and NCOR1/2-mediated transcriptional repression. (A) t-SNE plot of +/Y and Mecp2/Y AE2 cells after single-cell RNA sequencing, n = 2000 cells, collected at P18. (B) Volcano plot showing genes with altered expression in Mecp2/Y AE2 cells after single-cell RNA sequencing. The top 20 significantly altered genes are labeled. Significant genes have $-\log_{10}(P\text{-value}) < 0.05$. (C) SREBP1 expression in the +/Y and Mecp2/Y lung at P21 and P56. The 68 kDa band (shown) is the active, cleaved protein, whereas the 120 kDa inactive precursor was not visualized. GAPDH was used for normalization. (D) TBL1XR1 is associated with NCOR1/2 co-repressor complex members NCOR1, MECP2 and HDAC3 in the mouse lung, as shown by co-immunoprecipitation (co-IP) assay. This complex forms without MECP2 in Mecp2/Y lung tissue. i, input (nuclear protein); ip, immunoprecipitated protein. Anti-TBL1XR1 and anti-H3K4me3 (positive control) ChIP-qPCR of sites surrounding the (E) Acot1 promoter and (F) Hmgcs1 promoter, n = 3. Data are expressed as mean ± SEM. Statistics were assessed using Student’s t-test. *$P < 0.05$, **$P < 0.01$. FC, fold change.

stimulates TG synthesis, is decreased, whereas Srebf2, which stimulates sterol synthesis, is unchanged. A western blot assay in whole lung tissue confirmed that nuclear SREBP1 expression is decreased at P21 but is normal by P56 (Fig. 3C). In contrast, nuclear SREBP2 expression is unchanged at both time points (Supplementary Material, Fig. S5D). SREBP1c transcriptionally activates the SRE-containing genes Fasn, Scd1 (41), Scd2 (42) and ATP citrate lyase (Acly) (43), which have decreased expression in Mecp2/Y AE2 cells.

PCs represent the largest percentage of lipids in pulmonary surfactant. Synthesis of PCs by the cytidine diphosphate (CDP):choline pathway begins with the uptake of exogenous choline from the bloodstream predominantly by sodium-dependent choline transporters. Choline is phosphorylated by choline kinase (CK) to form phosphocholine, which is activated by cytidine triphosphate (CTP):phosphocholine cytidylyltransferase (CCT) to make CDP-choline, which is finally joined to a diacylglycerol (DG) backbone by choline/ethanolamine phosphotransferase to form PC. In Mecp2/Y AE2 cells, the CK, choline kinase alpha (Chka) and the CPT, phosphate cytidylyltransferase 1 choline alpha (Pcyt1a) are significantly underexpressed (Chka FC: $-0.549$, P-value: $5.87E-22$; Pcyt1a FC: $-0.262$, P-value: $3.77E-5$). The PC transporter Abca3 is expressed at lower levels in Mecp2/Y AE2 cells (FC: $-0.506$, P-value: $5.42E-9$). Mutations in ABCA3 are associated with surfactant deficiency and fatal respiratory distress (44). Decreased expression of
Table 1. MeCP2 deficiency alters lipid metabolism gene expression in lung AE2 cells

| Gene                                    | Log FC (Down/Up) | P-value  |
|-----------------------------------------|------------------|----------|
| **Transcriptional regulation of lipid metabolism** |                  |          |
| Cebpα                                   | ↓ 0.66157        | 2.75E-32 |
| Srebf1                                  | ↓ 0.48646        | 1.58E-25 |
| Nr1d1                                   | ↓ 0.39904        | 2.18E-25 |
| Foxa2                                   | ↓ 0.29705        | 1.31E-11 |
| **Sterol biosynthesis**                 |                  |          |
| Pmvk                                    | ↑ 0.34146        | 3.15E-16 |
| Hmgcs1                                  | ↑ 0.28520        | 2.45E-08 |
| Srebf1                                  | ↓ 0.52903        | 9.72E-24 |
| Raly                                    | ↓ 0.25489        | 0.000109 |
| **Fatty acid biosynthesis**             |                  |          |
| Cyp5a                                   | ↑ 1.08181        | 1.88E-10 |
| Scd1                                    | ↓ 0.74866        | 2.11E-44 |
| Scd2                                    | ↓ 0.54825        | 2.27E-15 |
| Fasn                                    | ↓ 0.28478        | 5.45E-05 |
| Acox                                    | ↓ 0.55099        | 5.37E-11 |
| **Fatty acid regulation**               |                  |          |
| Acot1                                   | ↑ 1.13401        | 3.10E-123|
| Eci2                                    | ↑ 0.41057        | 2.34E-33 |
| Acot2                                   | ↑ 0.28115        | 6.12E-14 |
| Acox                                    | ↓ 0.46970        | 1.78E-14 |
| **Lipid storage**                       |                  |          |
| Plin2                                   | ↑ 0.31851        | 2.27E-17 |
| **Phospholipid biosynthesis**           |                  |          |
| Chka                                    | ↓ 0.54883        | 5.87E-22 |
| Dgkg                                    | ↓ 0.31762        | 7.60E-13 |
| Lpcat1                                  | ↓ 0.48220        | 2.39E-06 |
| Pyc1α                                   | ↓ 0.26249        | 3.77E-05 |
| Sphingolipid synthesis                  |                  |          |
| Spf2                                    | ↑ 0.37772        | 2.04E-14 |
| Sgms1                                   | ↑ 0.32411        | 1.33E-09 |
| **Lipid rafts**                         |                  |          |
| Cde2                                    | ↑ 1.31228        | 9.21E-78 |
| Cfr                                     | ↑ 0.27103        | 5.74E-11 |
| Mt1c                                    | ↑ 0.36050        | 4.80E-09 |
| Peroxisomal lipid metabolism            |                  |          |
| Prdx6                                   | ↑ 0.59145        | 2.18E-39 |
| Gpx1                                    | ↑ 0.58170        | 1.92E-34 |
| Acod                                   | ↓ 0.56540        | 4.54E-27 |
| Mitochondria (nuclear)                  |                  |          |
| Ndufa6                                  | ↑ 0.55456        | 8.27E-31 |
| Ndufa7                                  | ↑ 0.29279        | 2.91E-09 |
| Uqcr1                                   | ↑ 0.29871        | 4.72E-11 |
| Cox7a2L                                 | ↑ 0.29685        | 6.65E-10 |
| Mitochondria (mt-expressed)             |                  |          |
| Ndufa3                                  | ↓ 0.28295        | 0.000335 |
| mt-nd1                                  | ↓ 0.84693        | 1.21E-11 |
| mt-nd2                                  | ↓ 0.87795        | 3.75E-23 |
| mt-nd3                                  | ↓ 0.92690        | 6.98E-45 |
| mt-nd4                                 | ↓ 0.52441        | 0.006614 |
| mt-nd4                                 | ↓ 0.82727        | 3.84E-14 |
| mt-nd5                                 | ↓ 0.57768        | 2.96E-16 |
| mt-cyb                                 | ↓ 0.66476        | 5.31E-06 |
| mt-co1                                 | ↓ 0.53783        | 0.046432 |
| mt-co2                                 | ↓ 0.80265        | 1.68E-11 |
| mt-co3                                 | ↓ 0.78379        | 8.45E-09 |
| mt-atp6                                 | ↓ 0.58252        | 0.022863 |

Genes are classified by lipid metabolism pathway and sorted by P-value. LogFC, Log2-fold change.

PC-producing enzymes is likely responsible for the remarkably low levels of PCs in MeCP2/Y BAL fluid. Notably, Pyc1α and Abca3 are major targets of SREBP-induced activation (7,45); therefore, increased TGs could inadvertently reduce SREBP activity, limiting FC synthesis.

**MECP2 regulates the expression of lung lipid metabolism enzymes through interaction with the NCOR1/2 co-repressor complex**

The NCOR1/2 complex includes HDAC3, G protein pathway suppressor 2, TBL1X and TBL1XR1, the latter of which are direct binding partners of MECP2. NCOR1 and HDAC3 are master regulators of lipid biosynthesis in the liver (28,29). We previously showed that decreased binding of NCOR1/2 in a state of MeCP2 deficiency leads to the upregulation of lipid biosynthesis genes in the liver (30). In mouse lung, co-immunoprecipitation of TBL1XR1 revealed an association with MECP2, as well as with NCOR1/2 co-repressor complex partners NCOR1 and HDAC3 (Fig 3D). The formation of this complex in the lung suggests its role in the direct regulation of lipid metabolism gene targets. As the MECP2–NCOR1/2 complex primarily acts as a transcriptional repressor, genes with increased expression in MeCP2/Y AE2 cells were chosen as putative targets of...
MECP2-directed transcriptional regulation for chromatin immuno-precipitation (ChIP)-qPCR. ChIP was performed using an anti-TBL1XR1 antibody. Histone 3 lysine 4 trimethylation (H3K4me3) and immunoglobulin G (IgG) (Supplementary Material, Fig. S5E and F) were assessed as positive and negative controls, respectively. Loss of Mecp2 significantly hinders the binding of TBL1XR1 to Acot1 (Fig. 3D) and Hmgcs1 (Fig. 3F), both of which have increased expression in Mecp2/Y AE2 cells. These results suggest that MECP2, in concert with the NCOR1/2 co-repressor complex, directly regulates the expression of Acot1 and Hmgcs2 in the mouse lung. In contrast, TBL1XR1 does not bind to the promoter region of the peroxiredoxin-6 (Prdx6) gene, which is also expressed at high levels in Mecp2/Y AE2 cells (Supplementary Material, Fig. S5G).

Fluvastatin treatment reduces lung TG accumulation and improves breathing symptoms in male Mecp2/Y mice

As aberrant lung lipids likely contribute to respiratory symptoms in Mecp2-mutant mice, we sought to test the effects of pharmacological correction of lung lipid metabolism. Statins are commonly prescribed pharmaceuticals that reduce systemic cholesterol and TGs by interfering with lipid metabolism through competitive inhibition of HMG-CoA-reductase. Previously, two classes of statins, lovastatin and fluvastatin, were shown to improve overall health, motor activity and metabolic health in 129.Mecp2-mutant mice (33). A second study found that lovastatin was unable to improve these parameters in B6.Mecp2/Y mice (46). As lovastatin is very labile and requires careful activation and handling for its use in mice, fluvastatin was used in this study.

Weekly fluvastatin treatment reduced serum lipids in Mecp2/Y mice while improving subjective health scores and motor coordination, as previously shown (33) (Fig. 4A, Supplementary Material, Fig. S6A-D). It also lowered lung TGs (Fig. 4B), but lung cholesterol was unchanged across all groups (Fig. 4C). Remarkably, fluvastatin treatment normalized breathing frequency in Mecp2/Y mice and modestly, but not significantly, improved respiratory apneas (Fig. 4D-F).

Therefore, to validate the therapeutic relevance of metabolism-targeted treatment for human RTT patients, the effects of statin treatment on respiratory symptoms in female 129.Mecp2m1.1839f/+ mice were assessed (Fig. 4G). Fluvastatin treatment reduced serum cholesterol and improved subjective health scores and rotarod performance in Mecp2/+ mice, as previously shown (33) (Supplementary Material, Fig. S6E-H). Fluvastatin treatment also lowered lung TG levels in Mecp2/+ mice (4.14 ± 0.9 mg/g in vehicle-treated +/+; 14.70 ± 3.01 in vehicle-treated Mecp2/+; 7.54 ± 0.72 in treated Mecp2/+; P = 0.0229) (Fig. 4H). Lung cholesterol was unchanged across the four groups (Fig. 4I). Thus, low doses of fluvastatin improve aberrant lipid accumulation. Fluvastatin treatment also normalized breathing frequency in Mecp2/+ mice compared with vehicle-treated Mecp2/+ mice (273.01 ± 8.93 breaths/min in fluvastatin-treated Mecp2/+; 316.81 ± 13.77 in vehicle-treated Mecp2/+; P = 0.046; 273.03 ± 10.42 in vehicle-treated +/+; P = 0.012) to wild-type levels (0.023 ± 0.02 in vehicle-treated +/+; Fig. 4I). Therefore, statin treatment can lower lung lipids and improve respiratory symptoms in mouse models of RTT.

MECP2 directly directs lipid metabolism with the NCOR1/2 co-repressor complex, resulting in abnormal lung surfactant components

Mecp2/Y mouse lungs have a global perturbation of lung lipid metabolism, which is also present in female Mecp2/+ mice, suggesting heterozygous mosaic loss of Mecp2 is sufficient to alter lung lipids. Lipid metabolism abnormalities are evident in Mecp2-mutant mouse models and RTT patients. Symptomatic Mecp2/Y male mice have increased brain cholesterol, serum cholesterol and TGs, and develop fatty liver disease (30,33). A subset of RTT patients has increased serum cholesterol and TGs (47,48).

MECP2 is required for the NCOR1/2 co-repressor complex to bind to a subset of its gene targets, although these targets may differ across different tissues. As a master regulator of metabolism, a direct target of the MECP2–NCOR1/2 complex in the liver is squalene epoxidase (Sqle), a rate-limiting enzyme in cholesterol synthesis (30,47). In Mecp2/Y AE2 cells, the NCOR1/2 complex protein TBLX1R1 does not readily bind to Acot1 and Hmgcs1 genes, which are consistently expressed at higher levels (Fig. 5A), suggesting that they are direct targets of this repressor complex. ACOT1 is essential for directing the metabolic fate of fatty acids and its upregulation suggests decreased substrate production for beta-oxidation by shutting fatty acids away from degradation. HMGCS1 produces HMG-CoA, the substrate for the first rate-limiting step of cholesterol biosynthesis. Importantly, much of the lung’s cholesterol content is derived from the serum; therefore, increased serum cholesterol in Mecp2/Y mice may contribute to the lung cholesterol phenotype shown here. However, BAL fluid cholesterol, made exclusively by AE2 cells, is increased at P21 in Mecp2/Y mice, and lung TGs are elevated in AE2-cKO mice, in spite of the lack of elevated serum lipids. Similarly, BAL fluid PCs are decreased in AE2-cKO mice, suggesting...
Figure 4. Fluvastatin treatment reduces lung lipids and improves respiratory symptoms in MeCP2-mutant mice. (A) Male mice were administered fluvastatin or vehicle control from 4 to 8 weeks of age. HPLC analysis of lung (B) triglycerides and (C) cholesterol, n = 4. Respiratory parameters were measured: (D) breathing frequency, (E) tidal volume and (F) apneas, n = 8. (G) Female mice were administered fluvastatin or vehicle control from 6 to 32 weeks of age. HPLC analysis of lung (H) triglycerides and (I) cholesterol, n = 4. Respiratory parameters were measured: (J) breathing frequency, (K) tidal volume and (L) apneas, n = 7. Data are expressed as mean ± SEM. Statistics were performed using one-way ANOVA with Tukey’s test for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001.

decreased surfactant PCs and excess lung TGs are mechanistically linked. DGs are required for PC synthesis and are incorporated via phospholipid:diacylglycerol acyltransferase enzymes; the reverse reaction reduces the pool of PCs to make DGs, which become TGs via diglyceride acyltransferase (DGAT) enzymes. Although these genes were not identified in our single-cell RNA sequencing experiment, it is likely that loss of MECP2’s transcriptional repression at other genes that regulate lipid homeostasis via the NCOR1/2 co-repressor complex alter lipid biosynthesis and storage in the MeCP2-deficient lung. Identifying other
lipid-regulating targets of MECP2 in AE2 cells, and other cells in the lung, will be necessary for a full understanding of the cause of lung lipid abnormalities.

The drastic reduction of surfactant PCs in Mecp2-mutant mice is an important finding. Single-cell RNA sequencing revealed that Chka and Pcyt1a, two key enzymes of the PC synthesis pathway, are underexpressed. It is possible that loss of MECP2 may indirectly shut down PC synthesis through SREBP1, preventing normal surfactant production (Fig. 5). Another possibility is that TGs are shuttled toward storage, reducing the pool of DGs for PC synthesis. In addition, as BAL fluid PCs, but not lung PCs, are significantly decreased, surfactant exocytosis could be deficient, trapping PCs in AE2 cells. The resolution of these possibilities will require further exploration. Changes in phospholipid composition in the lung surfactant of Mecp2-deficient animals alone could be a major driving force of the respiratory symptoms associated with RTT. Reduced surfactant PCs cause surfactant dysfunction and impair the lung’s ability to facilitate gas exchange. In addition, in vitro experiments have shown that excess cholesterol and/or neutral lipids disrupt the surface tension-lowering capability of surfactant (49). Notably, surfactant dysfunction causes ground glass opacities and bronchial thickening in computed tomography scans, both of which are present in RTT patient lungs (50).

Respiratory symptoms upon Mecp2 deletion are a result of lung- and brain-autonomous events

The comparison of respiratory symptoms in mice with AE2 cell-specific deletion of Mecp2 to mice with a hindbrain neuron-specific deletion of Mecp2 revealed that AE2 cell-specific loss of Mecp2 elevated lung TGs, reduced BAL fluid PCs and caused respiratory symptoms. These findings demonstrate that loss of Mecp2 in the lung directly contributes to respiratory symptoms in RTT, challenging the view that RTT symptoms result solely due to neuron-specific loss of Mecp2 (51).

Breathing frequency was increased in mice with an AE2-cell deletion of Mecp2, whereas tidal volume was changed in mice with a hindbrain neuron-specific deletion of Mecp2, suggesting loss of Mecp2 from either the lung or hindbrain imparts distinct respiratory symptoms. Respiratory apneas are considered the most striking respiratory symptom of RTT as they can lead to cyanosis and fainting (52). Intriguingly, mice with AE2 cell-specific and hindbrain neuron-specific deletions of Mecp2 both display an elevation in the number of respiratory apneas. However, apneas in Mecp2Δ/Y mice were far more severe. Our data suggest that both neuronal- and lung-autonomous processes influence respiratory apneas, and loss of Mecp2 from both areas likely acts in an additive manner to produce the dramatic apneas evidenced in RTT patients and Mecp2-mutant mouse models.

Lung metabolic defects impact long-term patient care

Here, we show that Mecp2 is a crucial regulator of lipid metabolism in the lung, and that its loss imparts consequences on lung homeostasis and respiratory symptoms. RTT patient death is most often caused by lung infection, asphyxiation and respiratory failure (11), and at least 17% of RTT patients develop pneumonia (52). Surfactant proteins bind to pathogens and facilitate their clearance by alveolar macrophages (49); surfactant deficiency or dysfunction can therefore weaken the lung’s immune response to foreign pathogens. In addition, accumulated lung lipids can interfere with macrophage clearance efforts, thereby decreasing their efficacy in preventing infection (53). Low doses of fluvastatin improve lung lipid accumulation and respiratory symptoms in both male and female Mecp2-mutant mice. TGs are the primary elevated lipid in the Mecp2-deficient lung. Statins, while an HMGCR inhibitor, reduce serum
crossing Mecp2tm1Bird. A deletion of the last two exons (exons 3–4) of Mecp2, which were also on a C57BL/6J background. AE2 cell-specific female mice to male mice heterozygous for Cre transgenes, Mecp2tm1Bird were obtained from The Jackson Laboratory. These mice harbor a knock-in mutation of an Enhanced Green Fluorescent Protein (EGFP) sequence in the 3’UTR of the Mecp2 gene. Female Mecp2tm1Bird mice were bred to wild type male mice to produce male Mecp2tm1Bird offspring.

**Materials and Methods**

**Animals**

All animal procedures were approved under animal use protocol 21-0251H by the Animal Use Committee at the Canadian Council on Animal Care (CCAC)-accredited animal facility. The Center for Phenogenomics (TCP). Congenic 129.Mecp2tm1Blh/Y mice feature a deletion of the last two exons (exons 3–4) of the Mecp2 transcript, resulting in a null allele. Male Mecp2/Y (null) and +/Y (wild type), and female Mecp2+/+ and +/- mice were obtained by crossing Mecp2tm1Blh/+ females to males of the 129SveEv56/Tac strain for >10 generations. Mice were fed a standard diet (Harlan Teklad 2918) ad libitum, consisting of 18% protein, 6% fat and 44% carbohydrates. Mice were housed in a 12 h light/dark cycle and were euthanized between the hours of 9 a.m. and 12 p.m. (ZT 2–5) to control for circadian rhythm fluctuations. 129.Mecp2/Y male mice develop behavioral phenotypes, including hindlimb clasping and hyporeactivity, at 4 weeks of age, followed by a rapid decline in health and death by 8–10 weeks. 129.Mecp2/+ female mice develop neurological phenotypes between 4 and 6 months of age with symptom variability due to random X-chromosome inactivation.

Conditional deletions of Mecp2, and respective controls, were obtained by crossing B6.Mecp2tm1Blh+/+(Mecp2-flx) heterozygous female mice to male heterozygous for Cre transgenes, which were also on a C57BL/6J background. AE2 cell-specific deletion of Mecp2 was achieved by crossing Mecp2-flx mice to B6.Sftpctm(cre/ERT2)Blh mice, which have a Tmx-inducible Cre under the control of the surfactant protein C promoter. Sftpctm(cre/ERT2)Blh/Mecp2tm1Blh/+ (AE2-cKO) mice were given three 75 mg/kg bodyweight intraperitoneal injections of Tmx at 3 weeks of age over a period of 5 days (every other day) to induce Cre excision. Hindbrain-specific deletion of Mecp2 was achieved by crossing Mecp2-flx mice to mice with Cre under the control of the atonal BHLB transcription factor 1 promoter (B6.Cg-Tg(Atoh1-cre)18frj). Atoh1 regulatory elements drive constitutive Cre expression in precursors of granule cell neurons of the cerebellum, dorsal hindbrain and spinal cord (hindbrain-cKO). To achieve whole-body deletion of Mecp2 on the C57BL/6J background, two-cell embryos were retrieved from pregnant Mecp2tm1Blh+/+ mice, incubated in a solution containing Histidine-Transactivator of transcription (TAT)-Nuclear localization signal-tagged Cre recombinase and transferred to a pseudo-pregnant female recipient, as previously described (54), by the Mouse Model Services Core at TCP. Female C57BL/6 Mecp2tm3.1Bird mice were obtained from The Jackson Laboratory. These mice harbor a knock-in mutation of an Enhanced Green Fluorescent Protein (EGFP) sequence in the 3’UTR of the Mecp2 gene. Female Mecp2tm3.1Bird mice were bred to wild type male mice to produce male Mecp2tm3.1Bird offspring.

**Electron microscopy and immunohistochemistry**

Mice were anesthetized with an intraperitoneal injection of 100 mg/kg bodyweight of ketamine and 10 mg/kg bodyweight xylazine in saline. Once unresponsive, a needle was inserted into the left ventricle of the heart and mice were perfused with 10 U/ml heparin in phosphate-buffered saline (PBS) at a rate of 3 ml/min for 10 min. Lungs were fixed via intratracheal administration of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The trachea was tied with sutures and the lung was dissected out of the chest cavity and immersed in the above fixative for 16 h at 4°C. Tissues were post-fixed in osmium tetroxide, dehydrated in an ascending series of acetone and embedded in Epon Araldite prior to polymerization at 60°C overnight. Thin sections were cut, mounted on grids, and stained with uranyl acetate and lead citrate prior to microscopy. Images were captured with a charge-coupled device camera (AMT Corp.) and an electron microscope (JEOL JEM1011). A minimum of 15 images were taken from each animal.

For immunohistochemistry, lungs of P21 Mecp2tm3.1Bird mice were perfused with 10 U/ml heparin in PBS as above and fixed via intratracheal administration of 4% paraformaldehyde (PFA). Excised lungs were immersed in 4% PFA for 16 h at 4°C. Tissues were washed in PBS, embedded in paraffin, cut at a thickness of 5 μm and dried on a slide warmer at 37°C. Mounted sections were deparaffinized in a graded series of ethanol washes. Antigen retrieval was performed in 0.1 M citrate buffer, pH 6.0. Slides were blocked with 5% goat serum, 1% bovine serum albumin and 0.1% Triton X-100 in PBS. Slides were incubated with primary and secondary antibodies: GFP (ab13970, 1:500), surfactant protein C (SPC) (ab90716, 1:500), Alexafluor 488 (Thermo A-11039, 1:400), Alexafluor 594 (Thermo A-11012, 1:400). Slides were mounted using ProLong Gold Antifade Mount with 4’,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher) and imaged on a Nikon A1R confocal laser microscope equipped with NIS Elements software. For histology, lungs were perfused as described above. A mixture of 1:1 optimal cutting temperature compound and PBS was administered intratracheally. Excised lungs were immersed in a cryo mold and frozen on dry ice. Sections were sliced using a cryostat and oil red O stain was applied.

**Lipid analysis**

Blood was drawn from mice using cardiac puncture between the hours of 2 p.m. and 5 p.m. (ZT 7–10) and serum was separated in BD serum separation tubes following manufacturer instructions. Serum and tissue samples were stored at −80°C until analysis by the Diabetes and Endocrinology Center at Baylor College of Medicine (Houston, TX). Lipids were isolated from tissue using CHCl3:CH3OH extraction, followed by drying of the organic phase under N2 pressure. Serum and tissue cholesterol and TG concentrations were assessed by HPLC.

After sacrifice, a blunt tip 20 G needle was inserted into the trachea and the lungs were lavaged with sterile PBS (500 μl...
for P21 mice and 1 ml for mice P56 or older; retrieval volume was generally 75–80% of administration volume). BAL fluid was stored at −80°C until further processing. Measurements of BAL cholesterol and PC were performed by LC-MS/MS at the Analytical Facility for Bioactive Molecules at The Hospital for Sick Children (Toronto, Canada). BAL samples (200 μl) were transferred to siliconized glass tubes containing 800 μl of ultra-pure water and were spiked with internal standards. Lipid extraction was performed using chloroform/methanol extraction. Organic phases were dried and reconstituted in 100 μl ethanol with 0.2% formic acid and transferred to siliconized vials for analysis. LC/MS–MS was performed on an Agilent 1290 Series binary pump (Agilent Technologies Inc.) coupled to an API4000 triple-quadrupole mass spectrometer (SCIEX). Quantitative analyses were based on the calibration curve for each analyte and analyzed by LC/MS–MS in the same conditions.

**Subjective health assessments**

Mice were assayed for general health once per week from 4 to 10 weeks of age. Scoring was blinded by genotype. Mice were scored using a published assessment method with slight modifications (33,55). In this scoring method, mice are given a score between 0 and 2 based on the severity of the phenotype assessed (Supplementary Material, Table S2). Mice were assessed for limb clamping, tremors, activity, grooming, hypotonia and body weight, for a combined possible score of 0–12.

**Rotarod**

Motor coordination was measured using the rotating rod (Stoelt ing Ugo Basile Mouse Rota-Rod). Mice were placed on the grooved rotating rod facing the opposite direction of rotation. The rev olution rate increased from four rotations per minute (RPM) to 40 RPM over the course of 5 min. The length of time that each mouse remained on the rod was recorded for eight trials over two consecutive days (four trials per day), with a minimum of 30 min between each trial. A trial ended for a mouse when it fell from the rod, stayed stationary on the rod while it spun for two revolutions, or when it successfully stayed on the rod for 5 min.

**Open field activity**

Locomotor activity was measured by free activity in an open field chamber (Accuscan Instruments). Open field chambers were cleaned thoroughly with Clidox prior to use. One mouse was placed in the center of each 40 × 40 cm open field chamber illuminated at 200 lux and left undisturbed for 20 min. Movement was tracked using the VersaMax software (Accuscan Instruments). Mice were removed from the open field chamber and each chamber was thoroughly cleaned with Clidox between experiments.

**Social behavior**

Social behavior was assessed in a 60 × 40 cm three-chamber apparatus with two clear plexiglass partitions. The apparatus was thoroughly disinfected with Clidox prior to use. A novel object (small orange cup) or a stranger mouse (wild-type mouse of the same sex and genetic background as the subject) were placed in containment cups in the left or right chambers. Subject mice were placed in the middle chamber with no access to other chambers for a 5 min acclimation period. Subjects were then allowed to freely move around the chambers for a period of 10 min. Following this, subjects were returned to the middle chamber with no access to the other chambers. The stranger mouse was moved with its containment cup to the opposite chamber. A novel stranger mouse was inserted in a clean containment cup in the chamber where the original stranger mouse was located. Access to all chambers was restored and subjects could explore freely for another 10 min. Movement was recorded using Ethovision XT, which generated tracking coordinates for each subject, analyzed as time spent within each chamber with a custom script. The three-chamber apparatus was thoroughly disinfected with Clidox between each subject.

**Plethysmography**

Respiration was monitored using a Buxco Whole Body Plethysmography (WBP) apparatus (Data Sciences International) according to manufacturer’s instructions. All testing was conducted between the hours of 9 a.m. and 12 p.m. Mice were placed in plethysmography chambers and allowed to acclimate for 30 min, until motionless. Baseline breathing rates were measured for a period of 5 min. Breathing frequency, tidal volume and enhanced pause (PenH) were analyzed using Buxco FinePoint Software. Apneas were defined as cessation of breathing for over 1 s (two respiratory cycles) and were assessed manually over a period of baseline breathing.

**Single-cell isolation and flow cytometry**

P18 mouse lungs were collected from +/Y and Mecp2/Y mice at 9 a.m. A single-cell suspension was made as published, with modifications (56). Briefly, lungs were excised and incubated in fresh Hank’s balanced salt solution (HBSS) with 5 U/ml dispase, 0.1% collagenase I and 0.002% DNase I for 30 min at room temperature. The lung was then disintegrated using forceps in a 6 cm petri dish. The cell suspension was filtered through 100, 70 and 30 μm nylon cell strainers. Red blood cells were lysed using RBC lysis buffer (Miltenyi Biotec). Cells were pelleted and re-suspended in 500 μl staining media [SM: HBSS, 2% fetal bovine serum (Wisent), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.2]. Cells were counted using the TC10/TC20 cell counter (BioRad). Approximately 3 million cells per sample were stained with fluorochrome-conjugated antibodies from BD Biosciences for BD Biosciences for 30 min on ice in the dark. Antibodies used were as follows: CD45.2 (558702), CD31 (561814), CD326 (563478), I-A/I-E (553623) and Podoplanin (566390). Cells were sorted using a MoFlo Astrios (Beckman Coulter) cell sorter. AE2 cells were as follows: CD45.2−, CD31−, CD326+, I-A/I-E+, Podoplanin−. Following sorting, cells were pelleted and re-suspended in fresh SM.

**Single-cell RNA sequencing**

For single-cell RNA sequencing, AE2 cells were isolated using flow cytometry from one +/Y and one Mecp2/Y mouse. Following cell sorting, over 95% of cells were negative for trypan blue (Invitrogen). Drop-seq was carried out at the Princess Margaret Genomics Facility (Toronto, Ontario) as previously described (57), using 2000 cells from each sample. Flow rates of 3000 p/s (cells and beads) and 13000 p/s (cell) were used. Following Drop-seq droplet collection, complementary DNA (cDNA) amplification and library preparation were carried out as described (57). Libraries were sequenced on an Illumina NextSeq 500. FASTQ sequencing reads were processed, aligned to the mouse genome (mm10) and converted to digital gene expression matrices using
STAR aligner (STAR v2.5.2b). The CELLRANGER (v3.0.2) pipeline was used to obtain two types of gene-barcode matrices: the first is an unfiltered gene-barcode matrix and the second is the filtered gene-barcode matrix. The matrices were loaded into R (v3.5.1) for the final graphical output of results and statistical analysis using SCATER (v1.2.0), CELLRANGERKIT (v1.1.0), SCRN (v1.2.2), RTSNE (v0.11), SC3 (v1.3.14), EDGEr (v3.16.5), SEurat (v2.2.0) and PCAMETHODS (v1.50.0). After cell quality control, 1559 +/Y cells and 1524 MeepCrypt2/Y cells remained. Low-abundance genes were filtered out and the dataset was normalized as previously published (58). Clustering and differential expression analyses were performed using a K-nearest neighbor algorithm and binary classifiers.

**RNA extraction and RT-qPCR**

Isolated AE2 cells or whole-lung RNA was used for RT-qPCR analysis. For whole-lung RNA, a 5 mm stainless steel bead (Qiagen, 69989) was added to chilled tubes containing lung tissue, which was then homogenized in 400 μl of Qiaseq using a Qiagen Tissuelyser II. RNA extraction was carried out using the RNeasy Lipid Tissue Mini Kit (Qiagen, CA, USA) and stored at −80°C. Reverse transcription of RNA was performed using the Superscript VILO cDNA synthesis kit (Invitrogen, CA, USA). Gene primers for RT-qPCR were designed to span exon–exon junctions of the gene of interest. RT-qPCR was performed using Power SYBR Green PCR Master Mix (Invitrogen) and the Viia7 Gene primers for RT-qPCR were designed to span exon–exon binding protein (Mecp2/Y) cells remained. Low-abundance genes were filtered out and the dataset was normalized as previously published (58). Clustering and differential expression analyses were performed using a K-nearest neighbor algorithm and binary classifiers.

**Protein extraction and western blotting**

Mouse lungs were transferred to round bottom tubes with a 5 mm stainless steel bead (Qiagen, 69989) and 300 μl of radioimmunoprecipitation assay buffer (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0 and fresh protease inhibitors (Sigma-Aldrich, 1187358001)). Tissues were lysed in a Tissuelyser II (Qiagen, 85300) at 50 Hz for 2–3 min. Protein concentration was determined using a Bradford assay (Thermo Fisher, 17526). Proteins were separated in a 4–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel (BioRad, 4561084). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot SD semi-dry transfer cell (BioRad). Following transfer, membranes were blocked in 5% milk-Tris-buffered saline, 0.1% Tween 20. The following antibodies were used: anti-SREBP1 (Santa Cruz Biotechnology, sc-366; 1:500), anti-SREBP2 (abcam, ab30682; 1:500), anti-glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling, 5174, 1:2000), peroxidase-AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, 111-035-144). Proteins were visualized using Clarity western ECL (BioRad, 170-5060) and imaged on a Chemi-Doc (BioRad).

**Drug administration**

As lipid metabolism follows a circadian rhythm, each treatment was administered at the same time of day, between 9 a.m. and 10 a.m. (ZT 2–3). Fluvastatin (Sellekchem) was dissolved

**ChIP**

ChIP was performed as published (59). Briefly, frozen lungs were crosslinked by incubation in Solution A (1% formaldehyde, 50 mM HEPES-KOH, 100 mM NaCl, 1 mM ethylenediamintetraacetic acid–EDTA, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N’-tetraacetic acid–EDTA) for 20 min at room temperature, after which 2.5 M glycine was used to quench the reaction. Tissue was washed and dounced in ice-cold PBS with a loose and tight pestle. Dounced cells were filtered through a 100 μm cell strainer and centrifuged. Protein G-Dynabeads (Invitrogen) were pre-blocked with 0.5% bovine serum albumin–BSA in PBS and bound to an anti-TBL1XR1 (TBLR1) antibody (Bethyl Laboratories, A300-408A) by overnight incubation at 4°C. Crosslinked and input DNA are listed in Supplementary Material, Table S2.

**Immunoprecipitation**

Immunoprecipitation was performed using the MilliMop Catch and Release Kit (Millipore). Following manufacturer’s instructions, 500 μg of nuclear lysate was incubated with an anti-TBLR antibody (Bethyl Laboratories, A300-408A) for 3 h at 4°C. Following elution, half of the immunoprecipitated sample was loaded into each lane of a 4–20% gradient SDS-PAGE gel. Following transfer to a PVDF membrane, the following antibodies were used for western blotting: anti-MEC2 (Sigma-Aldrich, M7443), anti-HDAC3 (abcam, ab7030) and anti-NCOR1 (Bethyl Laboratories, A301-145A). Input samples represent 25 μg of nuclear extracted protein.

**RNA extraction and RT-qPCR**

Isolated AE2 cells or whole-lung RNA was used for RT-qPCR analysis. For whole-lung RNA, a 5 mm stainless steel bead (Qiagen, 69989) was added to chilled tubes containing lung tissue, which was then homogenized in 400 μl of Qiaseq using a Qiagen Tissuelyser II. RNA extraction was carried out using the RNeasy Lipid Tissue Mini Kit (Qiagen, CA, USA) and stored at −80°C. Reverse transcription of RNA was performed using the Superscript VILO cDNA synthesis kit (Invitrogen, CA, USA). Gene primers for RT-qPCR were designed to span exon–exon junctions of the gene of interest. RT-qPCR was performed using Power SYBR Green PCR Master Mix (Invitrogen) and the Viia7 instrument (ABI). PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. Single product amplification was confirmed by dissociation curves. Each sample was amplified three times for precision. The average cycle threshold (Ct) of these technical replicates was used to calculate expression. Gene expression was normalized to TATA-box binding protein (Tbp) internal loading control and analyzed using the 2-ΔΔCT method. The primers used are included in Supplementary Material, Table S2.

**Nuclear protein extraction and immunoprecipitation**

Nuclear extraction was performed as published (23). Briefly, frozen tissue was dounced with 1 ml of NE1 buffer (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM MgCl2, 0.1% Triton X-100, 20% glycerol, protease inhibitor). Cells were pelleted and washed twice with NE1. Cells were then resuspended in 500 μl of NE1 with 10 μl of benzonase (Millipore 70746-3). Pellet was gently shaken at room temperature for 15 min. An equal volume of NE300 buffer (NE1 buffer with 5 mM NaCl) was added to the tubes and gently rotated for 30 min at 4°C. Tubes were spun at 16 000 g for 20 min at 4°C. Nuclear lysates (supernatant) were transferred to new tubes.

Drug administration

As lipid metabolism follows a circadian rhythm, each treatment was administered at the same time of day, between 9 a.m. and 10 a.m. (ZT 2–3). Fluvastatin (Sellekchem) was dissolved
in 100% Dimethylsulfoxide-DMSO at 6 mg/ml. On the day of administration, aliquots of fluvastatin were diluted in sterile saline such that the desired dose for a 20 g mouse was given in 100μl. Male mice were injected intraperitoneally with a twice weekly 1.5 mg/kg body weight dose from ages P28 to P56. Female mice were injected intraperitoneally with a weekly dose of 3 mg/kg body weight from 6 to 32 weeks of age. Vehicle controls (1:10 DMSO:saline) were administered at the same rate.

**Statistics**

The significance of the differences in mean values across two groups was evaluated by two-tailed Student t-tests. The statistical difference between four groups was evaluated using one-way or two-way analysis of variance with the Tukey post hoc test for multiple comparisons. Survival was assessed using the Logrank (Mantel–Cox) test. All statistical analyses were performed in GraphPad Prism (Version 7). P-values <0.05 were considered statistically significant.

**Supplementary Material**

Supplementary Material is available at HMG online.

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