Mammalian Genome (2018) 29:281–298
https://doi.org/10.1007/s00335-018-9743-x

Loss of the *zona pellucida*-binding protein 2 (*Zpbp2*) gene in mice impacts airway hypersensitivity and lung lipid metabolism in a sex-dependent fashion

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Received: 3 December 2017 / Accepted: 8 March 2018 / Published online: 13 March 2018

Abstract

The human chromosomal region 17q12–q21 is one of the best replicated genome-wide association study loci for childhood asthma. The associated SNPs span a large genomic interval that includes several protein-coding genes. Here, we tested the hypothesis that the *zona pellucida-binding protein 2* (*ZPBP2*) gene residing in this region contributes to asthma pathogenesis using a mouse model. We tested the lung phenotypes of knock-out (KO) mice that carry a deletion of the *Zpbp2* gene. The deletion attenuated airway hypersensitivity (AHR) in female, but not male, mice in the absence of allergic sensitization. Analysis of the lipid profiles of their lungs showed that female, but not male, KO mice had significantly lower levels of sphingosine-1-phosphate (S1P), very long-chain ceramides (VLCCs), and higher levels of long-chain ceramides compared to wild-type controls. Furthermore, in females, lung resistance following methacholine challenge correlated with lung S1P levels (Pearson correlation coefficient 0.57) suggesting a link between reduced AHR in KO females, *Zpbp2* deletion, and S1P level regulation. In livers, spleens and blood plasma, however, VLCC, S1P, and sphingosine levels were reduced in both KO females and males. We also find that the *Zpbp2* deletion was associated with gain of methylation in the adjacent DNA regions. Thus, we demonstrate that the mouse ortholog of *ZPBP2* has a role in controlling AHR in female mice. Our data also suggest that *Zpbp2* may act through regulation of ceramide metabolism. These findings highlight the importance of phospholipid metabolism for sexual dimorphism in AHR.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00335-018-9743-x) contains supplementary material, which is available to authorized users.

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Introduction

Asthma is a common complex disease with more than 300 million people affected world-wide. Current statistics estimates than 7.6% of world's adults and 8.4% of children (defined as individuals younger than 18 years) suffer from asthma (Centers for Disease Control and Prevention, https://www.cdc.gov/nchs/fastats/asthma.htm). Complexity of the genetic architecture of asthma and variability of phenotypes, interaction between genetic background and environment, age and sex differences in predisposition—all these factors add to the challenges that asthma presents for a genetic study. Genome-wide genetic association studies (GWAS) of asthma identified several dozen loci across the genome. For many of these regions however, the causality of specific polymorphisms has yet to be demonstrated. One of the best replicated and actively studied GWAS regions associated with childhood asthma is the chromosomal region 17q12–q21 (Moffatt et al. 2007, 2010). It spans about 200 kb and encompasses 5 protein-coding genes: IKAROS family zinc finger 3 (*IKZF3*), *zona pellucida*-binding protein 2 (*ZPBP2*), gasdermin B (*GSDMB*), ORMDL sphingolipid biosynthesis regulator 3 (*ORMDL3*), and gasdermin A (*GSDMA*) (Supplementary Fig. S1). It has been suggested that association with disease predisposition in this region was due to regulatory polymorphisms that cause changes in gene expression levels (Acevedo et al. 2015; Moffatt et al. 2007; Verlaan et al. 2009). Indeed, *cis*-regulatory SNPs associated with *GSDMB* and *ORMDL3* expression levels map to a 160-kb long region that largely overlaps with the asthma-associated interval (Ge et al. 2009; Verlaan et al. 2009). Moreover, expression of human *ORMDL3* and *GSDMB* transgenes in mice is associated with lung hypersensitivity and allergic asthma (Das et al. 2016; Miller et al. 2014). However, contribution from other 17q12–q21 genes cannot be ruled out (Hao et al. 2012; Naumova et al. 2013; Verlaan et al. 2009).

ZPBP2 encodes a protein that contains an immunoglobulin-like domain, is highly expressed in testis and male germ cells and has a function in sperm maturation and fertilization (Lin et al. 2007). Recent data suggest that ZPBP2 harbors a Link-like hyaluronic acid (HA)-binding domain, which may facilitate the binding of the spermatozoa to the oocyte's *zona pellucida* (Torabi et al. 2017). However, *ZPBP2* expression is not restricted to testis. *ZPBP2* RNA and protein are also detected in human somatic cells (lymphoblastoid cell lines, airway epithelium, intestinal epithelium, and T lymphocytes) albeit at lower levels (Berlivet et al. 2012; Carreras-Sureda et al. 2016; Ge et al. 2009; Moussette et al. 2017; Soderman et al. 2015; Verlaan et al. 2009). *ZPBP2* harbors a common polymorphism rs11557467 in its 5th exon that causes a non-synonymous amino-acid change from serine to isoleucine. However, due to the limited understanding of the relationship between structure and function for ZPBP2, the functional impact of this common non-synonymous variant remains unclear. The *ZPBP2* allele from the asthma-risk haplotype is expressed at lower levels than the allele from the non-risk haplotype, suggesting that reduced expression of *ZPBP2* may be a contributing factor in predisposition to asthma. Surprisingly however, higher methylation of the *ZPBP2* promoter that is also associated with lower transcription of the gene, is protective against asthma in females, but not males (Al Tuwaijri et al. 2016). Therefore, the ensemble of emerging evidence suggests that *ZPBP2* may be a contributing factor in the pathogenesis of asthma, and would likely act in a sex-specific fashion. However, the biological basis of such a relationship remains elusive.

To understand the role of *ZPBP2* in predisposition to asthma, we examined the phenotypes of mice that carry a deletion of the mouse ortholog of the *ZPBP2* gene—*Zpbp2*. We find that loss of *Zpbp2* in female mice leads to a decrease in airway hypersensitivity (AHR) to methacholine in the absence of allergic sensitization (naïve mice). Several lines of evidence implicate sphingolipid metabolism in asthma, the regulation of AHR and inflammation (Parker et al. 2016; Worgall et al. 2013), and reviewed in Ono et al. (2015) and Worgall (2017). Lower levels of VLCCs and higher levels of LCCs are associated with inflammation and increased cell migration (Espaillat et al. 2017). Our analysis of sphingolipid profiles of KO and wild-type mice, show that the *Zpbp2* deletion is associated with lower levels of C24:0 and sphingosine-1-phosphate (S1P) and higher levels of LCCs (C14:0, C16:0, and C18:0) in female lungs as well as higher IgE levels in the blood of female mice. The ablation of *Zpbp2* also results in lower levels of VLCCs (e.g., C24:0, C24:1, C26:0), Sph, and S1P in the livers, spleens and blood plasma of both females and males. Thus, our data suggest that ZPBP2 may contribute to AHR and its effect may be mediated through changes in lipid metabolism.

Materials and methods

Accession IDs

Organism (mouse)—Taxon ID 10090
Gene (*Zpbp2*)—Gene ID 69376
Gene (*Nr4a1*)—Gene ID 15370
Gene (*Lpin1*)—Gene ID 14245
Gene (*Ppara*)—Gene ID 19013

RNA-seq data have been submitted to NCBI with accession number GSE98513

Mice and crosses

The mouse strains C57BL/6J and B6;129S7-*Zpbp2*^{tm1Zuk}/J (Lin et al. 2007) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). The B6;129S7-*Zpbp2*^{tm1Zuk}/J mice were backcrossed to C57BL6/J for ten generations (N10) to place the deletion on a C57BL/6J inbred genetic background. N_{10} mice were intercrossed to generate congenic homozygous mutant mice. The strain is annotated as B6.129S7-*Zpbp2*^{tm1Zuk}/Mmjax and is now available from MMRRC (stock number MMRRC #42297). Homozygous B6.129S7-*Zpbp2*^{tm1Zuk}/Mmjax mice will be referred to as KO from this point on. Mice were maintained on a 12 h light/12 h dark cycle and fed ad libitum the irradiated 2918X Teklad Global rodent diet.

In the $N_{10} \times N_{10}$ crosses, the mutation was transmitted in Mendelian proportions suggesting that it did not cause embryonic/fetal lethality (Supplementary Table S1). Loss of *Zpbp2* expression in KO mice was confirmed using RT followed by PCR or quantitative PCR (qPCR). To determine body weight and growth, mice were weighed at the ages of 4, 12 weeks, 6, 9, and 12 months. Livers, spleens and gonadal fat pads were collected from 12 months old KO and WT mice of both sexes and weighted. All procedures were conducted in accordance with the guidelines set by the Canadian Council of Animal Care (Ottawa, ON, Canada) and were approved by Animal Care Committee of the McGill University Health Center (Montreal, QC, Canada).

Genotyping

DNA was extracted from mouse tissues using a standard proteinase K/phenol–chloroform procedure. Genotypes were determined by PCR using primers oIMR8314 and oIMR8315 to amplify the wild-type allele (Lin et al. 2007), and IMR781 and IMR782 for the mutant allele (Kessova and Cederbaum 2007) (Supplementary Table S2).

Expression analysis

For expression analysis, organs were collected from embryonic day 18.5 (E18.5), postnatal day 5 (P5), 4-week old (P28), 3 months old (12–14 week old, 3 mo) and 10 months old (10 mo) C57BL6/J mice. Total RNA was isolated using TRIzol® Reagent (Thermo Fisher Scientific) and purified using the RNeasy® MinElute® Cleanup Kit (Qiagen, Inc). CDNA was synthesized using 1 µg of RNA, Oligo (dT) 12–18 primers (Thermo Fisher Scientific) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) (Thermo Fisher Scientific; 37 °C for 50 min and 70 °C for 15 min). Relative gene expression levels were determined by quantitative RT-PCR (RT-qPCR) using Power SYBR® Green PCR master mix (Applied Biosystems by Thermo Fisher Scientific). Gene expression was normalized to the housekeeping gene elongation factor 2 (*Eef2*). The relative expression levels were calculated using the cycle threshold (Ct) values and the delta–delta CT method (Livak and Schmittgen 2001). Primers used for expression analysis are listed in Supplementary Table S3.

RNA-sequencing assays were conducted on RNA from livers of three C57BL/6J and three KO males. RNA-seq was performed by the McGill University and Genome Quebec Innovation Centre (Montreal, QC, Canada). The standard edgeR differential expression pipeline was used for identification of differentially expressed genes with a minimum $\log_2$ fold change cutoff (logFC) of 1.5 and a false discovery rate (FDR) corrected p value < 0.05. (Robinson et al. 2010). Two high quality samples (determined by Spearman's rank correlation) were identified in each of the KO and WT sets and used in the analysis. The edgeR goanna(), and kegga() functions were used to determine highly enriched pathways and gene ontology terms. The enrichment p value was calculated using Fisher's exact test. The heat map of the differentially expressed gene (DEG) expression values utilized $\log_2$ transformed counts per million mapped reads (logCPM) values.

Sensitization and challenge protocol for allergic asthma

To determine lung response to allergen, 8-weeks old males and females were sensitized weekly for three consecutive weeks by intraperitoneal injections with 100 µg of ovalbumin (OVA; Sigma, St. Louis, MO, USA) allergen adsorbed to 1.5 mg of aluminum hydroxide (Imject Alum, Pierce, Rockford, IL, USA) in 0.2 ml of sterile PBS. After sensitization, the mice were split into two groups: non-allergic (PBS) and allergic (OVA). One week following the third sensitization, the OVA mice were challenged with 1% ovalbumin solution for 30 min, whereas the PBS mice were exposed to PBS only.

Measuring pulmonary resistance

Mice were anesthetized with a cocktail of ketamine (100 mg/kg), xylazine (10 mg/kg), and acepromazine (3 mg/kg). The depth of anesthesia was monitored by checking the pedal reflex. Anesthetized animals were tracheotomized and connected to a ventilator, and the baseline resistance values were measured. A nebulizer was used to administer PBS or increasing doses of methacholine (ranging between 25 and 100 mg/ml) directly to the lungs through the tracheostomy tube. The maximum resistance in response to the PBS or methacholine exposure for each mouse was determined

using a Buxco plethysmograph system (Buxco Research System, Wilmington, NC, USA) and Harvard Apparatus ventilators (Harvard Apparatus, Holliston, MA, USA). Once data collection was complete, anesthetized animals were euthanized by cardiac puncture.

Airway resistance was tested in naïve females and males in three independent experiments performed for each sex separately: 10 KO, 16 HZ, 10 WT (littermates of the mutant mice), and 6 C57BL/6J naïve females; and 14 KO, 20 HZ, 8 WT (littermates of the mutant mice), and 15 C57BL6/J naïve males.

Airway resistance was measured in the following groups of OVA-sensitized animals: 8 KO-OVA females, 9 KO-OVA males, 5 KO-PBS females, 8 KO-PBS males, 12 WT-OVA females, 11 WT-OVA males, 10 WT-PBS females, and 8 WT-PBS males. Female and male mice were tested separately in at least two independent experiments per sex. The C57BL/6J mice display relatively low responsiveness to methacholine compared to other strains of mice, even after they are sensitized and challenged with allergens (Ackerman et al. 2005; Camateros et al. 2009; Kanagaratham et al. 2017; Moisan et al. 2006).

Assessment of lung histology

After completion of airway resistance tests, lungs were inflated with 10% buffered formalin (Fisher Scientific, Nepean, ON, Canada) for 48 h and embedded in paraffin (Leica Biosystems Inc., Concord, ON, Canada). Paraffin blocks were sectioned into 4 µm thick sections, and then slides were deparaffinized, hydrated, and stained. Hematoxylin and eosin stain (H&E) was done to quantify the leukocyte infiltration as described previously (Kanagaratham et al. 2014).

IgE measurements

Blood was collected into tubes containing EDTA. Samples were spun at 3000 rpm for 10 min to separate the plasma. Total IgE in the plasma was measured by enzyme-linked immunosorbent assay (ELISA) using the mouse BD OptEIA kit (BD Biosciences) following the manufacturer's instructions. IgE was measured in plasma of 16 WT, 9 HZ, and 8 KO naïve males; 8 WT-PBS, 10 WT-OVA, 8 KO-PBS, and 10 KO-OVA-sensitized and challenged males; 8 WT, 6 HZ, and 8 KO naïve females, and 8 WT-PBS, 9 WT-OVA, 5 KO-PBS, and 8 KO-OVA-sensitized and challenged females.

Measurements of lipids and markers of oxidation

Approximately 20 mg of mashed tissue (lung, liver, spleen) or 50 µl of plasma was preserved in 1 ml of 1 mM butylated hydroxyanisole in chloroform:methanol solution (2:1 vol/vol) (Folch et al. 1957; Van Handel and Zilversmit 1957). Samples were stored in − 80 until analysis. Lipids were isolated as described (Folch et al. 1957). Levels of ceramide species and S1P were measured using high performance liquid chromatography (HPLC) tandem mass spectrometry (MS) as previously described (Guilbault et al. 2008; Veltman et al. 2016).

Pyrosequencing methylation assay

DNA samples (500 ng–1 µg) were treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's protocol with modifications. Primers for the mouse *Zpbp2* promoter were designed using the PyroMark Assay Design 2.0 Software (Qiagen) to assay 5 CGs in the *Zpbp2* promoter that are not removed by the deletion (Supplementary Table S2). PCR amplification was performed using one standard primer and one HPLC-purified 5′ biotinylated primer (IDT, USA). PCR products were immobilized on Streptavidin Sepharose High Performance beads (GE Life Sciences) and analyzed using the PyroMark Q24 Advanced platform (Qiagen) and PyroMark Q24 Advanced CpG Reagents. Results were analyzed by the PyroMark Q24 Advanced software (Qiagen).

Sodium bisulfite sequencing methylation assay

The sodium bisulfite sequencing assays were performed on DNA from tails of KO and C57BL6/J males. The assay targeted 28 CGs located within the *Zpbp2* promoter region that was not removed by the deletion (for primers see Supplementary Table S2). Mutant mice had one additional CG due to a polymorphism between the C57BL/6J and 129/S7 strains. On average, 24 clones per sample were sequenced. Clones with more than 2% of non-converted cytosines were excluded from analysis. Sequencing results were analyzed using the BiQ Analyzer software (http://biq-analyzer.bioinf.mpi-inf.mpg.de).

Statistical analysis

Differences in expression levels determined by qPCR were analyzed using a two-tailed *t* test. Most phenotyping data were analyzed with GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA). Lung physiology data and lipid profiles were analyzed using two-way ANOVA followed by multiple *t* tests. Statistical significance was determined using the Holm–Sidak method, with alpha = 0.05. Comparisons between two groups were done using unpaired *t* test. Data are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Differences in body and organ weight were analyzed using a two-tailed *t* test.

For comparisons between two groups, effect sizes were calculated using Hedges' g, as most comparison groups contained different numbers of samples and often had different SD (Hedges 1981).

Results

Age and tissue specific regulation of *Zpbp2* expression

Since age and sex influence the genetic association between asthma and 17q12-q21 alleles in humans (Bouzigon et al. 2008; Hrdlickova and Holla 2011; Moffatt et al. 2007; Naumova et al. 2013), we examined *Zpbp2* expression levels in the lungs of female and male mice at different ages starting from embryonic day 18.5 (E18.5) to 10 months after birth (Fig. 1a). Age had a statistically significant effect on *Zpbp2* RNA levels ($p < 0.0001$, two-way ANOVA) with significant increase after P5 in both sexes. No significant differences between males and females were detected.

Next, to determine if allergic sensitization and challenge with allergen ovalbumin (OVA) influenced *Zpbp2* regulation, RNA levels were measured in the lungs of C57BL/6J wild-type mice sensitized with OVA and challenged with either PBS or OVA, 48 h after the challenge. We observed a 30–40% upregulation of *Zpbp2* in the lungs of OVA sensitized and subsequently challenged with OVA allergic compared to naïve males ($p < 0.05$, two-tailed t test), but no significant difference in *Zpbp2* expression levels was observed between OVA sensitized and subsequently challenged with PBS vs those challenged with OVA males (Fig. 1b). No differences were found between different treatment groups of female mice. Hence, our data show that *Zpbp2* expression levels in C57BL/6J mice vary with age and change in response to allergen sensitization and challenge in males. *Zpbp2* RNA levels in lungs are however very low and the observed differences are very small.

Phenotypic characterization of the *Zpbp2* KO mice

Fertility and growth

To elucidate the role that *Zpbp2* plays in predisposition to asthma, we used mice that carry a targeted mutation of the gene (Lin et al. 2007). In the *Zpbp2* knock-out mice (B6;129S7-*Zpbp2*^{tm1Zuk}/J), exons one to three of *Zpbp2* are deleted and replaced by an *Pgk1-HPRT* cassette. This results in ablation of *Zpbp2* mRNA and protein (Lin et al. 2007). Male carriers have reduced fecundity in an F_1 (C57BL6/J x 129/Sv7) genetic background (Lin et al. 2007). We transferred this targeted *Zpbp2* mutation on to the C57BL/6J genetic background and confirmed that the fecundity of KO males was severely reduced, while KO females were fertile (Fig. 1c). The *Zpbp2* deletion was transmitted in Mendelian proportions in F_2 crosses, which ruled out the possibility that paternal transmission caused embryonic lethality (Supplementary Table S1). We next examined the growth of the *Zpbp2* KO and wild-type (WT) mice between the ages of 4 weeks and 12 months (Fig. 1d, e). KO animals were heavier than wild-type controls across all ages. At the age of 12 months, the average weight of KO males and females was 147 and 127%, respectively, of that of control WT animals ($p < 0.00005$, and $p < 0.005$, for males and females, respectively; two-tailed t test) (Fig. 1d, e). KO females had significantly smaller livers and larger gonadal fat pads than WT females ($g = 3.0$ and 1.7, for liver and fat pad weights, respectively, $p < 0.05$, two-tailed t test), whereas no significant differences in organ weight normalized by body weight were observed in KO males (Fig. 1f, g).

KO females have reduced lung hypersensitivity and sphingosine-1-phosphate levels in the absence of allergic sensitization

Lung response to methacholine was measured in naïve KO, heterozygous (HZ) and WT mice. Naïve KO and HZ female mice showed lower AHR than WT females ($g = 1.77$ and 1.43, respectively, $p < 0.01$, two-tailed t test), whereas naïve KO and HZ males showed no change in AHR compared to WT controls (effect sizes $g = 0.18$, and 0.26, respectively) (Fig. 2a and Supplementary Fig. S2a, b). No significant differences in AHR were found between WT and C57BL/6J mice. Hence, loss of *Zpbp2* caused changes in AHR in a sex-dependent fashion.

S1P signaling influences AHR even in the absence of allergic sensitization through action on vagal neurons (Chiba et al. 2010; Roviezzo et al. 2007; Trankner et al. 2014). Since the *Zpbp2* KO females had an attenuated AHR to methacholine in the absence of allergic sensitization, we hypothesized that they also had lower levels of S1P compared to WT female mice. Sphingolipid profiles in the lungs, livers, spleens and blood plasma of naïve KO, HZ and WT mice were assayed. Significantly lower S1P levels were observed in the lungs of naïve KO and HZ females compared to WT females ($g = 6.3$ and 3.9, respectively, $p < 0.0001$, two-tailed t test), whereas in the lungs of naïve HZ males the S1P levels were higher than in WT controls ($g = 0.8$, $p < 0.01$, two-tailed t test), but the difference between KO and WT males was not significant (Fig. 2b and Supplementary Fig. S2). VLCC C24:0 levels were lower, and LCC C14:0, C16:0, C18:0, and C20:4 levels were higher in the lungs of KO females compared to controls ($p < 0.005$, unpaired t tests) (Fig. 2c). In male lungs, sphingosine (Sph) levels were higher in HZ compared to controls ($p < 0.0001$, unpaired t tests), but no other significant differences were

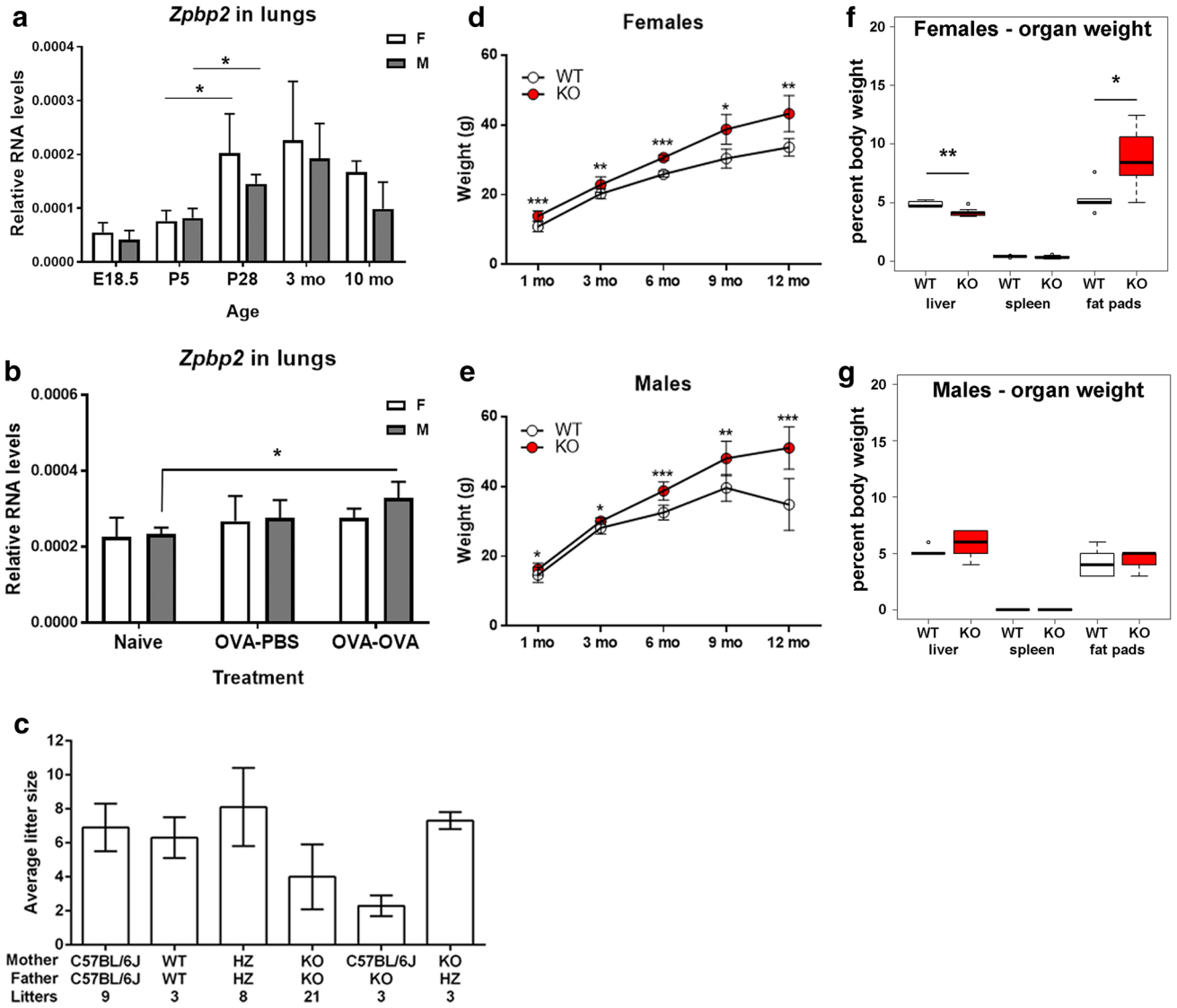


Fig. 1 *Zpbp2* expression and effect of the *Zpbp2* deletion on fertility and body weight. **a** Age influences *Zpbp2* expression in the lungs of C57BL/6J mice. *Zpbp2* expression was measured in the lungs of E18.5, P5, P28, 3 months old and 10 months old male and female mice ($n = 6$–10 mice per group). *Zpbp2* RNA levels were normalized to *Eef2* levels. **b** Sensitization and challenge with allergen ovalbumin (OVA) causes increase in *Zpbp2* RNA levels in adult C57BL/6J male mice. **c** Effect of the *Zpbp2* deletion on mouse fecundity. **d, e** Genotype and age effect on body weight in female (**d**) and male (**e**) mice. **f** KO female mice have lower liver weight, and increased fat accumulation in gonadal fat pads at 12 months of age. **g** No significant differences are observed in the weights of livers, spleens and gonadal fat pads between KO and WT male mice at 12 months of age. Significant differences between groups are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

observed between KO and WT animals (Fig. 2d). Moreover, lung resistance (RI-max) at methacholine dose 50 mg/ml was positively correlated with the lung S1P levels in naïve female mice (Pearson correlation coefficient $R = 0.574$). The correlation was also present in males and remained significant when data from both sexes were combined (Pearson correlation coefficients 0.2375 and 0.4553, respectively) (Supplementary Fig. S2c–e).

Unlike in lungs, in livers, spleens and blood plasma S1P, Sph, as well as the levels of VLCC C24:0, C24:1, C26:0 were significantly lower in KO mice of both sexes compared to controls (adjusted $p < 0.01$, multiple t tests) (Fig. 3 and Supplementary Fig S3). We also tested the levels of malonyldialdehyde (MDA), a marker of lipid peroxidation and cellular stress. KO mice had higher levels of MDA in their livers, spleens and plasma, compared to WT mice ($p < 0.005$, unpaired t tests) (Supplementary Fig. S3). In lungs, however MDA levels were higher in KO females ($g = 4.0$, $p < 0.01$, unpaired t test), but not males, compared to WT mice (Supplementary Fig. S3). It is therefore possible that upregulation

of the lipid peroxidation pathways may contribute, at least in part, to the lower levels of VLCCs in KO mice.

Lower levels of VLCCs and S1P and higher levels of MDA that were observed in KO mice suggest that the *Zpbp2* locus influences ceramide metabolism.

Acute allergic asthma model

We tested lung response to sensitization and challenge with allergen ovalbumin (OVA) in KO and WT animals. In females, AHR differences between WT-OVA and WT-PBS as well as between KO-OVA and KO-PBS mice showed modest effect sizes ($g = 0.33$ and 0.64, respectively), and did not reach statistical significance (Fig. 4a). AHR tended to be lower in KO-OVA females compared to WT-OVA ($g = 0.28$; $p > 0.05$, two-tailed t test) and in KO-PBS compared to WT-PBS ($g = 1.02$; $p = 0.08$, two-tailed t test) females. In males, higher AHR was observed in WT-OVA compared to WT-PBS mice ($g = 1.17$, $p < 0.05$, one-tailed t test) and in KO-OVA compared to KO-PBS mice ($g = 1.44$; $p < 0.05$, one-tailed t test). In OVA-challenged males, the genotype had a modest effect on AHR ($g = 0.65$; $p > 0.05$, two-tailed t test) (Fig. 4b).

Airway infiltration by leukocytes following OVA sensitization and challenge was measured using H&E staining. Both KO and WT mice showed significant increase in leukocyte infiltration. No statistically significant differences between KO and WT female nor male mice were found with respect to airway infiltration with leukocytes (Fig. 4c–e).

Sphingolipid and MDA levels were measured in the lungs of allergic mice (Fig. 5). In WT female lungs, allergen sensitization and challenge were associated with lower levels of Sph and S1P in WT-OVA compared to WT-PBS (adjusted $p < 0.00001$ and $p < 0.001$, respectively, multiple t tests) (Fig. 5a). No significant differences were however observed between KO-OVA and KO-PBS female lungs. KO-PBS females had lower levels of C24:0, Sph and S1P in their lungs compared to WT-PBS females (adjusted $p < 0.0005$, $p < 0.0005$, and $p < 0.00005$, respectively, multiple t tests), but no significant differences were found between WT-OVA and KO-OVA female lungs.

In male lungs, no significant changes in sphingolipid levels were found in KO-OVA versus KO-PBS, or WT-OVA versus WT-PBS mice (Fig. 5b). KO-PBS male lungs had lower levels of C24:0, C24:1, C26:0, and higher levels of C20:4 compared to WT-PBS male lungs (adjusted $p < 0.005$, multiple t tests), whereas the only significant difference between WT-OVA and KO-OVA male lungs was found in the levels of C20:4 (adjusted $p < 0.000005$, multiple t tests) (Fig. 5b).

MDA levels were increased in the lungs of WT-OVA mice of both sexes compared to the lungs of WT-PBS mice ($g = 2.0$, $p < 0.01$, and $g = 1.5$, $p < 0.05$ for females and males, respectively; p value determined using two-tailed t test). The lungs of KO-OVA mice of both sexes also showed higher MDA levels compared to KO-PBS lungs ($g = 1.4$, $p < 0.05$, and $g = 1.6$, $p < 0.005$ for females and males, respectively, p value determined using two-tailed t test). KO-OVA male lungs had significantly higher MDA levels than WT-OVA male lungs ($g = 2.0$, $p < 0.001$, two-tailed t test) (Fig. 5). Differences between KO-OVA and WT-OVA female lungs did not reach statistical significance ($g = 1.1$, $p = 0.0868$, two-tailed t test).

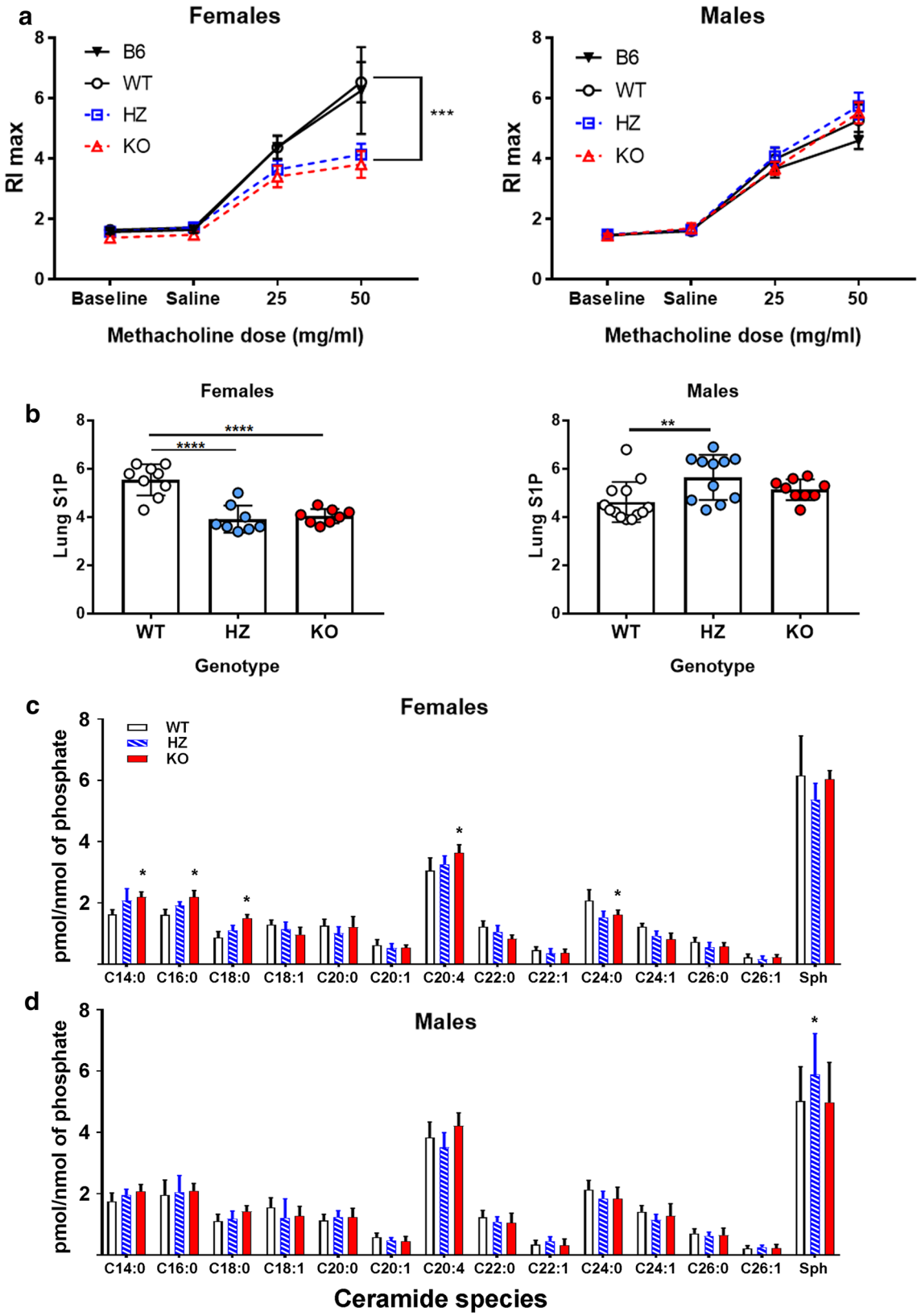
IgE levels

Plasma IgE levels were significantly higher in KO than in WT naïve female mice (Fig. 6a), whereas in naïve males no differences were observed between genotypes (Fig. 6b). In the acute allergic asthma model, plasma IgE levels were increased upon OVA challenge in males and females of both genotypes (Fig. 6c, d).

The liver transcriptome of *Zpbp2* KO mice shows changes in lipid metabolism and MAPK signaling pathways

While in lungs of naïve mice sex-specific factors influenced the impact of the deletion on ceramide metabolism, the relationship between genotype and ceramide levels was independent of sex in their livers. We reasoned that liver would be the more informative tissue for identifying the biological pathways affected by the *Zpbp2* deletion and causing reduction in ceramide levels. We conducted RNA-seq analysis of the liver transcriptomes of KO and WT mice. A total of 26 differentially expressed genes (DEGs) with log_FC ≥ 1.5 and adjusted edgeR p value ≤ 0.05 were identified, 13 were upregulated and 13 were downregulated in KO mice (Fig. 7a, b). Functional annotation analysis showed highest enrichment of the lipid metabolism and MAPK signaling pathways and GO terms for regulation of lipid biosynthesis (Supplementary Tables S4 and S5). The effect of the mutation on the expression of two differentially expressed genes, lipin 1 (*Lpin1*) and nuclear receptor subfamily 4, group A, member 1 (*Nr4a1*), was validated by RT-qPCR (Fig. 7c). Thus, the sum of our data suggests that deletion of *Zpbp2* is associated with changes in the expression of genes involved in the lipid metabolism pathways.

We hypothesized that reduced levels of VLCCs and increased lipid peroxidation (MDA levels shown in Supplementary Fig. S3) in the *Zpbp2* KO mice led to downregulation of transcription of the peroxisome proliferator activated receptor alpha (*Ppara*) gene, which is highly expressed in the liver and plays a pivotal role in lipid metabolism and adipocytokine signaling (Aoyama et al. 1998; Watanabe et al. 2000). We therefore tested *Ppara* expression levels in


a
Females
Males
B6
WT
HZ
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RI max
***
Baseline
Saline
25
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Methacholine dose (mg/ml)
b
Females
Males
Lung S1P
****
****
**
WT
HZ
KO
Genotype
c
Females
pmol/nmol of phosphate
C14:0
C16:0
C18:0
C18:1
C20:0
C20:1
C20:4
C22:0
C22:1
C24:0
C24:1
C26:0
C26:1
Sph
d
Males
Ceramide species

◂**Fig. 2** Impact of *Zpbp2* deletion on lung response to methacholine and sphingolipid levels. **a** Lung resistance in response to increasing doses of inhaled methacholine in naïve animals. Naïve KO and HZ female mice have lower lung resistance than WT females. No effect of genotype on AHR is present in naïve males. **b** S1P (sphingosine-1-phosphate) levels in the lungs (**c**) of naïve WT, HZ and KO animals. Error bars show standard error of the mean (SEM). Significant differences between groups are shown as $*p<0.05$, $**p<0.01$, $****p<0.0001$. **c, d** Levels of different species of ceramides in the lungs of WT, HZ and KO females (**c**) and males (**d**). KO female mice have higher levels of LCC (C14:0, C16:0, C18:0), and C20:4 and lower levels of VLCC C24:0 compared to WT mice. Error bars show standard error of the mean (SEM). No statistically significant differences in ceramide levels were detected in male lungs. HZ male lungs have elevated Sph compared to WT lungs. Asterisks indicate statistically significant differences in ceramide levels $*p<0.05$

mouse livers. *Ppara* RNA levels were lower in the livers of KO mice representing about 76% of the WT levels ($g=1.3$, $p<0.05$, two-tailed Student's t test) (Fig. 7d).

Deletion of *Zpbp2* is associated with increased methylation of the promoter/enhancer region, but has no significant effect on *Ormdl3* levels

Zpbp2 is expressed at low levels in most non-gonadal mouse tissues. This led us to hypothesize that the impact of its deletion on lipid metabolism may be due to a *cis*-regulatory effect on the transcription of a neighboring gene rather than loss of the *Zpbp2* gene product (Berlivet et al. 2012; Naumova et al. 2013). The 5′ region of the *Zpbp2* gene harbors an enhancer region with several DNAse I hypersensitive sites (DHS) and enrichment of enhancer-specific histone marks H3Kme1 and H3K27ac (Fig. 8a). The deleted region is replaced with a *Pgk1-HPRT* cassette (Lin et al. 2007). Since insertion of foreign DNA often triggers methylation of the integrated DNA and may spread to the adjacent host DNA (Watanabe et al. 2015), we tested the methylation levels of the intact part of the *Zpbp2* promoter/enhancer (P/E) region. The *Zpbp2* P/E region was hypomethylated in the DNA of WT mice, but gained methylation in the DNA of KO animals (Fig. 8b, c), reminiscent of the variation in *ZPBP2* P/E methylation levels observed in human cells (Al Tuwaijri et al. 2016; Berlivet et al. 2012; Moussette et al. 2017; Naumova et al. 2013). The *Zpbp2* enhancer region has been shown to interact with several distant genes in *cis* (He et al. 2014; Schmiedel et al. 2016). Therefore, we tested the hypothesis that deletion of *Zpbp2* and increase in its P/E methylation levels influenced regulation of neighboring genes, *Ormdl3, Ikzf3*, and growth factor receptor bound protein 7 (*Grb7*) that interact with the *Zpbp2* locus in mouse and human tissues (He et al. 2014; Schmiedel et al. 2016) (Supplementary Fig. S1).

Ormdl3 encodes a putative negative regulator of de novo ceramide biosynthesis (Oyeniran et al. 2015), [reviewed in Ono et al. (2015) and Worgall (2017)] and resides 30 kb distal to *Zpbp2* on mouse Chr 11. Mice that overexpress the human *ORMDL3* transgene have reduced levels of S1P in their lungs, whereas mice with an airway epithelium-specific deletion of *Ormdl3* have increased S1P levels (Miller et al. 2014, 2017a, b). If our hypothesis were correct and the deletion and methylation of the *Zpbp2* enhancer influenced *Ormdl3* transcription, we would observe upregulation of *Ormdl3* in the KO livers and spleens as well as lungs from KO female mice. We found no changes in *Ormdl3* expression in lungs and spleens of KO mice (Fig. 8d). In livers, *Ormdl3* RNA levels were lower in KO mice, however these differences did not reach statistical significance ($g=0.8$, $p>0.05$, two-tailed t test). Thus, our results do not support the hypothesis and we conclude that reduced levels of S1P in the organs of KO mice did not result from a *cis*-effect of the *Zpbp2* deletion on *Ormdl3* expression.

Ikzf3 encodes a transcription factor with a well-established role in B-cell maturation. Its transcriptional start site (TSS) is located 5 kb proximal to the *Zpbp2* TSS. We found a modest but statistically significant increase in *Ikzf3* expression in the spleens of both KO male and KO female mice compared to WT animals ($g=1.0$, $p<0.05$, two-tailed t test) (Fig. 8e and Supplementary Fig. S4). No effect was observed in lungs. *Ikzf3* was not expressed in livers.

Grb7 encodes an adapter protein involved in several signaling pathways. *Grb7* levels were significantly higher in the lungs of KO mice compared to controls ($g=1.0$, $p<0.05$, two-tailed t test) (Fig. 8f). However, we found no effect of the *Zpbp2* deletion on *Grb7* expression neither in spleens, nor in the livers of KO mice. Thus, our data show that the *Zpbp2* deletion is associated with modest increase in the expression of *Ikzf3* in spleens and *Grb7* in lungs, but has no significant effect on *Ormdl3* levels.

Discussion

In this study, we demonstrate that deletion of the *Zpbp2* gene in mice causes changes in AHR to acetylcholine receptor agonist methacholine in a sex-dependent fashion: the deletion reduces AHR in females, but not males, in the absence of allergic sensitization (Fig. 2a). Our data are in agreement with the role of 17q12 alleles in bronchial sensitivity and non-allergic asthma (Bouzigon et al. 2008; Granell et al. 2013). The deletion is also associated with increased methylation levels of the promoter/enhancer region adjacent to the deletion. Studies in humans show that increased methylation of the *ZPBP2* promoter (and presumably lower expression of the gene) in peripheral blood cells is associated with a reduced risk of asthma in females, but not males (Al Tuwaijri et al. 2016). Therefore, the *Zpbp2* knock-out mouse model recapitulates several aspects of the complex

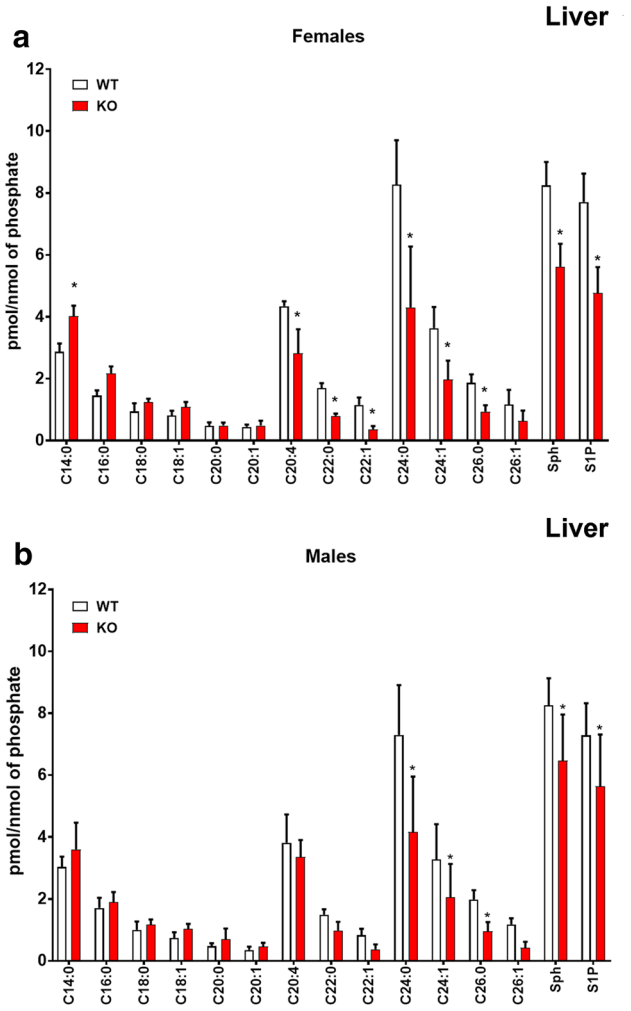


Fig. 3 The *Zpbp2* deletion influences sphingolipid profiles in livers. Levels of different species of sphingolipids in the livers of WT and KO females (**a**) and males (**b**). Error bars show SEM. Asterisks indicate statistically significant differences in ceramide levels with $p < 0.05$

interplay between sex, *ZPBP2* regulation, DNA methylation, and asthma that was observed in humans with the caveat that the mechanisms that cause variation in DNA methylation in knock-out mice may differ from those that act in the human locus.

The deletion did not have a significant effect on AHR in the acute allergic asthma model (Fig. 4). OVA sensitization and challenge led to increase in AHR in both KO and WT male mice. In females, AHR tended to increase following allergen sensitization and challenge, but the increase was not significant (Fig. 4a). AHR differences between WT-OVA and KO-OVA mice were also not significant. In contrast, the same OVA-challenged mice showed a pronounced inflammatory response measured as airway infiltration with leukocytes and increase in IgE levels. The low AHR response to OVA sensitization and challenge in our mice is likely due to the overall resistance to methacholine and low sensitivity of the C57BL/6J strain to allergen challenge (Ackerman et al. 2005; Camateros et al. 2009; Kanagaratham et al. 2017; Moisan et al. 2006). The same deletion placed in a genetic background conveying higher allergen sensitivity may show a more pronounced protective effect. This hypothesis is currently being tested.

Importantly, we find that the deletion affects lung lipid metabolism in a sex-specific fashion: female KO mice have reduced levels of S1P and anti-inflammatory ceramide species C24:0 and increase in pro-inflammatory C14:0, C16:0, and C18:0 in their lungs, whereas in males differences in ceramide levels between KO and WT lungs are not significant. This correlates with the impact of the deletion on AHR in naïve females, but not males. Moreover, lung resistance of naïve *Zpbp2* KO female mice positively correlates with the levels of S1P in their lungs (Supplementary Fig. S2). As S1P is known to influence smooth muscle contractility independently of inflammation (Chiba et al. 2010, 2011), differences in S1P levels may be the key to understanding how loss of *Zpbp2* reduces AHR.

In contrast to lungs, lower levels of S1P, Sph, C24:0, C24:1, and C26:0 are found in livers and spleens of both KO males and females. Based on the lipid profiles across the three organs and lack of statistically significant sex differences in *Zpbp2* expression in WT mice, we hypothesize that while the *Zpbp2* deletion alters lipid metabolism and reduces VLCC and S1P levels, in the male lungs this decrease is compensated by factors that are either exclusively or predominantly expressed in male lungs and are involved in regulation of S1P metabolism. These factors have yet to be identified.

In liver, the *Zpbp2* deletion impacts expression of genes involved in the MAPK signaling pathway, which is known to be activated by S1P (Che et al. 2012; Wu et al. 1995), as well as expression of several genes implicated in lipid metabolism and transport, including *Lpin1, Nr4a1,* and *Ppara*, and those effects are independent of sex (Fig. 7c, d). LPIN1 is a phosphatidic acid phosphohydrolase involved in the regulation of lipid metabolism and pathogenesis of alcoholic fatty liver disease (Langner et al. 1991; Lee et al. 1995; Peterfy et al. 2001). Mice carrying the fatty liver dystrophy (*fld*) mutation, which deletes part of the *Lpin1* gene, have enlarged livers, decreased body weight and reduced fat pads as well as reduced levels of sphingomyelin (Langner et al. 1991; Peterfy et al. 2001). NR4A1 is a transcription factor involved in regulation of inflammation and lipid metabolism (Hanna et al. 2012; Pols et al. 2008). It is worth noting that NR4A1 deficient females, but not males, show increased susceptibility to diet-induced obesity (Perez-Sieira et al. 2013). NR4A1 deficiency is associated with enhanced allergic inflammation in mice (Kurakula et al. 2015). Moreover, in humans, the *NR4A1* gene is associated with bronchial

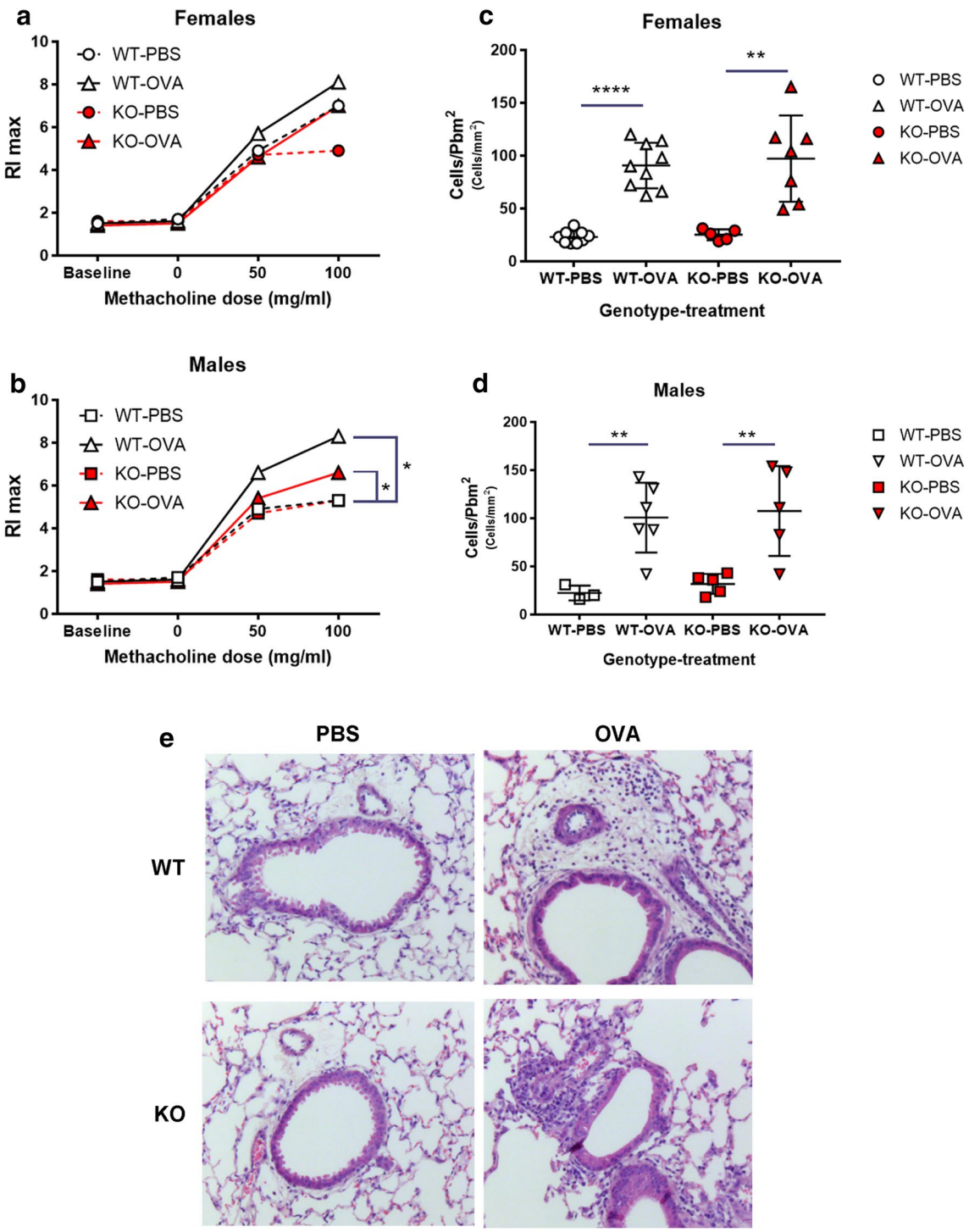


Fig. 4 Acute allergic asthma model. **a** Lung resistance in response to increasing doses of inhaled methacholine in allergic female mice. No significant differences between KO and WT females are observed. **b** Lung resistance in response to increasing doses of inhaled methacholine in allergic male mice. **c, d** Levels of infiltration of airways with leukocytes are similar in WT and KO female (**c**) and male (**d**) mice. Error bars show standard deviation. **e** Representative pictures for leukocyte infiltration in the lungs of allergic animals using H&E staining. Type of challenge is shown on top, genotype is shown on the left. Measurements were done on different paraffin-embedded slides and using at least four different airways in each section analyzed for each mouse from each group (WT-PBS, WT-OVA, KO-PBS, KO-OVA). Quantification was done by counting the number of inflammatory cells around each airway and normalizing it by division over the square of the perimeter "in millimeter" of the airway basement membrane. $N = 3$–9 per group. Significant differences between groups are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (Color figure online)

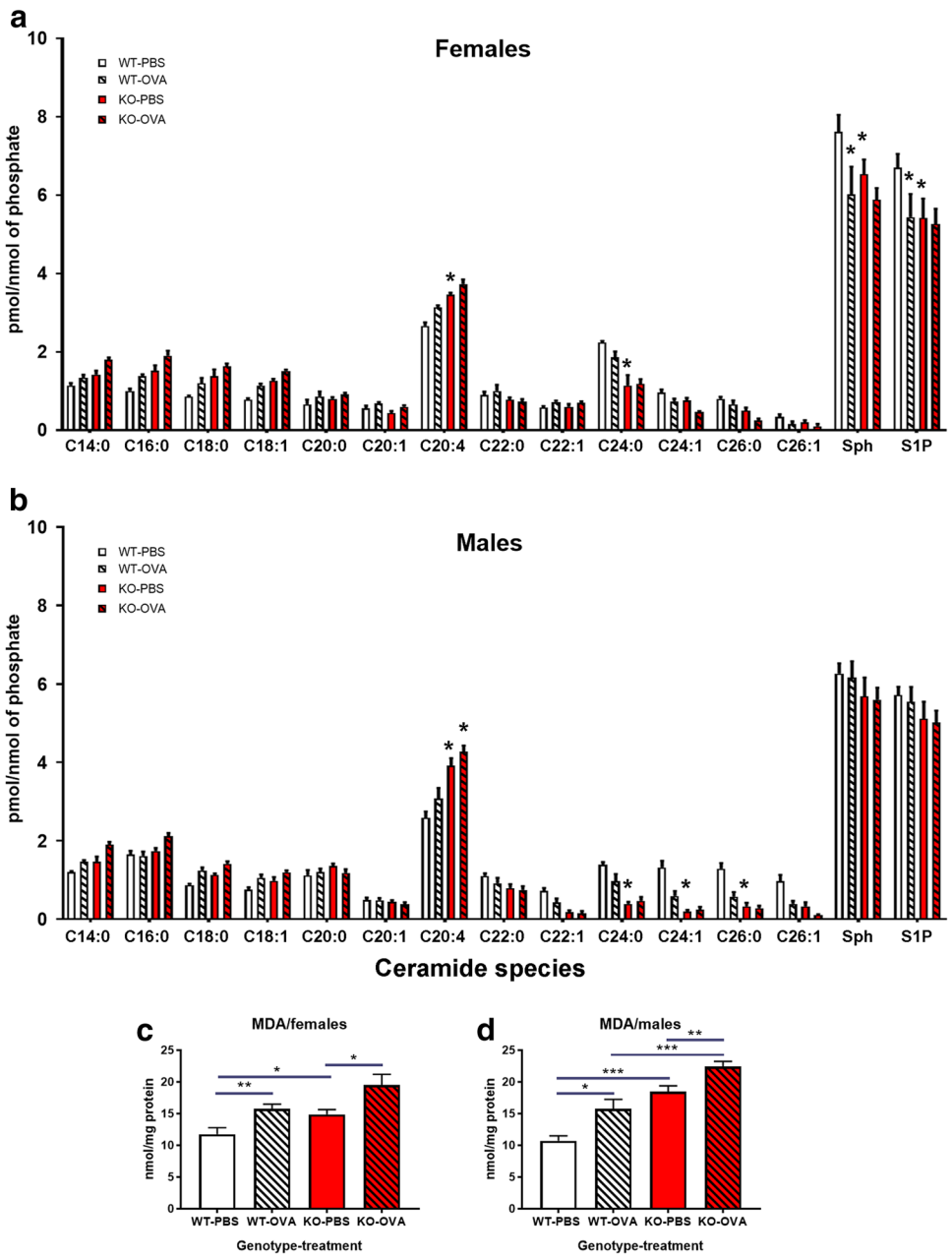


Fig. 5 Sphingolipid levels in allergic mice. **a, b** Levels of different species of ceramides in the lungs of OVA sensitized and challenged with PBS or OVA WT and KO female (**a**) and male (**b**) mice. **c, d** Levels of malonyldialdehyde (MDA) in the lungs of female (**c**) and male (**d**) mice. Error bars show SEM. Asterisks indicate statistically significant differences in ceramide levels $*p < 0.05$

sensitivity (Kurakula et al. 2015). Our mice had reduced levels of *Nr4a1*, but did not show higher inflammation compared to controls. PPARA is a transcription factor with a critical role in the regulation of hepatic genes and fatty acid metabolism (Akiyama et al. 2001). *Ppara* knock-out mice have increased body weight, enlarged livers, and increased gonadal fat pad weights (Akiyama et al. 2001; Aoyama et al. 1998; Lee et al. 1995). The increased body weight of aging KO mice and increased fat accumulation in adult KO females are in good agreement with the increased expression of *Lpin1* and reduced expression of *Nr4a1* and *Ppara* as well as altered lipid metabolism in our KO mice. Remarkably, a recent GWAS study of body fat distribution in humans shows an association between SNPs in the *ZPBP2-GSDMB* region and body fat distribution in women of European descent (Chu et al. 2017). All these lines of evidence support the mechanistic link between the *ZPBP2* locus and the lipid metabolism pathways. However, the nature of this link is still elusive. Furthermore, in this study we examined expression profiles of mouse livers and focused on the impact of the *Zpbp2* deletion on ceramide metabolism in the liver as it was similar in both sexes. Separate RNA-seq experiments to identify affected gene pathways in female and male lungs at baseline and after methacholine challenge would help elucidating how and why the deletion influences AHR and why this influence is limited to female lungs.

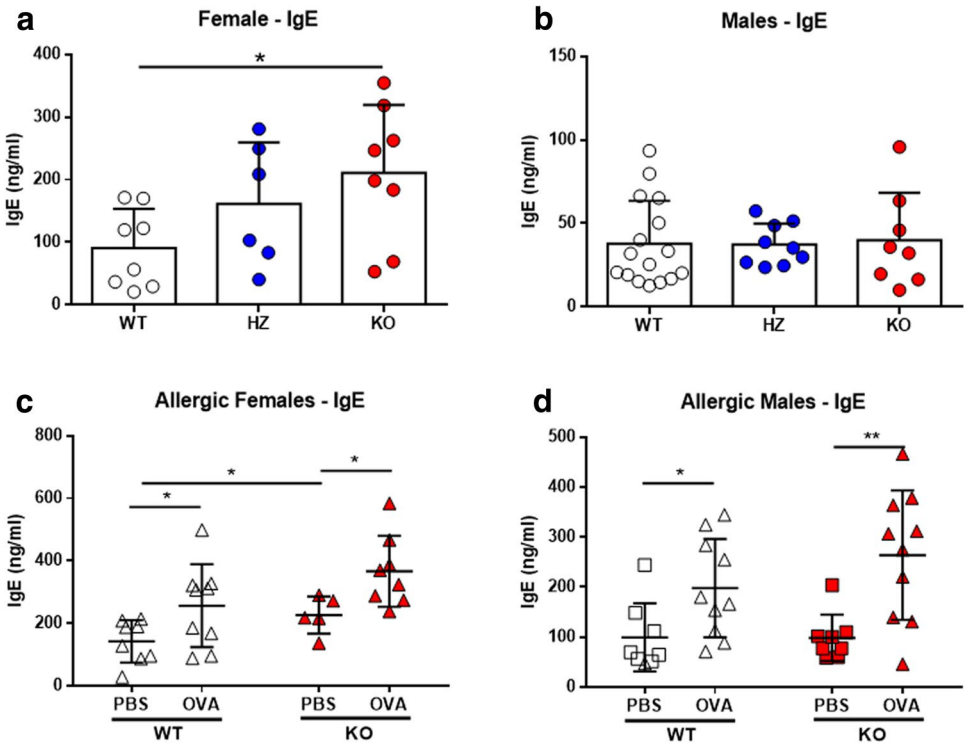


Fig. 6 Blood plasma IgE levels. **a, b** blood plasma IgE levels are higher in naïve KO females compared to WT females (**a**), but not different in naïve KO males compared to WT males (**b**). **c, d** blood plasma IgE levels increase after OVA challenge in KO and WT mice of both females (**c**) and males (**d**). Statistically significant differences are marked by $*p<0.05$, $**p<0.01$, $***p<0.001$

Methylation of mouse transgenes is a well-documented phenomenon resulting from host response to foreign DNA (Ratnam et al. 2017; Sapienza et al. 1987), reviewed in (Turker 2002), however the nature of DNA methylation of the host DNA region adjacent to a deletion/insertion is less understood (Jahner and Jaenisch 1985; Hollister and Gaut 2009; Turker 2002). In principle, in the *Zpbp2* KO mice it may result from spreading of DNA methylation from the inserted *Pgk1-HPRT* cassette or from lack of *Zpbp2* transcription (Jahner and Jaenisch 1985). Independent of the mechanism involved, methylation of the *Zpbp2* enhancer region could attenuate expression of neighboring genes that interact with this enhancer. Alternatively, if *Zpbp2* competes with neighboring genes for a common enhancer that is not affected by the deletion, this may lead to upregulation of neighboring genes due to loss of the competitor. We therefore tested the hypothesis that the KO phenotypes resulted from a *cis*-regulatory effect of the deletion; that is, that the loss of *Zpbp2* expression, or partial loss and increased methylation of the *Zpbp2* enhancer region modified transcription of neighboring genes. Indeed, we observed modest upregulation of *Ikzf3* in spleens and *Grb7* in lungs of KO mice. IKZF3 is important for the maturation of B lymphocytes, NK, and Th17 cells and plays a critical role in immunity (Holmes et al. 2014; Quintana et al. 2012; Wang et al. 1998). In principle, higher levels of *Ikzf3* expression in KO mice may contribute to the higher levels of IgE in KO females and the differences between KO and WT mice with respect to expression of the Th17 cell differentiation and IL17 signaling pathways genes (Supplementary Table S4 and S5). GRB7 is implicated in cell migration, respiratory syncytial virus infection and the dynamics of stress granules, however we found no evidence in the literature regarding its role in lipid metabolism or asthma pathogenesis (Han and Guan 1999; Tsai et al. 2008). To determine how the *cis*-regulatory effects of the deletion of *Zpbp2* impact the observed phenotypes in the KO mice, further studies are necessary.

Chromosomal region 17q12-q21 is associated with several common complex genetic diseases: childhood asthma, inflammatory bowel disease (IBD), primary biliary cirrhosis (PBC), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and type I diabetes (T1D) (Ayabe et al. 2016; Barrett et al. 2008; Bentham et al. 2015; Hirschfield and Siminovitch 2015; Moffatt et al. 2007, 2010; Stahl et al. 2010). The same haplotype that confers increased risk of asthma is protective for IBD, PBC, RA, SLE, and T1D, while the haplotype that confers increased risk of IBD, PBC, RA, SLE, and T1D is protective for asthma (Ayabe et al. 2016; Barrett et al. 2008; Bentham et al. 2015; Hirschfield and Siminovitch 2015; Moffatt et al. 2007, 2010; Stahl et al. 2010). Hence, genetic variants residing in the 17q12-q21 region may have a pleiotropic effect and play a role in the pathogenesis of all six conditions. Lipid metabolism affects lung sensitivity, inflammation, and immunity (Parker et al. 2016; Worgall et al. 2013). It represents a good candidate for the role of a common denominator in the pathogenesis of all these diseases. To date, multiple lines of evidence point to *ORMDL3* as

the top 17q21 asthma gene candidate with a function in *de novo* biosynthesis of ceramide (Miller et al. 2017a; Oyeniran et al. 2015; Worgall 2017). Here, we provide the first evidence that *ZPBP2* may be another 17q12 gene that influences lipid metabolism and bronchoconstriction.

It is important to note that the relationship between genetic variants in the 17q12-q21 region and lipid metabolism is likely to be complex as no correlation has been found between 17q12-q21 alleles and plasma long-chain sphingoid base levels (Zhakupova et al. 2016). This may be due to the strong influence of methylation levels on *ZPBP2* expression that may mask the effect of genotype (Berlivet et al. 2012; Moussette et al. 2017). Therefore, determining the relationship between genotype, *ZPBP2* methylation and levels of different ceramide species in human cells would be a necessary next step in the elucidation of the mechanism responsible for genetic association with disease in the 17q12-q21 region.

Fig. 8 *Cis*-regulatory effects of the *Zpbp2* deletion. **a** Map of the mouse *Zpbp2* locus. Enrichment for enhancer and promoter-specific histone marks are shown in the context of the UCSC browser (mm9). The red rectangle shows the deleted region. The black rectangle indicates the region assayed for DNA methylation levels. All features are shown in the context of the UCSC browser. **b** Sodium bisulfite sequencing analysis of *Zpbp2* promoter methylation in the tails of WT and KO mice. Each panel corresponds to one DNA sample. Each circle represents a single CG. Open circles represent unmethylated, filled circles represent methylated CGs. Each row corresponds to a single clone. **c** The *Zpbp2* deletion is associated with gain of methylation in lungs, spleens and livers of KO mice. Pyrosequencing methylation assays were done in 5–9 animals per genotype. Error bars show standard deviation. **d** The *Zpbp2* deletion has no significant impact on *Ormdl3* RNA levels in the lungs, spleens and livers of WT ($n=8$–11) and KO ($n=8$–12) mice. **e** *Ikzf3* RNA levels are higher in the spleens of KO mice compared to WT animals. *Ikzf3* is not expressed in liver. **f** *Grb7* RNA levels are higher in the lungs of KO compared to WT mice, but no difference is detected in spleens or livers. Significant differences between groups are shown as $*p<0.05$, $**p<0.01$. (Color figure online)

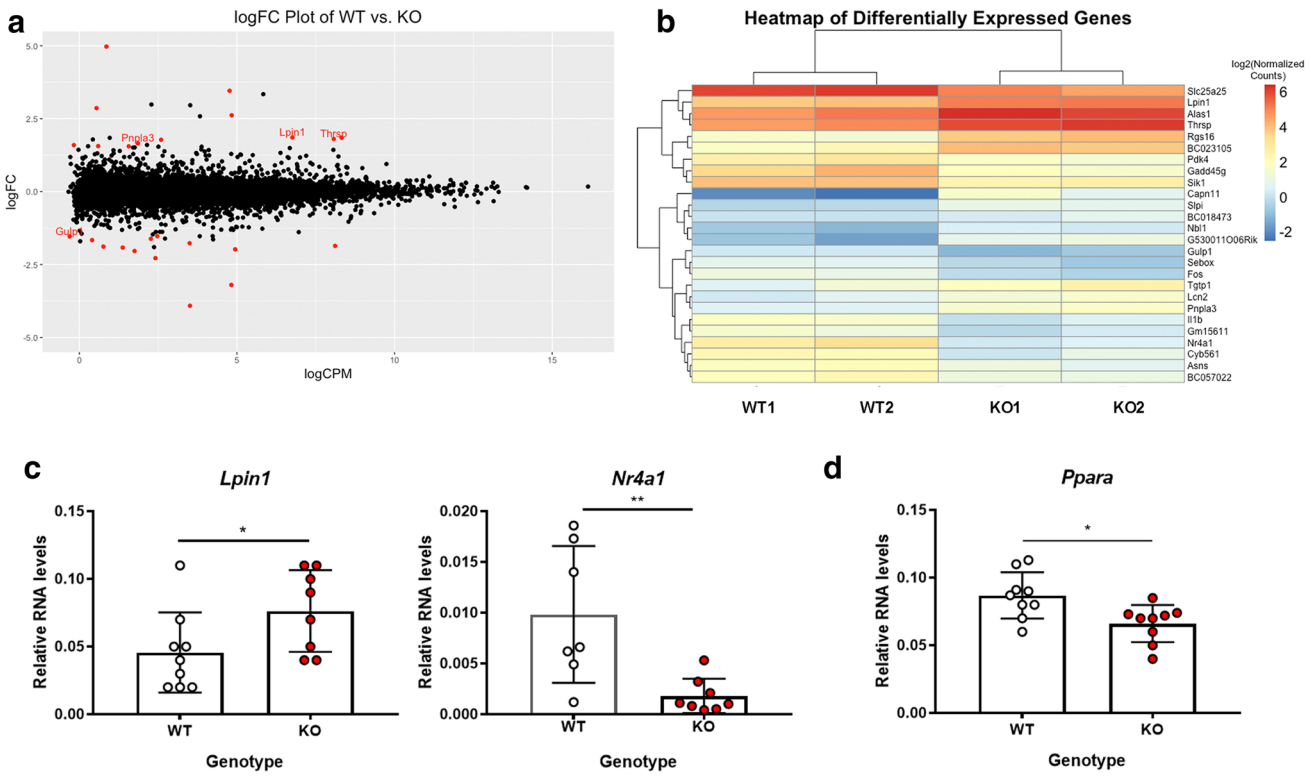
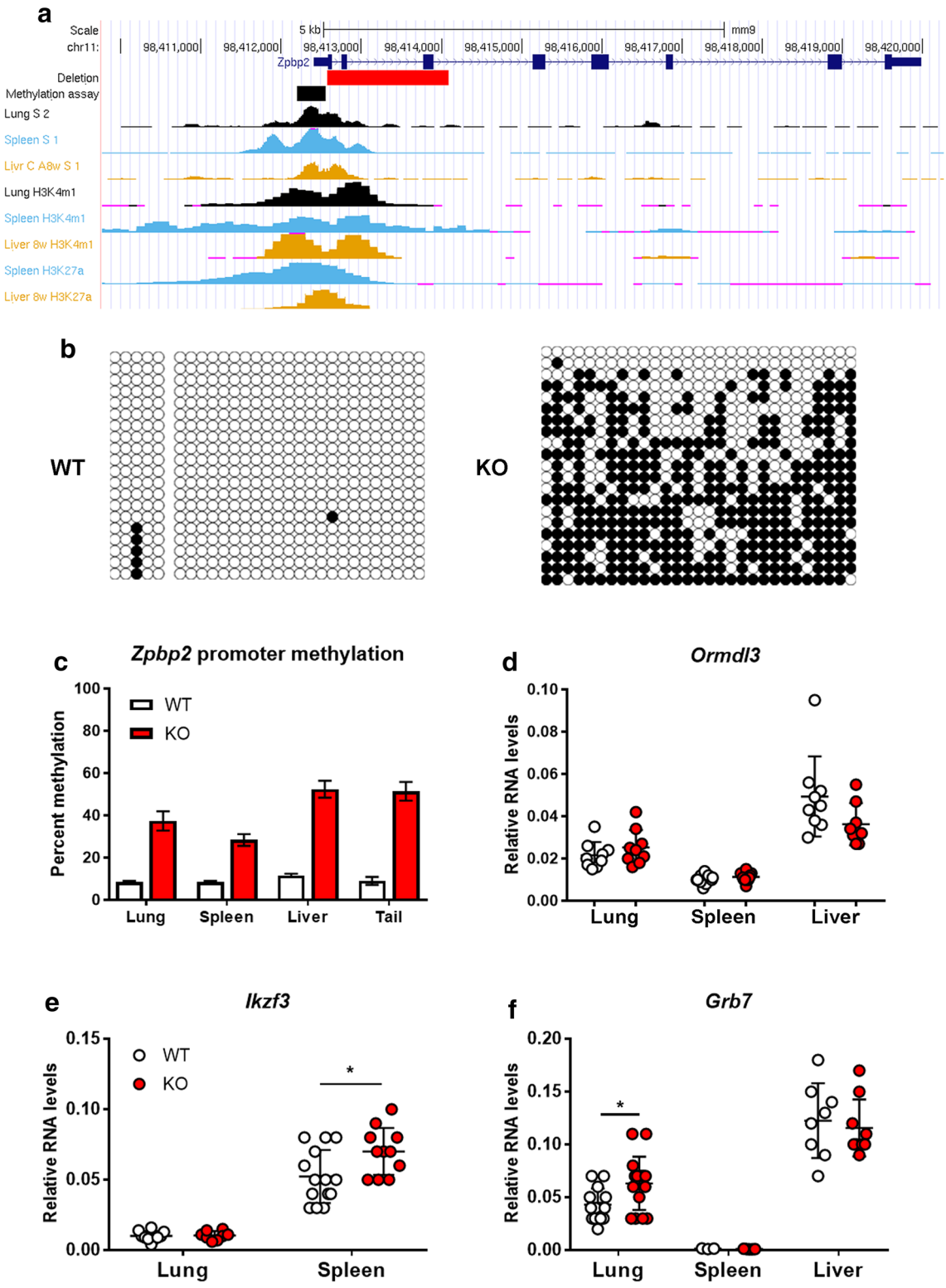


Fig. 7 *Zpbp2* deletion impacts expression of genes involved in lipid metabolism. **a** Comparison of WT and KO liver transcriptomes. Bland–Altman plot showing the $\log_2$ transformed counts per million mapped reads expression values and the $\log_2$ transformed fold change in expression values between the livers of WT and KO ($n_{WT}=2$, $n_{KO}=2$) obtained from the RNA-seq analysis. Differentially expressed genes are highlighted in red and lipid metabolism associated genes are labeled. **b** Heat map and dendrogram showing $\log_2$ transformed counts per million mapped reads values of differentially expressed genes. **c** Differences in the expression of *Lpin1* and *Nr4a1* were validated using quantitative qPCR ($n=8$–9, both sexes included). **d** Reduced expression of the *Ppara* gene in KO livers suggests an impact of the deletion on the adipocytokine signaling pathway ($n=8$–9, both sexes included). Significant differences between groups are shown as $*p<0.05$, $**p<0.01$. (Color figure online)



Acknowledgements The authors are grateful to Pamela Stroud, Amanda Baumholtz, and Wei Cui for technical assistance and Drs. Danielle Malo and Aimee Ryan for helpful discussion. This work was supported by funds from the Canadian Institutes of Health Research (to AKN and DR) and the Research Institute of the McGill University Health Centre (to AKN). DR is a member of Infectious Diseases and Immunity in Global Health Program, (IDIGH). VC has been a recipient of the Department of Human Genetics scholarship. CK has been a recipient of the Banting and Best Doctoral Training Award from CIHR.

Author contributions CK contributed to study design, data collection, statistical analysis, and writing of the manuscript. VC contributed to data collection, data analysis, and writing the manuscript. MY, BH, and SM contributed to data collection and analysis. DV contributed to data analysis, interpretation and writing of the manuscript. LJ contributed to data collection. GB contributed to data analysis and interpretation. JBdS contributed to data collection and data analysis. DR contributed to study design, data interpretation and edited the manuscript. AKN contributed to study design, data collection, analysis, data interpretation, and wrote the manuscript. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest Authors declare that they have no competing interest.

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