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# The KRAB Zinc Finger Protein RSL1 Modulates Sex-Biased Gene Expression in Liver and Adipose Tissue To Maintain Metabolic Homeostasis

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**Krüppel-associated box zinc finger proteins (KRAB-ZFPs) are a huge family of vertebrate-specific repressors that modify gene expression in an epigenetic manner. Despite a well-defined repression mechanism, few biological roles or gene targets of KRAB-ZFP are known. Regulator of sex-limitation 1 (RSL1) is a mouse KRAB-ZFP that enforces male-predominant expression in the liver, affecting body mass and pubertal timing. Here we show that female but not male *Rsl1*$^{-/-}$ mice gain more weight than wild-type mice on a high-fat diet (HFD) and that key liver and white adipose tissue (WAT) metabolic genes are altered in both *Rsl1*$^{-/-}$ sexes in response to dietary stress. Expression profiling of *Rsl1*-sensitive genes in liver and WAT indicates that RSL1 accentuates sex-biased gene expression in liver but greatly diminishes it in WAT. RSL1 expression solely in liver is sufficient to limit diet-induced weight gain and suppress lipogenic genes in WAT, indicating that RSL1 balances metabolism via liver-to-adipose-tissue communication. RSL1's effects on adult physiology exemplify a significant modulatory capacity of KRAB-ZFPs, in the absence of which there is widespread metabolic dysregulation. This ability to buffer against gene expression noise, coupled with extensive individual genetic variation, highlights the enormous potential of *KRAB-Zfp* genes as candidate risk factors for complex diseases.**

Metabolic homeostasis in the face of nutritional stress requires integrated control of gene expression within and between multiple organs. Many of the critical regulatory networks are known (1), but genetic factors that modulate responses to dietary perturbation are less well studied. Recently, a large class of transcriptional repressors, Krüppel-associated box zinc finger proteins (KRAB-ZFPs), were shown to have a significant capacity to modulate gene expression and to influence complex phenotypes, including metabolic homeostasis (2–5). These proteins are broadly expressed and counter gene activators in tissue- and sex-specific patterns (2), exemplifying the complex regulation also found in many metabolic responses (1).

*KRAB-Zfp* genes arose in tetrapods and have greatly expanded to nearly 400 genes in humans and mice, often clustered due to segmental duplications (6–9). Each protein has a C-terminal array of $C_2H_2$ zinc fingers that bind DNA and an N-terminal KRAB domain that recruits the corepressor KRAB-associated protein 1 (KAP1/TRIM28/TIF1β) (10–12). Tethered to chromatin via the KRAB-ZFP, KAP1 acts as a scaffold for histone deacetylases and histone and DNA methyltransferases that facilitate heterochromatinization to silence gene expression (11, 13, 14). *KRAB-Zfp* genes appear to have evolved to suppress retroviral activity in embryogenesis (15, 16), but additional roles in aspects of mammalian development and physiology, including metabolic homeostasis, have been acquired over time (2, 5, 17–20). Despite the enormity of the *KRAB-Zfp* family, remarkably few of its members have identifiable biological roles or gene targets.

We identified the *Regulator of sex-limitation 1* (*Rsl1*) gene as a mouse *KRAB-Zfp* involved in sex-biased liver gene expression (21, 22). In *Rsl1*$^{-/-}$ mice, the usually male-specific *sex-limited protein* (*Slp*) gene is expressed at high levels in female livers, and at levels even higher than wild-type (WT) levels in males, due to loss of the RSL1 repressor. Other sex-biased liver genes sensitive to *Rsl1* encode cytochrome P450s, major urinary proteins (MUPs), and some metabolic enzymes (2, 21, 22). RSL1 binding 2 kb upstream of *Slp* recruits KAP1, thus preventing expression in females (3). Repression is overcome in males by hormonal induction acting through STAT5b, the major effector of sexually dimorphic liver gene expression (3, 23). RSL1 binds upstream of *Slp* in both males and females, but in males STAT5b action is tempered by RSL1 by reciprocal occupation of the DNA binding site. This demonstration of RSL1 action on a *bona fide* target corroborates the mechanism of KRAB/KAP1-mediated repression *in vivo*. Moreover, KRAB-ZFP interactions in some cases are dynamic and thus may modify adult physiology in response to environmental cues, such as hormones or, in the present study, metabolites.

Sex bias in gene expression is a broad phenomenon, with the majority of transcripts in mouse liver showing some degree of sex bias that is similarly detected in humans (24–26). *Rsl1* affects 7.5% of the liver transcriptome, with over 400 genes altered >1.5-fold in *Rsl1*$^{-/-}$ mice. RSL1-affected genes include ones that are male predominant, female predominant, or not sex biased, and as many are activated as repressed, suggesting that RSL1 has a wide range of targets, many of which may be indirect (2). The enrichment of *Rsl1*-responsive liver genes in cholesterol, steroid, and metabolic pathways suggests that RSL1 acts in hepatic homeostasis and may be a factor linking sex differences to metabolism.

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In this study, we examined the broader impact of RSL1 on sex-specific metabolic gene regulation. RSL1 affected the fasting response more dramatically in males and diet-induced obesity (DIO) more dramatically in females. Given a sex-specific physiological effect on adipose tissue, dimorphism was sought at the level of gene expression. Comparative transcription profiling of liver and white adipose tissue (WAT) revealed that in the absence of RSL1 (*Rsl1*$^{-/-}$ mice), the extent of sex-biased gene expression was diminished in liver but exaggerated in WAT. In female mice expressing RSL1 only in liver, diet-induced weight gain was suppressed, as was expression in WAT of genes involved in *de novo* lipogenesis. These results suggest that RSL1 modulates systemic gene expression in accord with sex-specific physiological needs. Furthermore, the influence of *Rsl* does not drive one particular pathway, as reflected by widespread dysregulation in its absence. Genetic variation in KRAB-ZFP modulators is likely an important, albeit unassessed, determinant in differential susceptibility to complex diseases, such as obesity and diabetes, in humans.

## MATERIALS AND METHODS

**Mice and diets.** B10.D2 mice (WT) were purchased from Jackson Laboratory (stock 000463). Congenic B10.D2.PL-*rsl* (*Rsl1*$^{-/-}$) and liver-specific *Rsl1* transgenic (L-*Rsl1*-tg) mice were described previously (2, 27) (the L-*Rsl1*-tg gene is carried on the *Rsl1*$^{-/-}$ background). All protocols involving mice received prior approval from the University Committee on Use and Care of Animals at the University of Michigan. Mice were housed in an environmentally controlled room at 23°C with a 12-h light-dark cycle, with *ad lib* access to water and standard rodent chow (diet 5001; LabDiet, St. Louis, MO), except where noted. All mice were aged to 10 weeks prior to experimentation to allow sex-specific gene expression patterns that arise at puberty (4 to 6 weeks) to stabilize. For food restriction, mice were transferred to clean cages without food bins at 5:00 p.m. and euthanized at 9:00 a.m. the following day for collection of serum, liver, and abdominal WAT, which were quick-frozen in liquid nitrogen and stored at −70°C until analyzed. For high-fat feeding, WT and *Rsl1*$^{-/-}$ mice were transitioned to a high-fat diet (HFD) (45% kcal in fat) (D12451; Research Diets, New Brunswick, NJ) provided *ad lib*. While on HFD, mice were weighed three times per week and euthanized after 60 days for collection of serum, liver, and abdominal WAT (gonadal depot), and the tissues were frozen and stored as described above.

**Metabolic assessment.** Mice were placed in Comprehensive Laboratory Animal Monitoring System (CLAMS) metabolic chambers (Columbus Instruments, Columbus, OH) for 72 h to assess feeding behavior, activity, and gas exchange values ($VO_2$ and $VCO_2$). The respiratory exchange ratio (RER) was calculated as the $VCO_2/VO_2$, and heat production was calculated using the following equation: heat (kcal/h) = $[3.82 + (1.23 \times \text{RER})] \times VO_2$ (28). For glucose tolerance tests, mice were fasted overnight and injected intraperitoneally with D-glucose (2 g/kg body weight). Blood glucose was monitored from the tail vein (Freestyle blood glucose monitoring strips; Abbott Labs, Alameda, CA) 0, 30, 60, 90, 120, and 150 min after injection. Areas under the curves (AUC) were calculated with the aid of the StatsToDo website (http://www.statstodo.com/AUC_Pgm.php).

**Serum analysis.** For biochemical assays other than glucose assay, serum was isolated from whole blood collected either from the tail vein or via cardiac puncture at the time of euthanasia. Insulin was measured using an ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA) kit from Crystal Chem (Downers Grove, IL). Nonesterified free fatty acids (NEFA) were measured using a NEFA-HR (2) kit from Wako Diagnostics (Richmond, VA).

**RNA analysis.** Total RNAs were isolated from liver and abdominal WAT by use of RNeasy minikits (Qiagen) according to the manufacturer's instructions. For real-time quantitative reverse transcription-PCR (qRT-PCR), cDNA was synthesized from 2.5 μg total RNA with a High Capacity cDNA Archive kit (Applied Biosystems, Warrington, United Kingdom) and amplified by PCR in an Applied Biosystems 7500 thermocycler with Power Sybr green master mix (Applied Biosystems) according to the manufacturer's protocol. Primer sequences are reported elsewhere (2, 29) and are available upon request. RT reaction mixtures were pooled ($n \geq 3$ mice per pool), and PCR was performed in duplicate. For *Elovl6*, *Elovl3*, *MUP1*, and *Lcn2*, real-time PCR was repeated on individuals to verify results from pooled RT reaction mixtures. Cycle threshold ($C_T$) values were converted to relative expression levels by using the $2^{-\Delta\Delta CT}$ method (30), with 18S rRNA as the normalization standard. Data are expressed as the mean (± standard error [SE]) relative to that for WT males, and Student's *t* test identified significant differences from the WT within each sex.

**Microarray.** Gene expression profiles were obtained from abdominal WAT from both sexes of WT and *Rsl1*$^{-/-}$ mice by methods similar to those reported previously for profiling liver gene expression (2). Briefly, abdominal WAT RNAs from both sexes of WT and *Rsl1*$^{-/-}$ mice were placed into two pools per sex and genotype ($n = 5$ mice per pool). cDNA from each pool was synthesized and hybridized to a mouse MG-430 PM strip array by the University of Michigan MicroArray Core according to instructions from Affymetrix (Santa Clara, CA). Results were analyzed for quality, converted to relative expression values by the robust multiarray average (RMA) method of Irizarry et al. (31), and deposited into the NCBI Gene Expression Omnibus (GEO) database.

Functional annotation was performed using the DAVID Bioinformatics resource provided by the NIH (http://david.abcc.ncifcrf.gov/home.jsp). Genes that were significantly different between WT and *Rsl1*$^{-/-}$ mice (2-fold cutoff; $P < 0.05$) were analyzed independently by sex as "higher in *Rsl1*$^{-/-}$ than WT mice" and "lower in *Rsl1*$^{-/-}$ than WT mice." Annotation terms from the Gene Ontology (GO), Panther, and KEGG databases were used to cluster genes, and enrichment scores of >1.0 were considered significant. For Venn diagrams, numbers were determined by sorting gene lists with Microsoft Excel, and images were created with Microsoft PowerPoint.

**Primary mouse hepatocytes.** Primary mouse hepatocytes were isolated from adult (>8 weeks old) mice by using a two-step liver perfusion/collagenase digestion method as described previously (5). Isolated hepatocytes in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) were plated in 12-well dishes at a density of $2 \times 10^5$ per well and incubated for 3 to 4 h in a humidified incubator at 37°C and 5% $CO_2$. Attached cells were washed once in 1× phosphate-buffered saline (PBS) and switched to serum-free M199 medium. Half the wells were supplemented with 25 mM glucose plus 100 nM insulin. After an 18-h incubation, hepatocyte RNA was isolated by use of TRIzol (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions and analyzed by qRT-PCR as described above.

**Microarray data accession number.** Microarray data have been deposited in the NCBI GEO database under accession number GSE49075.

## RESULTS

***Rsl1*$^{-/-}$ females are prone to diet-induced obesity.** Mice homozygous for null mutations in the KRAB-Zfp gene *Rsl1* have metabolic dysregulation that is exacerbated by fasting, with some responses blunted and others amplified (2). In particular, hepatic phosphoenolpyruvate carboxykinase 1 (PEPCK; *Pck1*) and stearoyl coenzyme A desaturase 1 (*Scd1*) mRNA levels are elevated and reduced, respectively, relative to WT levels. The WT response to fasting (2-fold rise in *Pck1* and 5-fold drop in *Scd1*) is attenuated in *Rsl1*$^{-/-}$ males but not females. To test the impact of RSL1 in another paradigm of metabolic stress, adult WT mice, *Rsl1*$^{-/-}$ mice, and *Rsl1*$^{-/-}$ mice with a liver-specific *Rsl1* cDNA transgene (L-*Rsl1*-tg) were put on a high-fat diet (HFD). On normal chow, both sexes of *Rsl1*$^{-/-}$ mice weighed ~10% less than their WT or L-*Rsl1*-tg counterparts (Fig. 1A), primarily due to decreased body fat (2). The response to HFD is normally sexually dimorphic and

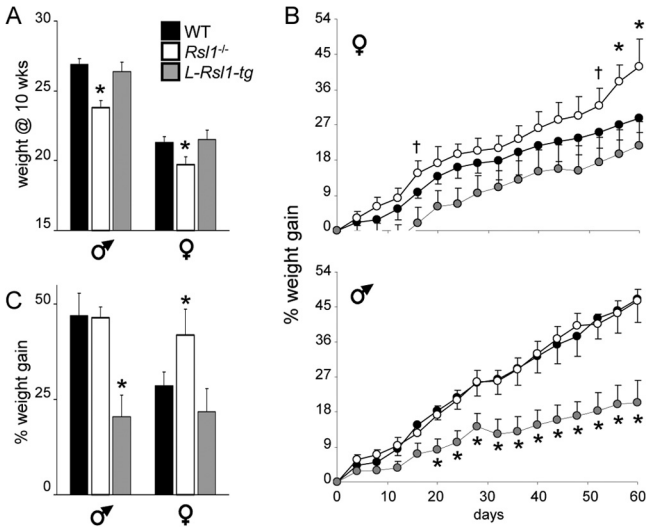



**FIG 1** RSL1 influences response to HFD. (A) Mean body weights and standard errors of the means (SEM) for mice fed standard lab chow from weaning to 10 weeks of age (for WT males, $n = 27$; for $Rsl1^{-/-}$ males, $n = 18$; for L-*Rsl1*-tg males, $n = 12$; for WT females, $n = 28$; for $Rsl1^{-/-}$ females, $n = 21$; and for L-*Rsl1*-tg females, $n = 13$). The *t* test for each group versus the WT determined differences within each sex. *, $P < 0.001$. (B) Percent weight gain throughout 60 days of HFD. Data are plotted as mean percentages ± SEM over time. WT males ($n = 8$), $Rsl1^{-/-}$ males ($n = 4$), L-*Rsl1*-tg males ($n = 5$), WT females ($n = 6$), $Rsl1^{-/-}$ females ($n = 6$), and L-*Rsl1*-tg females ($n = 10$) were studied. *t* tests evaluated differences from WT mice. †, $P < 0.10$; *, $P < 0.05$. (C) Mean percent weight gain ± SEM after 60 days of HFD. *, $P < 0.05$.

was affected further by RSL1, in a sex-dependent manner. After 8 weeks of HFD, there was no difference in percent weight gain among WT and $Rsl1^{-/-}$ males; however, $Rsl1^{-/-}$ females gained significantly more (~46%), on average, than WT mice (Fig. 1B and C). Furthermore, expression of RSL1 solely in liver strongly protected both sexes against diet-induced weight gain. L-*Rsl1*-tg males gained roughly half the weight of either WT or $Rsl1^{-/-}$ males, while L-*Rsl1*-tg and WT females had similar weights. Female-specific DIO also occurs in liver-specific *KAP1* knockout (L-KAP1-KO) mice (32), a complementation consistent with the role of KAP1 as an obligate corepressor of KRAB-ZFPs. This observation also suggests that among KRAB-ZFPs, RSL1 is critical in preventing female-specific DIO.

**Systemic metabolic dysregulation in $Rsl1^{-/-}$ mice.** Additional phenotypic differences were evident when we compared adult WT and $Rsl1^{-/-}$ mice in CLAMS metabolic chambers (Table 1). Over 3 days, $Rsl1^{-/-}$ mice ate about 20% less than WT mice. After day 1, when individual ambulatory behaviors stabilized, $Rsl1^{-/-}$ males exhibited significantly more lateral movement than WT mice (~1.7 times). $Rsl1^{-/-}$ females trended toward more lateral movements than those of WT mice, but all females were nearly twice as active as males, with much more individual variation. RER was significantly higher in both sexes of $Rsl1^{-/-}$ mice during the active phase, consistent with greater lateral movements and enhanced glucose metabolism. Heat production, determined from gas exchange values (28), indicated that $Rsl1^{-/-}$ females, to a greater extent than males, had repeated episodes of increased heat production (Fig. 2A). These events occurred during the first few hours of their active (a.m.) period and again late in their inactive period, around 2:00 p.m., suggesting cyclical bouts of nonshivering thermogenesis. The effects of RSL1 on several genes involved in circadian rhythm are intriguing in this regard (see below and reference 2).

To assess the stress response, metabolites were analyzed in sera from mice fed standard chow *ad lib*, fasted overnight (16 h), or given 8 weeks of HFD (Table 1). Glucose levels were significantly lower in $Rsl1^{-/-}$ than WT males on chow or HFD but did not

**TABLE 1** Metabolic phenotypes and serum profiles of WT and $Rsl1^{-/-}$ mice[a]

| Measurement | Value for: | | | |
|---|---|---|---|---|
| | Females | | Males | |
| | WT | $Rsl1^{-/-}$ | WT | $Rsl1^{-/-}$ |
| Total food intake (g) over 3 days | 14.3 ± 1.6 | 10.6 ± 0.5* | 15.0 ± 2.3 | 12.8 ± 1.7 |
| Lateral movement (days 2 and 3) | 2,058 ± 521 | 2,812 ± 814 | 965 ± 206 | 1,650 ± 287* |
| Respiratory exchange ratio (active period) | 0.73 ± 0.02 | **0.80 ± 0.03*** | 0.75 ± 0.02 | **0.83 ± 0.04*** |
| Glucose level (mg/dl) | | | | |
| Fed | 111.2 ± 4.3 | 114.0 ± 4.8 | 150.6 ± 8.4 | 128.0 ± 4.9* |
| Fasted | 91.5 ± 5.0 | 90.4 ± 7.0 | 97.0 ± 4.7 | 93.0 ± 9.2 |
| HFD | 111.0 ± 5.7 | 120.7 ± 8.4 | 144.3 ± 12.3 | 118.8 ± 3.5† |
| Free fatty acids (meq/liter) | | | | |
| Fed | 0.32 ± 0.02 | 0.34 ± 0.06 | 0.38 ± 0.02 | 0.37 ± 0.08 |
| Fasted | 0.72 ± 0.13 | 0.97 ± 0.09† | 0.45 ± 0.10 | 0.64 ± 0.12 |
| HFD | 0.68 ± 0.17 | 0.51 ± 0.05 | 0.72 ± 0.08 | 0.70 ± 0.08 |
| Insulin level (ng/ml) | | | | |
| Fed | 0.52 ± 0.06 | 0.45 ± 0.05 | 0.84 ± 0.08 | 0.70 ± 0.05† |
| Fasted | 0.33 ± 0.05 | 0.27 ± 0.03 | 0.28 ± 0.05 | 0.18 ± 0.04† |
| HFD | 0.53 ± 0.08 | **0.75 ± 0.08*** | 3.79 ± 0.62 | **2.03 ± 0.37*** |

[a] For CLAMS measurements (1st three measurements), $n = 4$; for all others, $n \geq 6$. Lateral movement is presented as the mean number of infrared beam breaks recorded per hour. The respiratory exchange ratio was calculated as the $VCO_2/VO_2$ ratio. Shaded values differ in only one sex. Values in bold differ in both sexes. Note that insulin levels after 8 weeks of HFD were significantly elevated relative to WT levels in $Rsl1^{-/-}$ females and significantly reduced in $Rsl1^{-/-}$ males. *, $P < 0.05$; †, $P < 0.10$. Data are means ± SEM.

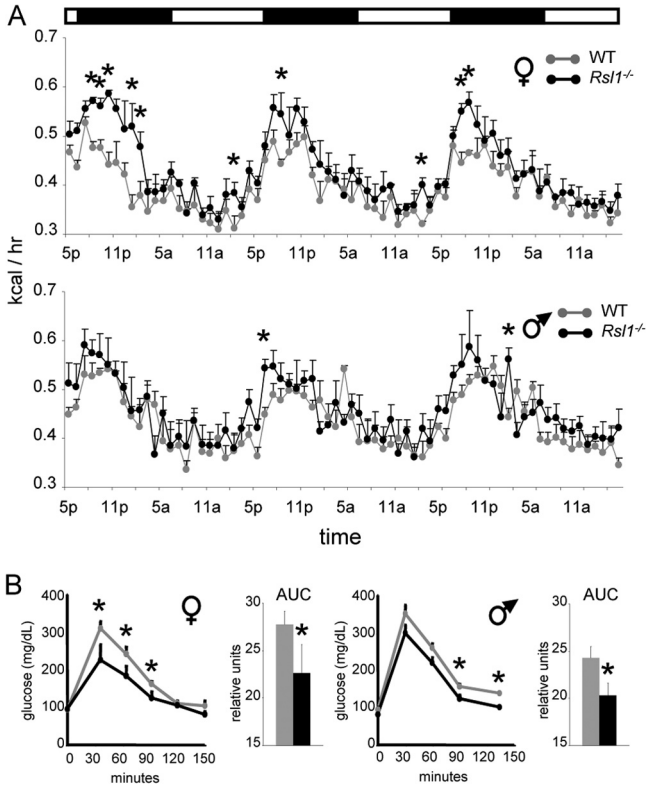



**FIG 2** Heat production in *Rsl1*$^{-/-}$ females is transiently greater than that in WT mice at the beginning of their active phase, and both sexes have enhanced insulin sensitivity. (A) Gas exchange values from the CLAMS metabolic chambers ($n = 4$ mice per sex and genotype) were used to calculate energy expenditure as heat, based on the following equation: heat (kcal/h) = [3.82 + (1.23 × RER)] × $VO_2$. Data are plotted as means ± SEM. Means were considered significantly different (*) if the difference in WT versus *Rsl1*$^{-/-}$ mice was ≥20% and the *t* test *P* value was <0.05. (B) For glucose tolerance tests, mice were fasted overnight and injected intraperitoneally with D-glucose (2 g/kg body weight). Blood glucose was monitored 0, 30, 60, 90, 120, and 150 min after injection. Data are plotted as means ± SEM over time. WT males ($n = 7$), *Rsl1*$^{-/-}$ males ($n = 6$), WT females ($n = 4$), and *Rsl1*$^{-/-}$ females ($n = 6$) were studied. *t* tests evaluated differences from the WT group. *, $P < 0.05$. AUC were calculated for each mouse and plotted as means ± SEM. *t* tests evaluated differences from the WT group. *, $P < 0.05$.

differ after fasting. WT and *Rsl1*$^{-/-}$ females, however, had similar serum glucose levels, regardless of feeding state. Serum-free fatty acids in fasted *Rsl1*$^{-/-}$ mice were similar to those in WT mice and exhibited a trend toward higher levels ($P = 0.08$) in females. After HFD, insulin levels relative to WT levels were 87% lower in *Rsl1*$^{-/-}$ males but elevated by 42% in *Rsl1*$^{-/-}$ females. Collectively, these data suggest that on a nutritionally balanced diet, the lack of RSL1 enhances energy expenditure in both sexes, but during HFD, *Rsl1*$^{-/-}$ females, but not males, gain excessive weight and develop hyperinsulinemia.

Because dysregulation of insulin can contribute to both lean and obese phenotypes, we examined RSL1's effects on systemic insulin signaling. Glucose tolerance tests (GTT) of age-matched adult mice demonstrated faster glucose clearance in both sexes of *Rsl1*$^{-/-}$ mice (Fig. 2B), indicating enhanced insulin sensitivity. This is consistent with their leaner body mass (2), lower blood glucose level (at least in males), and elevated RER. While a direct effect of RSL1 on insulin action cannot be ruled out, insulin signaling in *Rsl1*$^{-/-}$ mice appears to be intact and adaptive to differences in energy expenditure (Table 1). Thus, phenotypic variation in these mice is more likely due to altered gene expression in peripheral sites such as liver or adipose tissue.

**RSL1 alters liver response to dietary stress.** To identify RSL1 transcriptional targets affecting metabolic homeostasis, levels of genes with key roles in metabolism were examined, as well as those of genes shown previously to have altered expression in *Rsl1*$^{-/-}$ liver (2). Hepatic mRNA was measured in adult mice before and after fasting. Expression levels of both *Pck1* and the glucose-6-phosphatase gene (*G6pc*) (genes critical for hepatic gluconeogenesis) are normally similar in both sexes and increased upon fasting, but in *Rsl1*$^{-/-}$ mice, *Pck1* was higher in both sexes, and the response to fasting was lacking in males and blunted in females (reported previously [2] and replicated here with additional mice). *G6pc* was reduced in fed *Rsl1*$^{-/-}$ females and was attenuated by fasting in mice of both sexes (Fig. 3A). *Scd1*, which encodes the rate-limiting enzyme in lipogenesis, typically declines in response to fasting. In *Rsl1*$^{-/-}$ males, *Scd1* was low and unresponsive, and its decline in *Rsl1*$^{-/-}$ females was also somewhat attenuated. Levels of elongation of long-chain fatty acids 6 (*Elovl6*) and *Elovl3*, additional lipogenic genes, were higher and lower than WT levels, respectively, in fed *Rsl1*$^{-/-}$ males but were equivalent to WT levels after fasting. RSL1 affected neither the abundance nor response of hepatic *Elovl6* or *Elovl3* in females. Greater *Elovl6* and *Elovl3* differences in *Rsl1*$^{-/-}$ males could be due to *Ppara*, encoding a key moderator of hepatic metabolic functions (33), which exhibited decreased expression regardless of feeding. *Ppargc1a* and *Ppargc1b*, which encode the peroxisome proliferator-activated receptor (PPAR) coactivators PGC1α and PGC1β, also differed in fasted and fed males, respectively. No significant sex- or genotype-specific differences were seen in expression of two additional key metabolic regulators: sterol regulatory element binding transcription factor 1 (*Srebf1*) and carbohydrate-responsive element-binding protein (*Chrebp*) (not shown). In sum, the effects of food restriction on hepatic gene expression were both RSL1 and sex dependent, with a greater impact on the components of PPAR signaling in males than in females.

Differences in *Rsl1*$^{-/-}$ liver gene expression following 8 weeks of HFD were more striking in females than males, consistent with their DIO. For instance, *Pck1* mRNA levels remained higher than WT levels in males, regardless of diet, but in females the levels were nearly double the WT levels on normal chow and less than the WT levels after HFD (Fig. 3B). Conversely, *Scd1* and *Ppara* mRNAs were significantly reduced in *Rsl1*$^{-/-}$ males on normal chow and elevated in parallel with WT levels by HFD. Expression of these genes was similar in WT and *Rsl1*$^{-/-}$ females on normal chow but significantly attenuated in response to HFD in *Rsl1*$^{-/-}$ females (Fig. 3B).

Interestingly, liver gene expression consistent with a loss of RSL1 repression was evident in *Rsl1*$^{-/-}$ females on HFD. For instance, *Elovl3* levels were similar to WT levels in *Rsl1*$^{-/-}$ females on a chow diet but significantly upregulated on HFD (Fig. 3C). Furthermore, *Elovl3* is likely to play a key role in the phenotype of *Rsl1*$^{-/-}$ mice given that *Elovl3* knockout females are resistant to DIO (34). Major urinary protein 1 (*Mup1*) was also markedly upregulated in *Rsl1*$^{-/-}$ female livers for mice on HFD (Fig. 3C). MUP1, known to be a pheromone carrier, may also transport metabolic signals given its lipocalin-like structure (35). A related family member, lipocalin 2 (*Lcn2*), was also elevated in *Rsl1*$^{-/-}$ females, but unlike *Mup1*, *Lcn2* was slightly reduced by HFD in

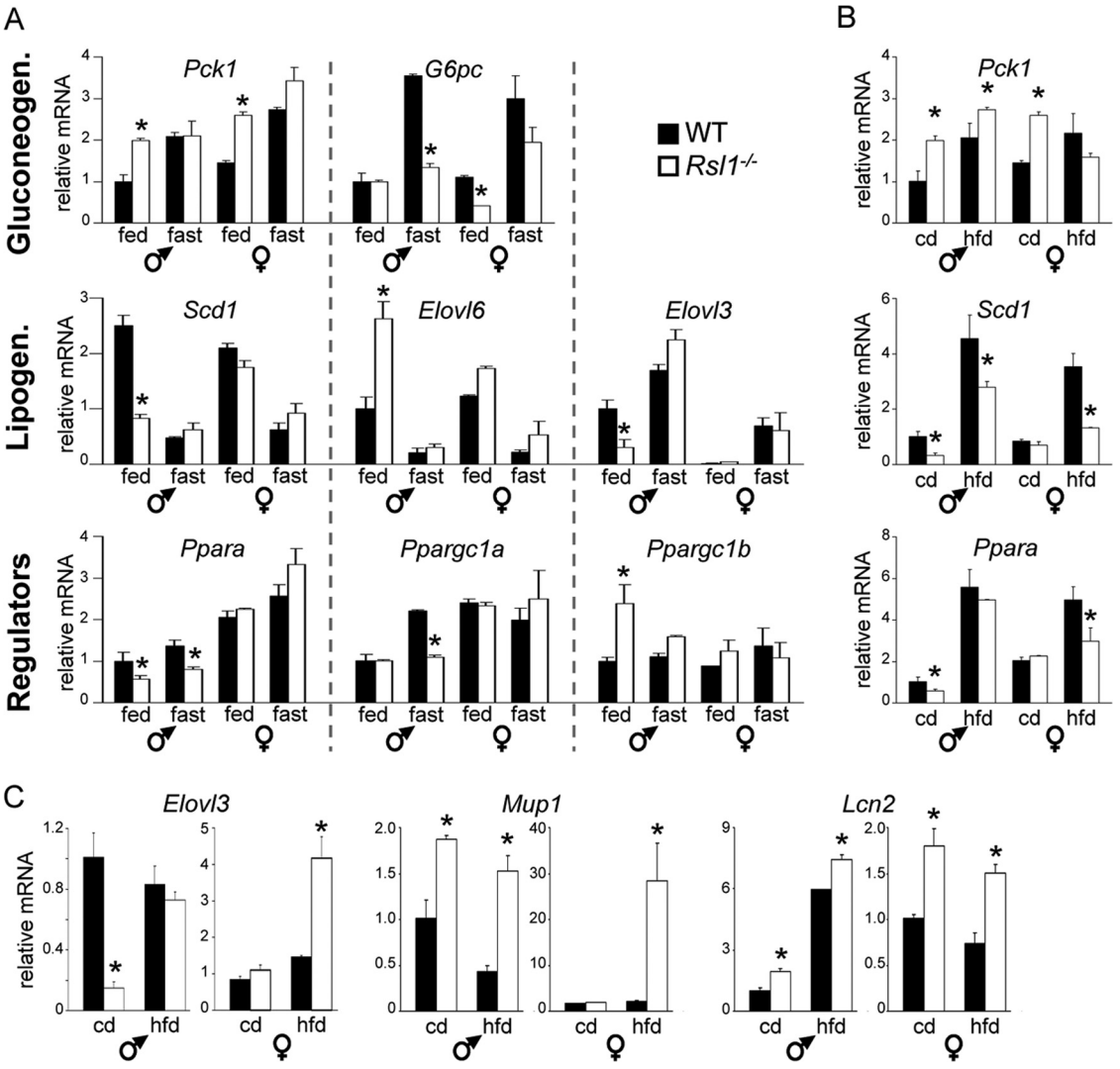



**FIG 3** Sex-specific effects of RSL1 on hepatic response to dietary stress. Hepatic mRNA abundance was measured by qRT-PCR (pools for ≥4 mice). (A) Mice were fed a chow diet *ad lib* (fed) or fasted for 16 h (fast). (B and C) Mice were fed a chow diet (cd) or high-fat diet (hfd) for 8 weeks. The data are plotted as the means and standard deviations (SD) for duplicate reactions relative to WT males. In panel C, note that the graphs for males and females differ substantially in scale. *t* tests evaluated differences from the WT group. *, $P < 0.05$.

WT and *Rsl1*$^{-/-}$ females (Fig. 3C). Each of these genes showed distinctly different expression patterns in males versus females. *Elovl3* was downregulated in *Rsl1*$^{-/-}$ males on chow but upregulated to the WT level by HFD. The *Mup1* level was nearly 2-fold higher than the WT level in *Rsl1*$^{-/-}$ males, regardless of diet, and *Lcn2*, which increased severalfold with HFD, remained elevated in *Rsl1*$^{-/-}$ males. Together, the data show that the hepatic expression of these genes is sex and RSL1 dependent and that the response to high-fat chow in females correlates with DIO.

**RSL1 action in white adipose tissue.** *Rsl1* is broadly expressed, and its levels in many tissues are higher than that in liver (unpublished data), suggesting that additional organs could contribute to the *Rsl1*$^{-/-}$ phenotype (lean on a standard chow diet, diminished hepatic response to fasting, and female DIO). In particular, differences in activity, respiration, and appetite in *Rsl1*$^{-/-}$ mice (Table 1) suggest that WAT is involved in dysregulation. WAT and liver share overlapping pathways (gluconeogenesis/glyceroneogenesis) and regulators (primarily PPARα in liver and PPARγ in WAT), albeit with tissue- and sex-specific differences. To first assess the extent to which RSL1 affects gene expression in WAT, select genes that were *Rsl1* responsive in liver were analyzed in WAT (Fig. 4). In *Rsl1*$^{-/-}$ females but not males, *Pck1* showed a 5-fold increase upon fasting but was unchanged on HFD. Levels of *G6pc* and *Scd1* in WAT were affected by RSL1 only in males, with mRNA levels about half the WT levels for mice on chow or HFD but equivalent after fasting. *Elovl6*, which is not sex biased in liver, was about 20 times higher in female than male WAT and was nearly twice the WT level in both sexes of chow-fed *Rsl1*$^{-/-}$ mice. For mice on HFD, *Elovl6* mRNA levels dropped to WT levels in *Rsl1*$^{-/-}$ males but remained elevated in *Rsl1*$^{-/-}$ females. *Elovl3* is a male-predominant gene in liver (~50-fold) but is much less sex biased (and favoring females) in WAT. Similar to *Elovl6*, *Elovl3* mRNA remained significantly higher in *Rsl1*$^{-/-}$ females on HFD (Fig. 4). ELOVL enzymes contribute, in part, to *de novo* lipogenesis (36).

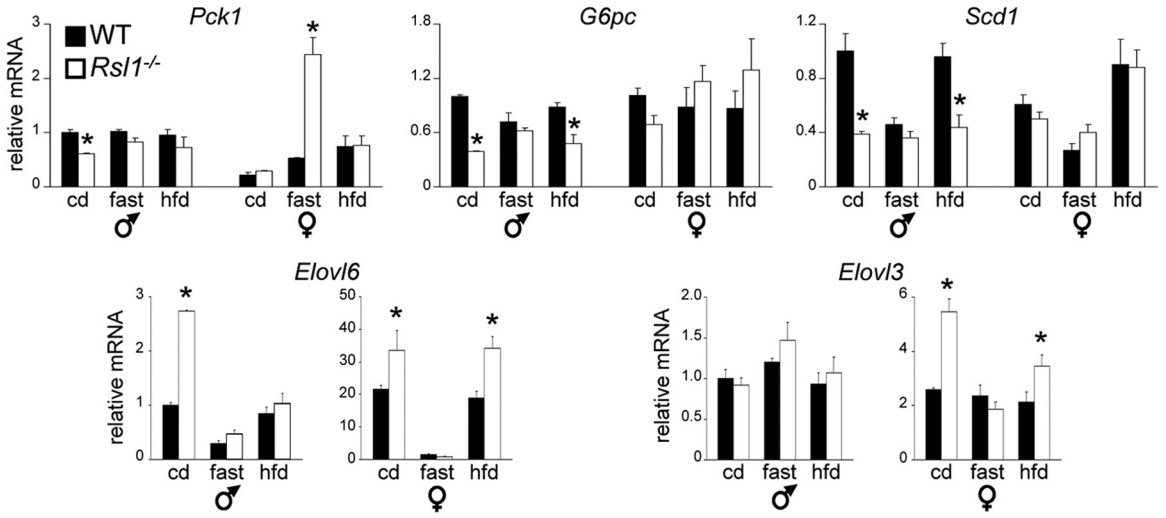



**FIG 4** RSL1 influences response to dietary stress in white adipose tissue. WAT mRNA abundance was measured by qRT-PCR (pools for ≥4 mice). Mice were fed a chow diet (cd), fasted for 16 h (fast), or given 8 weeks of high-fat diet (hfd). The data are plotted as the means and SD for duplicate reactions relative to WT males. Note that the results for *Elovl6* and *Elovl3* differ substantially in scale depending on sex. *t* tests evaluated differences from the WT group. *, $P < 0.05$.

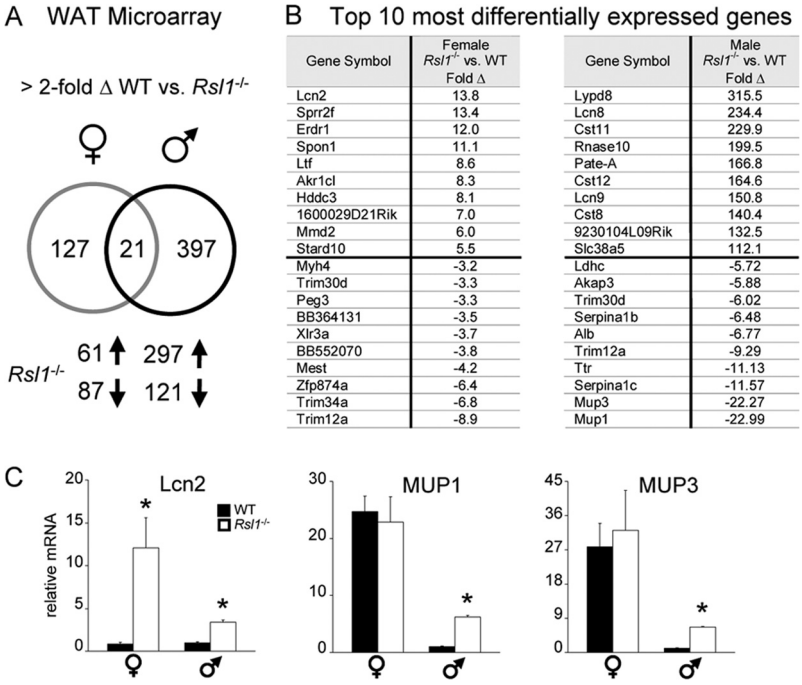
Thus, elevated mRNA levels in female *Rsl1*$^{-/-}$ WAT during HFD may be a key component underlying the diet-induced weight gain of these mice.

**WAT expression profiling.** The expression patterns of select genes in liver and WAT examined above highlight RSL1 effects that differ by sex and response to dietary challenge. To assess global gene expression differences, microarray analysis was performed on WAT RNAs from chow-fed adult mice, as done previously for liver (2). Out of 45,101 probe sets, 16,514 provided gene expression data. Like the case for liver, more genes differed in expression in males than in females (418 versus 148 at a cutoff of a 2-fold difference) (Fig. 5A). In both tissues, some genes gained and others lost sexually dimorphic expression and were reduced or elevated due to the absence of the RSL1 repressor. Prevalent among the genes most elevated in *Rsl1*$^{-/-}$ WAT were those encoding small secreted proteins, such as the *Lcn2*, small proline-rich protein 2F (*Sprr2f*), and lactotransferrin (*Ltf*) genes in females and *Lcn8* and *Lcn9* in males (Fig. 5B), suggesting altered systemic signaling in *Rsl1*$^{-/-}$ mice. As in liver (2), several members of the tripartite motif-containing (TRIM) family of multifunctional transcriptional modifiers were prevalent among the genes reduced in expression in *Rsl1*$^{-/-}$ mice. In addition, the MUP family members *Mup1* and *Mup3* were significantly increased in males when queried using gene-specific primers (29) (Fig. 5C). Interestingly, these MUP genes are male biased and RSL1 dependent in liver (21, 22) but exhibit a female bias of 5- to 20-fold that is not RSL1 dependent in WAT.

GO term and KEGG analyses of RSL1-affected WAT transcripts showed enrichment in pathways associated with secretory products ("extracellular region"), protease inhibitors, and cytoskeleton proteins (Table 2) rather than steroid and lipid metabolism as seen in liver. Categories enriched for RSL1-responsive genes in WAT were similar to those identified previously as sexually dimorphic in adipose tissue (including gene clusters for muscle contraction/development, cell motility and structure, and immune response) (26). Similarly annotated genes were reduced in expression in *Rsl1*$^{-/-}$ females and elevated in *Rsl1*$^{-/-}$ males (Table 2). The muscle-related genes may function in sexually dimorphic energy expenditure in adipose tissue (26), and their lower expression in *Rsl1*$^{-/-}$ females may relate to the vulnerability of these mice to caloric excess. Overall, the annotation terms for RSL1-affected genes in WAT suggest global adaptive and tissue remodeling responses typical of the hypertrophy and hyperplasia associated with adipocyte biology (37).

**Comparison of liver and WAT transcriptomes.** To examine the effects of RSL1 in multiple tissues, expression profiles from WAT were compared to data acquired previously from liver (2). At a differential gene expression threshold of 1.5-fold, 442 genes differed significantly between WT and *Rsl1*$^{-/-}$ livers. Remarkably, nearly 5 times more genes (2,333) surpassed this threshold in WAT. To view the global effect on sex-biased gene expression, heat maps were created by arranging transcripts that differed by 1.5-fold or more in WT males and females according to their sex bias (i.e., those highest in males [top, red] to those highest in females [bottom, blue]) (Fig. 6A). Previous results from liver (2) were filtered (differences of 1.5-fold or more) and replotted for direct comparison to WAT data (Fig. 6A). The global reduction in sex bias (i.e., lack of color in *Rsl1*$^{-/-}$ lane) was evident for the liver as discrete gene clusters throughout the heat map. In striking contrast, the loss of *Rsl1* greatly increased the number of sex-biased transcripts in WAT, with 1,926 for WT mice compared to 3,706 for *Rsl1*$^{-/-}$ mice. Venn diagrams (Fig. 6B) further indicate that the increased transcript number in *Rsl1*$^{-/-}$ WAT was the result of 2,210 genes acquiring, and 430 losing, sex-biased expression in the absence of *Rsl1*. To compare RSL1-dependent changes in the two tissues, genes were sorted by genotype and grouped into sex-biased genes in liver, WAT, or both. There were 75 more sex-biased genes in the overlap of liver and WAT in *Rsl1*$^{-/-}$ mice (155 in WT mice and 230 in *Rsl1*$^{-/-}$ mice) (Fig. 6C). This suggests that the loss of *Rsl1* diminishes sex-biased gene expression in liver but accentuates it in WAT (or, more importantly, RSL1 enhances liver sex bias and masks WAT sex bias).

Sexually dimorphic genes can be sorted into functional categories by the magnitude of their sex difference (26). For example, in liver, immune response genes differ 2- to 3-fold between males




| Gene Symbol | Female *Rsl1*$^{-/-}$ vs. WT Fold Δ |
|---|---|
| Lcn2 | 13.8 |
| Sprr2f | 13.4 |
| Erdr1 | 12.0 |
| Spon1 | 11.1 |
| Ltf | 8.6 |
| Akr1cl | 8.3 |
| Hddc3 | 8.1 |
| 1600029D21Rik | 7.0 |
| Mmd2 | 6.0 |
| Stard10 | 5.5 |
| Myh4 | -3.2 |
| Trim30d | -3.3 |
| Peg3 | -3.3 |
| BB364131 | -3.5 |
| Xlr3a | -3.7 |
| BB552070 | -3.8 |
| Mest | -4.2 |
| Zfp874a | -6.4 |
| Trim34a | -6.8 |
| Trim12a | -8.9 |

| Gene Symbol | Male *Rsl1*$^{-/-}$ vs. WT Fold Δ |
|---|---|
| Lypd8 | 315.5 |
| Lcn8 | 234.4 |
| Cst11 | 229.9 |
| Rnase10 | 199.5 |
| Pate-A | 166.8 |
| Cst12 | 164.6 |
| Lcn9 | 150.8 |
| Cst8 | 140.4 |
| 9230104L09Rik | 132.5 |
| Slc38a5 | 112.1 |
| Ldhc | -5.72 |
| Akap3 | -5.88 |
| Trim30d | -6.02 |
| Serpina1b | -6.48 |
| Alb | -6.77 |
| Trim12a | -9.29 |
| Ttr | -11.13 |
| Serpina1c | -11.57 |
| Mup3 | -22.27 |
| Mup1 | -22.99 |

**FIG 5** Genomewide identification of RSL1-sensitive genes in WAT. (A) Venn diagram of genes differentially expressed ≥2-fold in WT and *Rsl1*$^{-/-}$ abdominal WAT. Males had nearly three times as many differentially expressed genes as females; however, less than 15% of these genes were shared between the sexes. Data shown are the numbers of genes that were either elevated (upward-facing arrows) or reduced (downward-facing arrows) in *Rsl1*$^{-/-}$ compared to WT mice. (B) Top 10 genes with the greatest differential of up- and downregulated genes in female (left) and male (right) WAT. (C) Expression by qRT-PCR of genes selected from the microarray in WT and *Rsl1*$^{-/-}$ WAT ($n \geq 4$). *Mup* genes appeared to be decreased by microarray assay, where probes detected 10 *Mup* family members, but gene-specific primers for *Mup1* and *Mup3* revealed a male bias and RSL1 dependence in liver (21, 22) and a female bias unaffected by RSL in WAT. The data are plotted as means and SEM. *t* tests evaluated differences from the WT group. *, $P < 0.05$.

and females, whereas genes that encode steroid-metabolizing enzymes can differ by several orders of magnitude (2, 26). To examine global gene expression differences in liver and WAT in this manner, genes were sorted into bins in which the male-female differential was 1.5- to 2-fold, 2- to 3-fold, or >3-fold and plotted as a percentage of the total. In liver, the lack of RSL1 reduced the number of sex-biased genes only within the 1.5- to 2-fold range, by ~13% (Fig. 6D). Beyond a 2-fold differential, *Rsl1*$^{-/-}$ livers had a larger percentage of sex-biased genes than WT livers. In marked contrast, the percentages of genes in WAT within each fold range were nearly identical in WT and *Rsl1*$^{-/-}$ mice. Based on this analysis, genes that are most sex biased in liver are most affected by RSL1, whereas there is no apparent correlation in WAT between the presence or absence of RSL1 and the magnitude of sex bias.

**TABLE 2** Annotation of RSL1-responsive genes in WAT

| Group and annotation term | Enrichment score |
|---|---|
| Females | |
| Genes higher in *Rsl1*$^{-/-}$ mice | |
| Response to estradiol | 1.445 |
| Extracellular region | 1.175 |
| Genes lower in *Rsl1*$^{-/-}$ mice | |
| Proteolysis | 1.862 |
| Muscle contraction/development | 1.750 |
| Zinc binding | 1.098 |
| Males | |
| Genes higher in *Rsl1*$^{-/-}$ mice | |
| Protease inhibitor | 3.077 |
| Cell structure and motility | 1.953 |
| Cell adhesion | 1.486 |
| Cytoskeleton protein binding | 1.238 |
| Lipid biosynthesis | 1.133 |
| Genes lower in *Rsl1*$^{-/-}$ mice | |
| Extracellular region part | 2.008 |
| Regulation of lipase activity | 1.910 |
| Inflammatory response | 1.510 |
| Protease inhibitor | 1.434 |
| Response to heat | 1.180 |

[a] The threshold for a significant enrichment score was a value of >1.00.

For genes that differed >2-fold between WT and *Rsl1*$^{-/-}$ mice (545 in WAT and 206 in liver), more than 80% were RSL1 responsive in one tissue but not the other (Fig. 6E). Fewer than 20 genes in each sex overlapped, and 7 were expressed in reciprocal patterns, either elevated in *Rsl1*$^{-/-}$ liver and reduced in WAT or vice versa (e.g., *Mmd2*, *Dbp*, *Per2*, *Per3*, *Acot1*, *Nav2*, and *Cldn1*). Notably, 6 of the 7 reciprocally regulated genes were affected only in males. Among the RSL1-responsive genes in both liver and WAT, four are linked to peripheral circadian rhythms: *Per2*, *Per3*, *Dbp*, and *Usp2*. Only *Usp2* had a pattern consistent with derepression in both tissues, and this was evident only in females. In total, 21 genes were regulated in the same direction in liver and WAT, and 12 were elevated in *Rsl1*$^{-/-}$ mice (data not shown), suggesting direct regulation by the KRAB-ZFP repressor. Among the 12 elevated genes were genes associated with metabolism (e.g., *Hddc3*, *Mfsd2a*, *Elovl3*, and *Elovl6*) as well as paracine/autocrine signaling (e.g., *Prap1* and *Lcn2*).

**Liver-autonomous RSL1 action and impact on WAT lipogenic genes.** To test whether some RSL1 effects on metabolism are

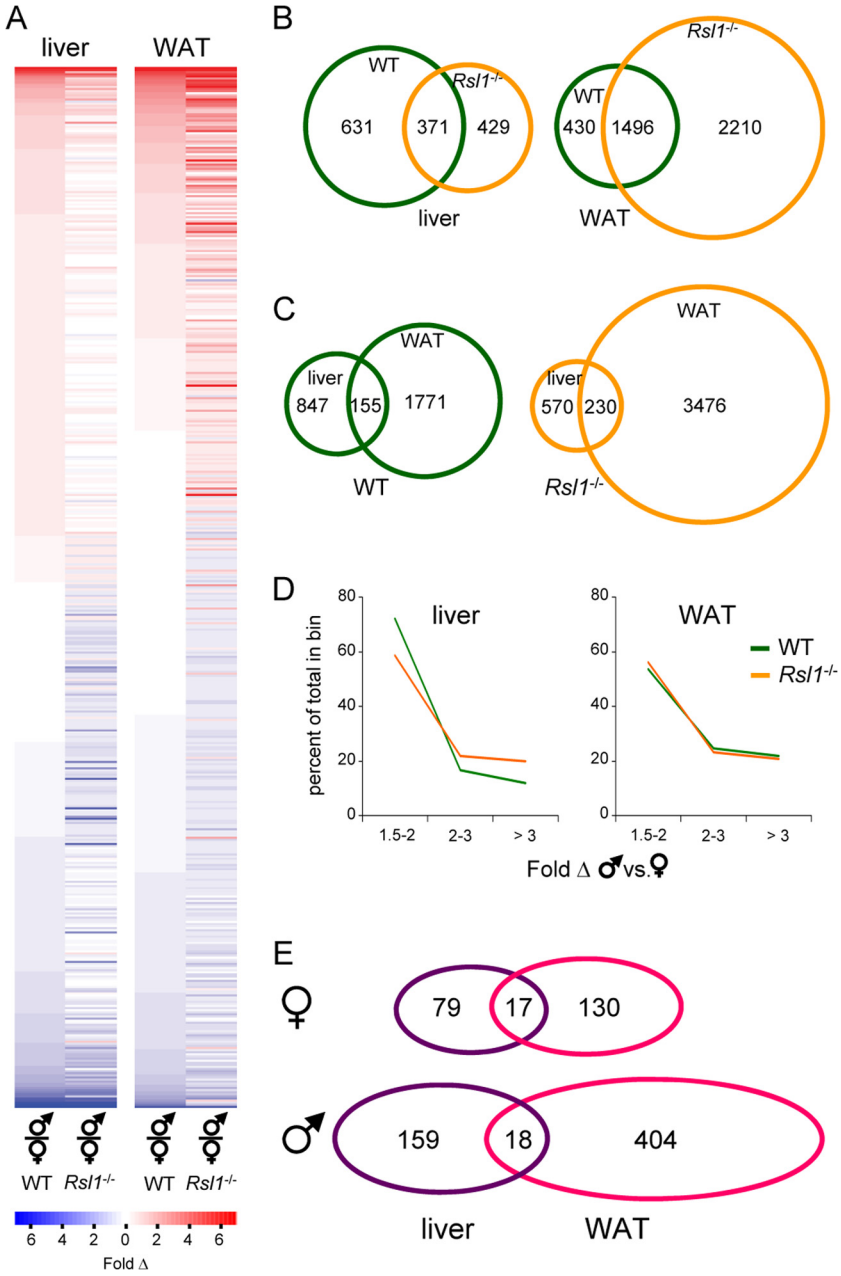



**FIG 6** Comparison of RSL1-sensitive genes in liver and WAT. (A) Heat maps highlighting genes that are sexually dimorphic (>1.5-fold difference between males and females) in liver and WAT. The intensity of color reflects the magnitude of the sex bias (red shows male-predominant genes, and blue shows female-predominant genes). The arrangement of genes in *Rsl1*$^{-/-}$ mice is identical to that in WT mice. (B and C) Venn diagrams of sexually dimorphic genes (>1.5-fold difference between males and females) in liver and WAT and their distributions among WT and *Rsl1*$^{-/-}$ mice. Circles are not to scale but are approximated to convey relative relationships among gene sets. (D) Sex-biased genes in liver and WAT were grouped into categories based on the magnitude of their sex bias (1.5- to 2-fold, 2- to 3-fold, and >3-fold) and plotted as percentages of the total. In WT liver, 71.9% of the sex-biased genes were in the 1.5- to 2-fold range (58.4% in *Rsl1*$^{-/-}$ liver), 16.2% were in the 2- to 3-fold range (21.8% in *Rsl1*$^{-/-}$ liver), and 12.0% were in the >3-fold range (19.9% in *Rsl1*$^{-/-}$ liver). In WAT, the differences between WT and *Rsl1*$^{-/-}$ mice were much smaller (in the 1.5- to 2-fold range, 53.5% for WT mice and 55.9% for *Rsl1*$^{-/-}$ mice; in the 2- to 3-fold range, 24.6% for WT mice and 23.2% for *Rsl1*$^{-/-}$ mice; and in the >3-fold range, 21.9% for WT mice and 20.8% for *Rsl1*$^{-/-}$ mice). (E) Venn diagrams of RSL1-responsive genes in liver compared to RSL1-responsive genes in WAT. A differential expression (WT versus *Rsl1*$^{-/-}$) threshold of 2-fold was used, with significance for *P* values of <0.05.

liver autonomous, primary hepatocytes were isolated from WT and *Rsl1*$^{-/-}$ mice, and responses to 18 h of medium supplemented with glucose and insulin were compared to mimic the conditions of being fed *ad lib*. For the gluconeogenic gene *G6pc*, basal mRNA levels differed by about 3-fold, and suppression by glucose plus insulin was evident only in WT mice (Fig. 7A). Other genes, including *Pck1*, *Elovl3*, and *Elovl6*, showed more complex responses (data not shown) indicative of additional interacting pathways that affect gene expression in liver. *Rsl1* mRNA levels in WT liver or hepatocytes did not change with fasting or insulin (data not

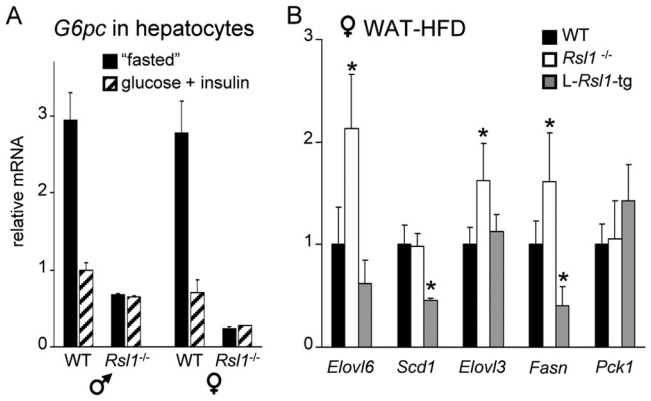



**FIG 7** Liver-autonomous RSL1 action and impact on WAT lipogenic genes. (A) *G6pc* expression in primary hepatocytes isolated from WT and Rsl1$^{-/-}$ livers and cultured for 18 h in starvation medium ("fasted") or starvation medium supplemented with glucose plus insulin. qRT-PCR results from duplicate experiments were normalized to that for WT males with glucose plus insulin, which was arbitrarily set to 1. Means and SD were plotted. (B) Genes involved in lipogenesis exhibit attenuated expression in WAT due to Rsl1 expression in liver. WAT mRNA abundance was measured by qRT-PCR ($n \geq 4$ mice per genotype). To ease comparisons for transcripts that vary in abundance, results were normalized and the means and SEM plotted relative to the level in WT females, which was arbitrarily set to 1. *t* tests evaluated differences from the WT group. *, $P < 0.05$.

shown). Therefore, at least for some metabolic genes, responses to nutritional cues in hepatocytes were similar to those seen in intact liver (Fig. 3A), supporting a liver-autonomous regulatory mechanism directly dependent on RSL1.

One of the most striking differences between WT and *Rsl1*$^{-/-}$ mice was the vulnerability of *Rsl1*$^{-/-}$ females to DIO and reversion of this feature by liver-restricted RSL1 (Fig. 1). In *Rsl1*$^{-/-}$ female WAT, genes that function in lipogenesis (e.g., *Elovl6* and *Elovl3*) were aberrantly elevated by HFD (Fig. 4), providing at least a partial explanation for the excessive weight gain in *Rsl1*$^{-/-}$ females. To examine the link between RSL1 in liver and lipogenesis in WAT, we measured the expression of lipogenic genes in WAT from L-*Rsl1*-tg females on HFD. Liver-specific expression of *Rsl1*-tg before and after HFD was verified by qRT-PCR (data not shown). In L-*Rsl1*-tg female WAT, *Elovl6*, *Elovl3*, *Scd1*, and fatty acid synthetase (*Fasn*) mRNAs were all markedly lower than those in *Rsl1*$^{-/-}$ female WAT (Fig. 7B). In contrast, levels of *Pck1*, responsible for glyceroneogenesis and fatty acid reesterification in WAT, did not differ significantly across genotypes. These results indicate that expression of key genes in lipid metabolism in females can be suppressed in WAT by RSL1's action in liver. In sum, these studies are the first to examine a KRAB-ZFP repressor in multiple tissues and to demonstrate their remarkable capacity to systemically modify adult physiology.

## DISCUSSION

Metabolic homeostasis is sensitive to distinct physiological needs of males and females and requires coordinated transcriptional regulation (38–42). The KRAB-ZFP RSL1 modifies liver metabolic gene expression in a sex-dependent manner (2) and is uniquely accessible to functional analysis in multiple adult tissues, thus unveiling a broad capacity of KRAB-ZFPs to modulate homeostasis. In the absence of RSL1, mice are generally lean and respond abnormally to dietary stress, and this is most pronounced in males upon fasting and females on HFD (Fig. 1). While sexually dimorphic liver gene expression is well studied, less is known about sex bias in WAT. Some key metabolic genes in liver and WAT that are influenced by sex and diet are also modulated by RSL1 (Fig. 3 and 4). Expression profiling of liver and WAT demonstrates that nearly twice as many genes are sex biased in WAT than in liver, and intriguingly, RSL1 enhances dimorphism in liver but suppresses sex bias in WAT (Fig. 6). While RSL1-sensitive liver genes are enriched in pathways for steroid, cholesterol, and lipid metabolism (2), RSL1-sensitive genes in WAT occur in pathways of secretion, protease inhibition, and cellular remodeling (Table 2), consistent with the widespread tissue and cellular adaptation that occurs in adipogenesis. RSL1 expression solely in liver attenuates lipogenic genes in WAT (Fig. 7), implying that RSL1-mediated liver-to-WAT communication is critical in females to protect against diet-induced weight gain. That fact that WT, *Rsl1*$^{-/-}$, and L-*Rsl1*-tg females appear healthy when provided proper nutrition highlights the role of RSL1 in modulating phenotypes that vary with environment. Thus, RSL1 helps to maintain homeostasis by buffering against fluctuations in gene expression. This is likely the case for other KRAB-ZFPs as well, given that the loss of KAP1 or DNMT3a, components of the general KRAB repression machinery, also leads to widespread dysregulation in gene expression (43, 44).

RSL1 modifies metabolism through mechanisms that likely include (i) direct KRAB-ZFP repression of target genes (e.g., *G6pc* in both sexes, *Pck1* in males, and *Elovl3* and *Mup1* in females), (ii) indirect action via transcription factor cascades (such as those directed by PPARα in male liver, and perhaps mediated via other KRAB-ZFPs, since they are known to cross-regulate [45]), and (iii) RSL1 control of systemic signaling (e.g., liver RSL1 effects on WAT gene expression via MUP1, lipocalins, etc.). Among the genes altered in *Rsl1*$^{-/-}$ mice, the derepression of *Pck1* and *Mup1* in liver may be particularly crucial for metabolism. Greater systemic energy utilization in *Rsl1*$^{-/-}$ mice on a balanced diet is suggested by an elevated hepatic *Pck1* level (Fig. 3), reduced feeding, greater movement, and a higher respiratory exchange ratio, without increased blood glucose (Table 1). Elevated MUP1 may be a significant contributor to the lean phenotype by leading to the repression of hepatic *Scd1* (35) (Fig. 8). MUP1, a member of the lipocalin family (46), is primarily a pheromone carrier (47), but its elevated synthesis and secretion from liver have been shown to benefit glucose metabolism (35). Lipocalins alter metabolic homeostasis via their paracrine/autocrine actions (35, 48, 49), and multiple lipocalin genes (e.g., *Lcn2*, *Lcn8*, and *Lcn9*) are RSL1 and sex dependent in WAT (Fig. 5B). Some appear to protect mice from metabolic stress, whereas others may facilitate damage (49–51). In either case, the diverse functions of lipocalins are linked to their ability to transport a wide variety of signaling molecules, including pheromones, retinols, fatty acids, and steroid hormones (46). While RSL1-mediated MUP1 effects may be specific to mice, members of the lipocalin and KRAB-ZFP families are abundant in humans, and transcriptional networks for metabolic balance are highly conserved. In particular, recent transcriptional profiling of the human liver found a significant sex bias in over 1,000 genes, including nearly 20 *KRAB-Zfp* genes and an enrichment for genes involved in lipid metabolism (24). Hence, there is a high probability that KRAB-ZFPs participate in sex-specific metabolic homeostasis in humans as well as mice.

Female-specific DIO is likely complex but may depend, in part,

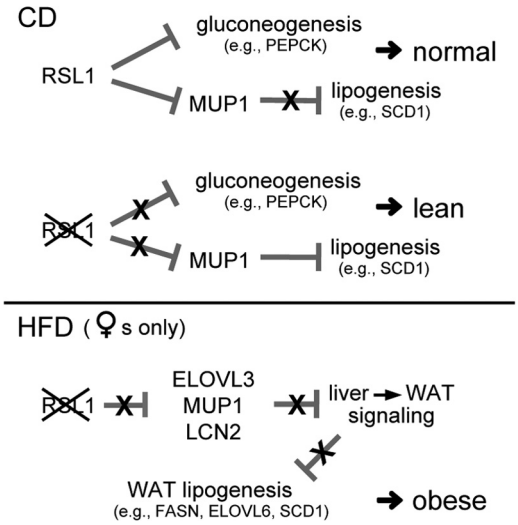



**FIG 8** RSL1-sensitive pathways in liver that may contribute to metabolic phenotypes. CD, chow diet; HFD, high-fat diet. "Lean" and "obese" refer to the state of *Rsl1*$^{-/-}$ mice relative to normal wild-type mice. RSL1 limits gluconeogenesis and MUP1 secretion, which in turn may derepress lipogenesis, leading to a normal phenotype. In the absence of RSL1, upregulation of these pathways promotes a lean phenotype. With HFD, the RSL1-deficient female liver fails to repress ELOVL3 and fatty acid transporters (e.g., MUP1 and LCN2), which triggers a cascade in systemic signaling, resulting in a failure to suppress WAT lipogenesis and in excessive weight gain.

on increased ELOVL3, MUP1, and LCN2 levels in female *Rsl1*$^{-/-}$ liver following HFD (Fig. 3C), which may trigger excess lipid storage in WAT. ELOVL3 knockout females are resistant to DIO, probably due to a lack of very-long-chain fatty acids (VLCFAs) that stimulate expansion of lipid droplets (34). In *Rsl1*$^{-/-}$ females, upregulated ELOVL3 in liver during HFD may increase synthesis of VLCFAs that could act in liver or be transported (possibly via lipocalins) to act in WAT to sustain lipogenesis and lead to weight gain (Fig. 8). Female and male adipocytes differ in insulin sensitivity (52), and small differences in insulin levels occur in *Rsl1*$^{-/-}$ mice on HFD (Table 1). This may affect female-specific DIO, but the mechanism awaits further study. Dimorphism in DIO is also linked to estrogen's ability to counter adiposity (53, 54). Altered estrogenic activity may occur in *Rsl1*$^{-/-}$ females, since several estrogen-activated enzymes are further upregulated in both liver and WAT (e.g., those encoded by *Fmo3*, *Cyp2b10*, *Cyp2b13*, *Cyp17a1*, and *Cyp3a41*) (2, 55) (Table 2). Some of these metabolize steroids and may alter systemic hormone levels. In fact, *Rsl1*$^{-/-}$ females enter puberty earlier than WT females, due either to increased estrogenic activity or to increased pheromone exposure via MUPs (29). Furthermore, on a standard chow diet, *Rsl1*$^{-/-}$ females have enhanced insulin sensitivity (Fig. 2B), unlike the obesity and insulin resistance seen in estrogen receptor α knockout (ERαKO) females (56). Collectively, these findings suggest that *Rsl1*$^{-/-}$ females may have mildly enhanced estrogen signaling that nevertheless fails to protect from HFD-induced adiposity.

In addition to actions during early development (57), KRAB-ZFPs have been implicated in cancer progression (58, 59) and two distinct pathways that lead to diabetes (5, 18). Here we demonstrate the ability of a KRAB-ZFP to modulate adult intermediary metabolism as well as responses to environmental stress, in a sex-dependent manner. For instance, in males, by supporting a proper fasting response among genes involved in glucose metabolism (e.g., *Pck1*, *G6pc*, etc.), RSL1 ensures a proper response to short-term energy demands that may be required for activities typical of male rodents, such as aggressive displays and territory defense, whereas in females, RSL1 has a greater impact on lipid metabolism, likely ensuring proper energy storage and mobilization during reproduction. Therefore, KRAB-ZFP-mediated epigenetic regulation is an important component in balancing metabolic demands that may be sex specific and essential for species survival and adaptation. Many of the *KRAB-Zfp* genes in mammalian genomes may have acquired similar roles in modulating gene-environment interactions, as evidenced by their rapid and species-specific expansion (60).

An intriguing finding is that sex-biased gene expression, which is well recognized in liver, is also evident in WAT, with even more genes affected. Furthermore, RSL1 accentuates this sex bias in liver but diminishes it in WAT (Fig. 6). This exemplifies a broad capacity of *KRAB-Zfp* genes to modify gene expression, essentially acting as expression quantitative trait loci (eQTL) (61). Fasting responses of several energy balance genes are exaggerated (e.g., *Elovl3*) or muted (e.g., *G6pc* and *Ppargc1a*) in RSL1-deficient mice, showing that RSL1 acts to lessen transcriptional noise and thus maintain homeostasis. Lack of RSL1 does not lead to a specific pathology but rather shifts the balance of regulation in both liver and WAT to a state that is weakened in its ability to manage environmental stress. This is corroborated by liver-specific *Kap1* ablation, which, similar to RSL1 deficiency in mice, leads to reduced sex bias in liver gene expression, dysregulation of metabolic genes, and female obesity on HFD (32). The fact that KAP1-deficient mice have more severe defects (hepatosteatosis and increased susceptibility to hepatic adenomas) likely reflects additional compromised KRAB-ZFPs, confirming that KRAB/KAP1-mediated regulation is integral to homeostasis. In fact, hypomorphic mutations in *Kap1* and *Dnmt3a*, both linked to KRAB-ZFP-mediated repression (14), increase the variability of many traits, affirming that regulatory effects of these factors in buffering gene expression increase phenotypic robustness (43, 44).

In conclusion, this study examines the effects of RSL1 in multiple tissues to highlight the role of a KRAB-ZFP in stabilizing complex phenotypes. *Rsl* reveals sex-specific gene regulation to be a tipping point for metabolic dysfunction, with loss of this epigenetic regulation predisposing females to obesity. *KRAB-Zfp* genes are highly polymorphic within a species (7, 8, 62) and are under positive selection (60), both of which may promote the emergence of new phenotypes. Hence, genetic variation in human *KRAB-Zfp* genes may be an important and understudied determinant of individual traits, including susceptibility to complex diseases. However, *KRAB-Zfp* genes are not assessed in genomewide association studies due to their repetitive nature and poor annotation. Given the broad regulatory effects of *Rsl* elucidated in this mouse model system, identifying functions for the hundreds of *KRAB-ZFP* genes extant in the human genome should have high priority for the potential to inform human health.

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