Published in final edited form as:

Hypertension. 2022 December ; 79(12): 2843–2853. doi:10.1161/HYPERTENSIONAHA.122.20169.

Cardiometabolic Consequences of Deleting the Regulator of G protein Signaling-2 (*Rgs2*) from Cells Expressing Agouti-Related Peptide or the Angiotensin II Type 1A Receptor in Mice

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Abstract

Background: Regulator of G protein signaling (RGS) family members catalyze the termination of G protein signaling cascades. Single nucleotide polymorphisms in the *RGS2* gene in humans have been linked to hypertension, preeclampsia, and anxiety disorders. Mice deficient for *Rgs2* ($Rgs2^{Null}$) exhibit hypertension, anxiety, and altered adipose development and function.

Methods: To study cell-specific functions of RGS2, a novel gene-targeted mouse harboring a conditional allele for the *Rgs2* gene ($Rgs2^{Flox}$) was developed. These mice were bred with mice expressing Cre-recombinase via the Agouti-related peptide locus (*Agrp*-Cre) to cause deletion of *Rgs2* from all cells expressing *Agrp* ($Rgs2^{Agrp\text{-}KO}$), or a novel transgenic mouse expressing Cre-recombinase via the ANG type 1A receptor (*Agtr1a* / AT_{1A}) promoter encoded in

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Disclosures

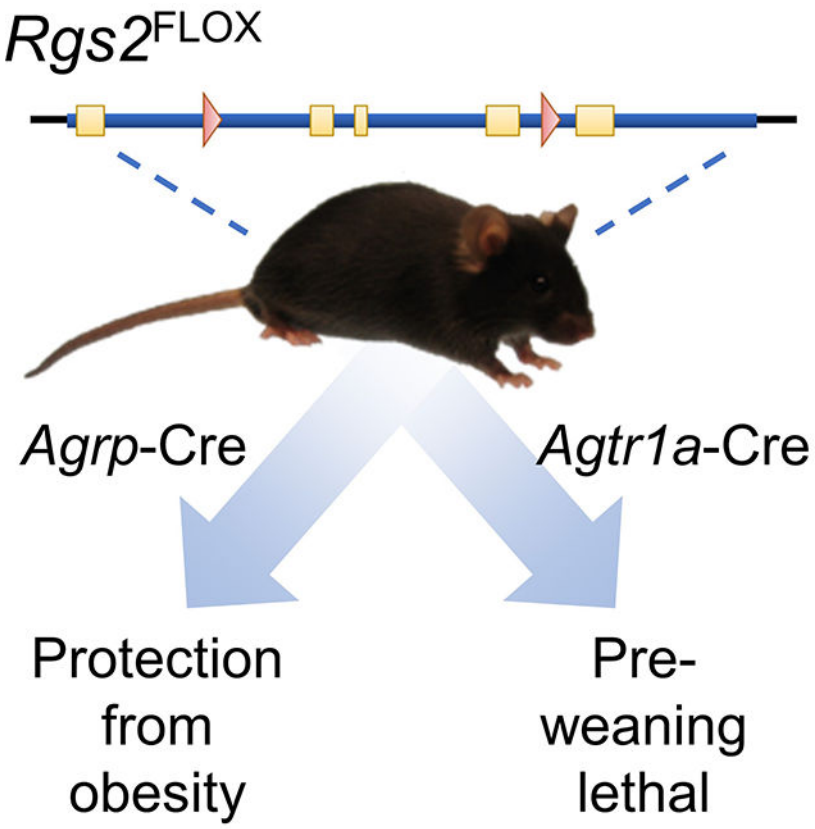
None.

a bacterial artificial chromosome (BAC-AT_{1A}-Cre) to delete *Rgs2* in all *Agtr1a*-expressing cells (*Rgs2*$^{AT1A\text{-}KO}$).

Results: Whereas *Rgs2*^{Flox}, *Rgs2*$^{Agrp\text{-}KO}$, and BAC-AT_{1A}-Cre mice exhibited normal growth and survival, *Rgs2*$^{AT1A\text{-}KO}$ exhibited pre-weaning lethality. Relative to littermates, *Rgs2*$^{Agrp\text{-}KO}$ exhibited reduced fat gains when maintained on a high fat diet, associated with increased energy expenditure. Similarly, surviving adult *Rgs2*$^{AT1A\text{-}KO}$ mice also exhibited increased energy expenditure. Surprisingly, given the hypertensive phenotype previously reported for *Rgs2*^{Null} mice and evidence supporting a role for RGS2 in terminating AT_{1A} signaling in various cell types, *Rgs2*$^{AT1A\text{-}KO}$ mice exhibited normal blood pressure, ingestive behaviors, and renal functions, both before and after chronic infusion of ANG (490 ng/kg/min, sc).

Conclusions: These results demonstrate the development of a novel mouse with conditional expression of *Rgs2* and illustrate the role of *Rgs2* within selected cell types for cardiometabolic control.

Graphical Abstract



Keywords

G protein; GTPase-activating protein; animal model; second-messenger; G protein coupled receptor

Background

The Regulator of G protein Signaling (RGS) family of proteins function as catalysts to terminate G protein signaling. There are currently at least 20 genes known to encode RGS proteins in mammals, and many encode multiple unique splice variants. Individual RGS proteins exhibit selectivity toward distinct G protein cascades and can be clustered into subfamilies with shared structures and functions. Ongoing research is aimed at clarifying the context-dependency of RGS protein and G protein interactions, the redundancy of distinct RGS proteins within individual cell types, and the implications of altered expression and function in health and disease.[1]

Regulator of G protein signaling 2 (RGS2) is a member of the B/R4 subfamily of RGS proteins. It functions to accelerate termination of Gαq and Gαi/o signaling and has been suggested to interact directly with adenylyl cyclase to also inhibit signaling secondary to the Gαs cascade.[1–5] The RGS2 gene (*RGS2*, *Rgs2*) has been associated with a number of cardiovascular, metabolic, and behavioral outcomes in humans and rodents. Single nucleotide polymorphisms and changes in expression of RGS2 have been associated with hypertension, preeclampsia, panic and anxiety disorders, antipsychotic-associated Parkinsonism, post-traumatic stress disorder, and suicide in humans (reviewed[6]). Expression of *RGS2* is inversely related to blood pressure (BP), as subjects with hypotension (i.e. Bartter's or Gitelman's syndromes) exhibit increased RGS2 but those with hypertension exhibit reduced RGS2 levels.[7] Rodent models support a critical role for RGS2 in the function of the vasculature,[8–11] control of blood pressure,[12] and the development and function of adipose[13] and placenta.[14]

Of relevance to cardiovascular, metabolic, and behavioral control, evidence supports a bidirectional relationship between RGS2 and the angiotensin II (ANG) type 1 receptor (AT_1 / *AGTR1* in humans, or AT_{1A}/*Agtr1a* in rodents). Second-messenger signaling from AT_1 is sensitive to termination by RGS2,[15, 16] and *RGS2* mRNA in cultured vascular smooth muscle cells is sensitive to ANG stimulation.[17] Our team recently demonstrated a critical role for AT_{1A}, localized to the subtype of neurons within the hypothalamic arcuate nucleus (ARC) that also express Agouti-related peptide (AgRP/*Agrp*), in the integrative control of resting energy expenditure.[18, 19] Previous studies have suggested that AgRP neurons express *Rgs2*, that *Rgs2* is among the most highly expressed RGS family members in this cell, and that feeding/fasting modulates *Rgs2* expression in this cell type.[20] These data prompt the hypothesis that *Rgs2* within AgRP neurons may influence energy expenditure, especially in response to activation of AT_{1A}.

Previous studies of RGS2 in rodent models have been largely accomplished using mice with global deletion (i.e. $Rgs2^{Null}$) mouse models. This approach has implicated RGS2 in the control of various endpoints, but because RGS2 is involved in the normal development of assorted tissue types (i.e. adipose,[13] placenta[14]), interpretation of outcomes is confounded due to compensatory physiology in these animals. The Blumer group previously developed a mouse model harboring a conditional (i.e. flox'ed) endogenous allele for the *Rgs2* gene, and used this model to investigate the function of RGS2 within endothelial and vascular

smooth muscle cell types.[10] That animal model was unfortunately lost, and subsequent investigations into the cell-specific functions of RGS2 have therefore been hindered.

The current study was carried out with four objectives. The first was to develop a novel mouse model that harbors a conditional endogenous allele for *Rgs2* (*Rgs2*^{Flox}). Second, we crossed these new *Rgs2*^{Flox} mice with a strain that expresses Cre-recombinase via the *Agrp* locus (*Agrp*-Cre[21]), to generate mice with selective deletion of the *Rgs2* gene in *Agrp*-expressing cells and explore metabolic consequences of such a disruption. Third, we developed a novel transgenic mouse model that expresses Cre recombinase via the *Agtr1a* locus encoded in a large BAC (BAC-AT_{1A}-Cre) to enable selective recombination of conditional genes within cells that express AT_{1A}. Fourth, we explored the cardiovascular and metabolic consequences of conditionally deleting the *Rgs2* gene only in cells that express *Agtr1a* after breeding the novel AT_{1A}-Cre and *Rgs2*^{Flox} models together. Together, the results reported here support a role for RGS2 in AT_{1A}-expressing cells in selected aspects of cardiometabolic function, and we anticipate that these two novel mouse models (BAC-AT_{1A}-Cre, *Rgs2*^{Flox}) will facilitate many future investigations in the fields of cardiovascular, metabolic, and psychiatric medicine.

Methods

All methods, including development of the novel *Rgs2*^{Flox} model (Figure 1A) are described in the Online Supplement.[22–29] Procedures were approved by the Medical College of Wisconsin and University of Iowa Institutional Care and Use Committees, and were in accordance with the expectations described in the Guide for the Care and Use of Laboratory Animals.[30] All data and supporting materials have been provided within the published article and associated supplemental files.

Results

Deletion of Rgs2 from Agrp-expressing cells

Agrp-Cre$^{+/+}$ sires were mated with *Rgs2*$^{Flox/Flox}$ dams and resulting F1 double-heterozygous (*Agrp*-Cre$^{+/-}$, *Rgs2*$^{Flox/WT}$) male offspring were bred with *Rgs2*$^{Flox/Flox}$ dams to generate targeted F2 "*Rgs2*$^{Agrp\text{-}KO}$" (mixed background, heterozygous *Agrp*-Cre$^{+/-}$ plus homozygous *Rgs2*$^{Flox/Flox}$) offspring and littermate controls. Deletion of *Rgs2* from *Agrp*-expressing cells was confirmed using amplification of genomic DNA isolated from the hypothalamic ARC. Amplification using primers that flank the conditional region result in detection of a recombined (i.e. deleted) allele only in samples from mice with Cre-recombinase expression (Figure 1B). Further, loss of expression of *Rgs2* from cells expressing endogenous *Agrp* was confirmed using in situ hybridization on coronal sections of hypothalamic ARC. While endogenous *Rgs2* and *Agrp* expression are colocalized within individual cells of the ARC, *Rgs2* expression was nearly ablated in ARC sections isolated from *Rgs2*$^{Agrp\text{-}KO}$ mice (Figure 1C). These results support deletion of the conditional *Rgs2* allele in *Agrp*-expressing cells of *Rgs2*$^{Agrp\text{-}KO}$ mice. Given the above breeding approach, we anticipated 25% *Rgs2*$^{Agrp\text{-}KO}$ offspring from this breeding paradigm. Of the n=84 female + 82 male F2 offspring that were born to this colony and survived to weaning at PD21, n=21 female + 14 male (21%, p=0.36 vs the expected 25% by Chi-square test) were *Rgs2*$^{Agrp\text{-}KO}$.

This suggests that deletion of the *Rgs2* gene from *Agrp*-expressing cells had no major consequences on early-life survival.

Metabolic impacts of deleting Rgs2 from Agrp-expressing cells

The dose-dependency of *Rgs2* gene copy number has been previously established,[14, 31] and therefore physiological phenotyping was only carried out on $Rgs2^{\text{Agrp-KO}}$ mice and littermate controls (i.e. *Agrp*-$\text{Cre}^{+/-}$, $Rgs2^{\text{WT/WT}}$; or *Agrp*-$\text{Cre}^{-/-}$, $Rgs2^{\text{Flox}}$). Littermates that harbored both the *Agrp*-Cre construct and a single conditional allele for *Rgs2* (i.e. *Agrp*-$\text{Cre}^{+/-}$, $Rgs2^{\text{Flox/WT}}$) were specifically excluded from phenotyping efforts.

Aerobic energy expenditure was assessed at 7 weeks of age in animals at rest at thermoneutrality (30°C) and in the post-absorptive state, therefore reflecting basal metabolic rate (BMR). While sex modified body mass and BMR, genotype had no effect on either endpoint (Table S1). At 8 weeks of age, significant interactions between sex and genotype influenced total body mass and fat-free mass (FFM), but not fat mass.

A subset of mice of each sex and genotype were then switched to a high fat diet (HFD; D12451, 45% kcal from fat). At week 10 (i.e. two weeks of HFD), control littermates exhibited significant increases in fat mass, but this effect was prevented in $Rgs2^{\text{AgRP-KO}}$ mice (diet x genotype interaction $p<0.01$) (Table S2). Although sex modified total fat gains, sex did not modify the interaction between diet and genotype; $p=0.14$). Food intake at this timepoint was suppressed by HFD, and this effect was prevented in $Rgs2^{\text{AgRP-KO}}$ mice (diet x genotype $p=0.03$). Feeding efficiency, an inverse metric of energy expenditure and digestive efficiency, was increased by HFD in control littermates (i.e. energy expenditure was decreased), and this effect was prevented in $Rgs2^{\text{AgRP-KO}}$ mice (diet x genotype $p=0.02$).

In week 11 (3 weeks of HFD), BMR was again assessed by respirometry. Unadjusted BMR was not altered by diet, genotype, or sex. Correction of week 11 BMR for FFM measured in week 10 uncovered a modulatory effect of sex, but not diet or genotype, upon BMR (Table S2).

Body composition was again assessed after 18 weeks. HFD increased fat and proportional fat masses, and although trends toward an attenuation of fat mass gains were observed in $Rgs2^{\text{AgRP-KO}}$ mice, such effects did not reach formal statistical significance (diet x genotype $p=0.06$) (Table S2). Interscapular brown adipose tissue (BAT), inguinal white adipose (iWAT), and perigonadal WAT masses were increased in control littermates, while iWAT gains were prevented in $Rgs2^{\text{AgRP-KO}}$ mice (diet x genotype $p=0.02$) (Table S3).

Finally, using caloric intake data from week 10 to estimate total caloric intake, and changes in body mass from week 8 to 18, feeding efficiency was again calculated. Feeding efficiency was increased in control littermates but not $Rgs2^{\text{AgRP-KO}}$ mice (diet x genotype $p=0.01$) (Figure 1D). These data support the conclusion that deletion of *Rgs2* from *Agrp*-expressing cells increases (or minimizes suppression of) total energy expenditure, to ultimately attenuate HFD-induced expansion of fat mass.

Lethality of RGS2 deletion from AT_{1A}-expressing cells on a mixed background

Heterozygous BAC-AT_{1A}-$Cre^{+/-}$ sires (harboring the novel transgene illustrated in Figure 2A) were mated with homozygous *Rgs2*$^{Flox/Flox}$ dams and resulting F1 double-heterozygous (BAC-AT_{1A}-$Cre^{+/-}$, *Rgs2*$^{Flox/WT}$) male offspring were bred with *Rgs2*$^{Flox/Flox}$ dams to generate targeted F2 "*Rgs2*$^{AT1A-KO}$" (mixed background, BAC-AT_{1A}-$Cre^{+/-}$, *Rgs2*$^{Flox/Flox}$) offspring. Thus, we anticipated 25% *Rgs2*$^{AT1A-KO}$ offspring from this breeding paradigm. Of the n=43 female + 32 male F2 offspring that were born to this colony and survived to weaning, only n=1 female + 1 male (3%, p=0.00006 vs the expected 25% by Chi-square test) were *Rgs2*$^{AT1A-KO}$ (Figure 2B). These results support the conclusion that genetic deletion of *Rgs2* from AT_{1A}-expressing cells in mice with a mixed background strain results in pre-weaning lethality. Histological analyses (hematoxylin & eosin staining) of the kidneys and hearts of the *Rgs2*$^{AT1A-KO}$ mice that survived to adulthood failed to reveal any robust structural abnormalities (Supplemental Figures S1 and S2). Body mass and body composition from wean to 20 weeks of age and tissue masses at 20 weeks of age were essentially indistinguishable between *Rgs2*$^{AT1A-KO}$ and littermate control mice. Thus, it is not immediately obvious why *Rgs2*$^{AT1A-KO}$ mice on a mixed background exhibited pre-weaning lethality.

Maintenance of pre-weaning lethality on the C57BL/6J background

Given the low survival rate of *Rgs2*$^{AT1A-KO}$ mice on a mixed background, it seems likely that analyses of these few adults was strongly influenced by a survivor bias. We hypothesized that the mixed background strain upon which the *Rgs2*^{Flox} strain was generated may contribute to this lethality phenotype, and therefore a subset of the *Rgs2*^{Flox} (mixed background) founder strain was serially backcrossed to the C57BL/6J strain (Jax laboratories 000664). After eight generations of crosses (*Rgs2*$^{Flox/WT}$ X C57BL/6J), siblings were again crossed to generate homozygous *Rgs2*$^{Flox/Flox}$ mice, now considered an N8 backcross to the C57BL/6J background strain. Resulting *Rgs2*$^{Flox/Flox}$ (N8) showed similar vitality and health as *Rgs2*$^{Flox/Flox}$ (mixed) mice. The *Rgs2*$^{Flox/Flox}$ (N8) mice were then bred with BAC-AT_{1A}-Cre mice to generate a new *Rgs2*$^{AT1A-KO}$ (N8) colony. Of the n=58 female + 53 male F2 offspring born to this new colony, n=4 female + 5 male (8%, p=0.0006 versus the expected 25% by Chi square) were *Rgs2*$^{AT1A-KO}$, indicating that deletion of *Rgs2* from AT_{1A}-expressing cells on a predominantly C57BL/6J background is subject to the same pre-weaning lethality that was observed on a mixed background (Figure 2B). This (N8) colony was used for all subsequent experiments.

Basal metabolic rate (BMR)

At 10-12 weeks of age, BMR was examined using gas respirometry. No differences in uncorrected BMR were noted between groups (Figure 2C). Generalized Linear Modeling (GLM) was then used to account for the variability added by the covariate of body mass. The GLM approach identified body mass as a significant covariate (p=0.02), and accounting for this covariate uncovered an effect of genotype upon BMR (p=0.01). No effect of sex (p=0.10) nor modulatory effect of sex upon genotype (p=0.70) were observed. With sexes combined, *Rgs2*$^{AT1A-KO}$ mice exhibited a 32% increase in BMR (p=0.03) relative to wildtype control littermates when the covariate of body mass was held at 21.96g (Figure

2D). These results indicate that deletion of *Rgs2* from *Agtr1a*-expressing cells results in a major increase in BMR in mice that survive beyond weaning.

Body composition & Metabolic cages

While FFM was indistinguishable between groups, proportional fat mass was greater in *Rgs2*$^{AT1A\text{-}KO}$ mice (Figure 3A, Table S4). These effects were surprising, given the increase in BMR in the *Rgs2*$^{AT1A\text{-}KO}$ model. Therefore, ingestive behaviors and digestive functions were assessed using metabolic caging. Water and food intake, digestive efficiency, and therefore total caloric absorption rates were largely unchanged in *Rgs2*$^{AT1A\text{-}KO}$ mice (Figure 3B–C, Table S5). Comparison of total daily caloric absorption versus FFM by regression modeling demonstrates a major impact of FFM upon caloric absorption (Figure 3E–F). Thus, the minor modulatory effects of genotype and sex noted here are likely all secondary to differences in FFM. Total daily sodium intake, urine volume, osmolality, sodium and potassium concentrations, and total daily elimination of sodium and potassium to urine were unaffected by sex or genotype.

Blood pressure

Systolic BP was assessed before and during chronic infusion of ANG. At baseline, no differences in systolic BP were noted between *Rgs2*$^{AT1A\text{-}KO}$ and littermate control mice, though heart rate was increased ($p<0.05$) when comparing only control and *Rgs2*$^{AT1A\text{-}KO}$ mice without *Rgs2*$^{AT1A\text{-}HET}$ littermates (Table S6). Chronic infusion of ANG caused expected increases in systolic BP and reductions in heart rate ($p<0.05$ versus zero by one-sample t-test), confirming the efficacy of the infusion, though no modulatory effect of genotype was noted. Thus, we conclude that deletion of *Rgs2* from *Agtr1a*-expressing cells had no major effect on baseline BP, nor BP responses to peripheral ANG infusion, but may impact heart rate.

Body composition effects of chronic ANG infusion

Total body mass, FFM, and fat masses diverged between male and female mice during chronic ANG infusion, but no modulatory effect of genotype was observed (Table S7). No effect of genotype was observed upon proportional fat mass, nor upon total body hydration status.

Metabolic cages after chronic ANG infusion

Following infusion of ANG, no effects of genotype were observed for food, fluid, or sodium intake behaviors, digestive efficiency, or daily caloric absorption rates (Table S8). Because male mice tended to lose weight during the chronic infusion (Table S7), energy efficiency was significantly reduced in males regardless of genotype (Table S8). These results are consistent with an increase in total energy expenditure in males, regardless of genotype, in response to chronic ANG infusion.

Urine volume, osmolality, and sodium and potassium concentrations were unaffected by genotype after ANG infusion. Total daily sodium elimination to the urine was unaffected by genotype, but an interaction between sex and genotype influenced total daily potassium elimination to the urine, driven largely by differences observed within females.

Tissue masses after chronic ANG infusion

With or without correction for total body mass, no effect of genotype was observed upon adipose tissues, heart ventricles, adrenal glands, or kidneys (Table S9). In contrast, perigonadal WAT, heart ventricles, and kidneys diverged between the sexes.

Adipose histology and gene expression

We next examined structure of adipocytes within iWAT of male mice after HFD feeding. Compared to control littermates, these adipocytes were much smaller in *Rgs2*$^{\text{AT1A-KO}}$ mice (Figure 4A–B). Together with tissue masses and NMR results, these results indicate that deletion of *Rgs2* from *Agtr1a*-expressing cells results in reduced adipose mass at least in part through reduced size of individual adipocytes.

Rgs2 mRNA was measured in BAT, iWAT and kidney of *Rgs2*$^{\text{AT1A-KO}}$ mice (Figure 4C). *Rgs2* was decreased in BAT of both *Rgs2*$^{\text{AT1A-HET}}$ and *Rgs2*$^{\text{AT1A-KO}}$ mice compared to control littermates, consistent with either of the BAC-AT_{1A}-Cre transgene in these tissues, or physiological feedback-mediated suppression of expression. Expression of *Rgs2* is much lower in iWAT compared to BAT, and there were no differences in *Rgs2* levels in iWAT among genotype groups. Finally, as expression of *Agtr1a* is strong in kidney, we compared adipose expression levels with *Rgs2* expression in kidneys of these mice. Renal expression of *Rgs2* was decreased in *Rgs2*$^{\text{AT1A-KO}}$ mice compared to control littermates. In contrast, no difference in expression of RAS genes in the kidney were observed between *Rgs2*$^{\text{AT1A-KO}}$ and control littermates (*Ren*: 0.94(0.66-1.32) fold of control (± SE), n=8 control versus 5 *Rgs2*$^{\text{AT1A-KO}}$, p=0.88; *Agt*: 0.92(0.75-1.14) p=0.75; *Agtr1a*: 0.97(0.82-1.15) p=0.86; *Agtr1b*: 1.02(0.97-1.08) p=0.92; and *Agtr2*: 1.05(0.76-1.46) p=0.89;).

Expression of Rgs2 in Agrp- and Agtr1a-expressing cells of the ARC

Given the increase in energy expenditure in both *Rgs2*$^{\text{Agrp-KO}}$ and *Rgs2*$^{\text{AT1A-KO}}$ mice, we explored the hypothesis that *Rgs2* may be deleted from an overlapping set of cells in these two models. Expression of *Agrp* is largely limited to the ARC of the hypothalamus, plus a small subset of cells within the adrenal gland.[32] In silico reanalysis of a published single-cell RNAseq dataset was used to examine expression of *Rgs2* across cell types of the ARC.[33] *Rgs2* was strongly expressed in a handful of cell types of this brain region, including the two cell type clusters identified as “AgRP neurons” (i.e. “GABA-14 (Npy, Agrp)” and “Sst-3, medium”) in the original study (Figure 5A). Next, we examined expression of the various RGS family members within these two *Agrp*-expressing cell types, and determined that *Rgs2* is one of the dominant RGS family members expressed in *Agrp*-expressing cells (Figure 5B). Interestingly, *Rgs2* expression is correlated with expression of *Agtr1a* in a number of cell types within the ARC including the “Sst-3, medium” Agrp-expressing cell type (Figure 5C). Consistent with this in silico reanalysis, in situ hybridization to detect *Rgs2* and *Agtr1a* within the mouse ARC highlights that there are cells present in this brain region that express *Rgs2*, *Agtr1a*, both, or neither (Figure 5D). Finally, using various methods, we have previously demonstrated that *Agtr1a* is expressed within a subset of *Agrp*-expressing neurons of the ARC.[18, 19] Again, consistent with those observations, *Agtr1a* expression within the acuate nucleus was correlated with expression of *Agrp*, predominantly within the “Sst-3, medium” subtype of *Agrp*-expressing cells (Figure 5E).

Together, these results support the co-expression of *Rgs2* and *Agtr1a* within a specific subset of *Agrp*-expressing cells in the ARC of the mouse hypothalamus.

Conclusions

Here we report the generation of a conditional $Rgs2^{Flox}$ mouse model and the cardiometabolic consequences of deleting *Rgs2* from cells that express *Agrp* or *Agtr1a*. Deletion of *Rgs2* from *Agrp*-expressing cells resulted in metabolic effects, and deletion of *Rgs2* from *Agtr1a*-expressing cells surprisingly resulted in pre-weaning lethality and failed to alter basic cardiovascular functions.

Deletion of *Rgs2* from *Agrp*-expressing cells resulted in reduced adiposity during HFD feeding, which is of interest for two reasons. First, $Rgs2^{Null}$ mice exhibit altered adipose development,[13] but those studies were unable to distinguish the contributions of the loss of *Rgs2* within the adipose versus other tissues. Our study informs interpretation of those results, as *Rgs2* deletion from *Agrp*-expressing cells caused a similar phenotype without notable changes in expression of *Rgs2* in iWAT. While not testing adipose-specific effects of *Rgs2*, our study positively implicates *Rgs2*, within *Agrp*-expressing cells, in the regulation of weight and adiposity in response to HFD. Second, the determination that *Rgs2* deletion caused adiposity phenotypes that were only unmasked after HFD feeding is consistent with its role in modulating G protein signaling in AgRP neurons. We previously demonstrated that genetic deletion of the AT_{1A} receptor from AgRP neurons causes changes in energy balance that are only unmasked after a stimulus such as HFD.[19] The demonstration here that *Agtr1a* and *Rgs2* are both expressed by the same subset of AgRP neurons, plus the discovery of a major role for *Rgs2* in *Agtr1a*-expressing cells for basal metabolic rate control, provides initial support for a working model in which AT_{1A} signaling within AgRP neurons, modulated by RGS2, mediates integrative control of energy expenditure.[34] Future studies utilizing tamoxifen-inducible versions of Cre-recombinase (i.e. CreERT2) to delete *Rgs2* from individual cell types in adulthood, after development is complete, will provide additional insight into the role of *Rgs2* within the brain versus adipose in the observed phenotypes.

We were surprised that deletion of *Rgs2* from AT_{1A}-expressing cells resulted in preweaning lethality. Both founder strains (BAC-AT_{1A}-Cre, $Rgs2^{Flox}$) exhibited normal survival, supporting the conclusion that it is the cell-specific deletion of *Rgs2* from AT_{1A}-expressing cells that caused the lethal phenotype. $Rgs2^{Null}$ mice exhibit normal fecundity and survival,[13, 14] and here we also demonstrate normal survival of mice with *Rgs2* deleted from *Agrp*-expressing cells. Similarly, deletion of *Rgs2* from endothelial and vascular smooth muscle cells was not reported to cause any major changes in survival.[10] These results hint at a precarious balance in G protein signaling within AT_{1A}-expressing, versus other, cell types in early development and survival. Future development of an inducible version of the BAC-AT_{1A}-Cre construct (e.g. to express a tamoxifen-dependent version of Cre-recombinase) may enable studies of the role of RGS2 within AT_{1A}-expressing cells while circumventing the pre-weaning lethality noted here.

Given the well-recognized role of ANG and its AT_1 receptor in hypertension in general, profound hypertension reported in male *Rgs2*^{Null} mice,[12, 31] and as RGS2 acts to buffer AT_1 receptor signaling in various cell types,[15, 16, 35, 36] we were surprised to discover that *Rgs2*$^{AT1A\text{-}KO}$ mice exhibited normal BP at baseline and relatively normal BP responses to ANG infusion. It is critical to appreciate that in the current study a tail-cuff plethysmography approach was utilized, as large changes in BP were anticipated.[12, 31] This methodology is generally acceptable when frank hypertension is expected, but is not recommended when subtle changes are of interest.[37] Thus, this approach may have failed to detect small changes in pressure or changes in diurnal rhythms, however, it is unlikely that this method would be unable to detect the magnitude of baseline BP increase previously reported for *Rgs2*^{Null} mice (~50 mmHg increase in BP). Further, it seems unlikely that this method would falsely detect an effect of ANG infusion upon BP when antagonism of AT_1 receptors in *Rgs2*^{Null} mice has been reported to have no effect on BP.[12] Assuming that the results obtained from the tail-cuff approach are at least qualitatively correct, it is unclear whether the lack of BP and renal phenotypes in the *Rgs2*$^{AT1A\text{-}KO}$ are due to desensitization of AT_{1A} receptors in relevant cell types, compensatory changes in other angiotensin receptors (such as AT_{1B}, AT_2, Mas, etc.) or endogenous RAS signaling cascades (production/degradation of ANG), RAS-independent functions of RGS2, or merely reflect a survivor bias. That RAS gene expression within the kidney was unchanged in the knockout animals provides initial evidence arguing against such effects, but many additional studies are warranted. Together with the previous report that genetic deletion of *Rgs2* from layers of the vasculature had no overt effect on BP,[10] the current study supports the conclusion that *Rgs2* expression in other tissues and cell types, or a combination of these with other cell types, is critically involved in BP control. The novel *Rgs2*^{Flox} mouse described herein will enable such investigations, especially when used in combination with inducible versions of Cre-recombinase to avoid developmental confounds such as the pre-weaning lethality documented herein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge assistance from the University of Iowa Genome Editing Facility, the MCW Biomedical Resource Center, and the MCW Rat Research Models Service Center.

Sources of Funding

This work was supported by the NIH (HL134850, HL084207, DK133121, HL007638, HL153101, HL144807, HL153274, HL127673), American Heart Association (18EIA33890055, 18PRE33960377, 19POST34380239, 903246, 826132, 898067), MCW Clinical & Translational Science Institute (UL1TR001436), and the Advancing a Healthier Wisconsin endowment.

Abbreviations

AgRP	Agouti-related peptide
ANG	angiotensin II

ARC	arcuate nucleus
AT_1	ANG type 1 receptor
BMR	basal metabolic rate
FFM	fat-free mass
HFD	high-fat diet
iWAT	inguinal fat
RAS	renin-angiotensin system
RGS	Regulator of G protein Signaling

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Novelty and Relevance

What is new?

Here we report the development and use of a new gene targeted mouse model with conditional expression of the gene encoding Regulator of G protein Signaling-2 (*Rgs2*^{Flox}).

What is relevant?

RGS2 is implicated in human hypertensive disorders, and *Rgs2*^{Null} animals exhibit an array of cardiometabolic and behavioral phenotypes. The lack of availability of a conditional (*Rgs2*^{Flox}) model, however, has limited investigation of cell-specific functions of this critical regulator of G protein signaling.

Clinical/Pathophysiological implications?

Conditional deletion of *Rgs2* from cells expressing Agouti-related peptide (AgRP) or the angiotensin AT_{1A} receptor both result in protection from diet-induced adipose gains, and deletion from AT_{1A}-expressing cells results in unexpected effects on pre-weaning survival and blood pressure.

Perspectives

As RGS2 is known to modulate G protein signaling in various cell types, and mutations in this gene are associated with various diseases, mechanistic dissection of the role of RGS2 within individual cell types is critically needed. Here we report development of a novel gene-targeted mouse with a conditional allele for *Rgs2* (*Rgs2*^{Flox}), and the consequences of selectively deleting *Rgs2* from *Agrp*- or *Agtr1a*-expressing cells upon selected cardiometabolic endpoints. Results positively implicate *Rgs2* within *Agrp*- and *Agtr1a*-expressing in the control of energy balance, and uncover an unexpected role of *Rgs2* in AT_{1A}-expressing cells in early life survival. The availability of this new *Rgs2*^{Flox} mouse strain will enable many future studies of the cell-specific functions of RGS2.

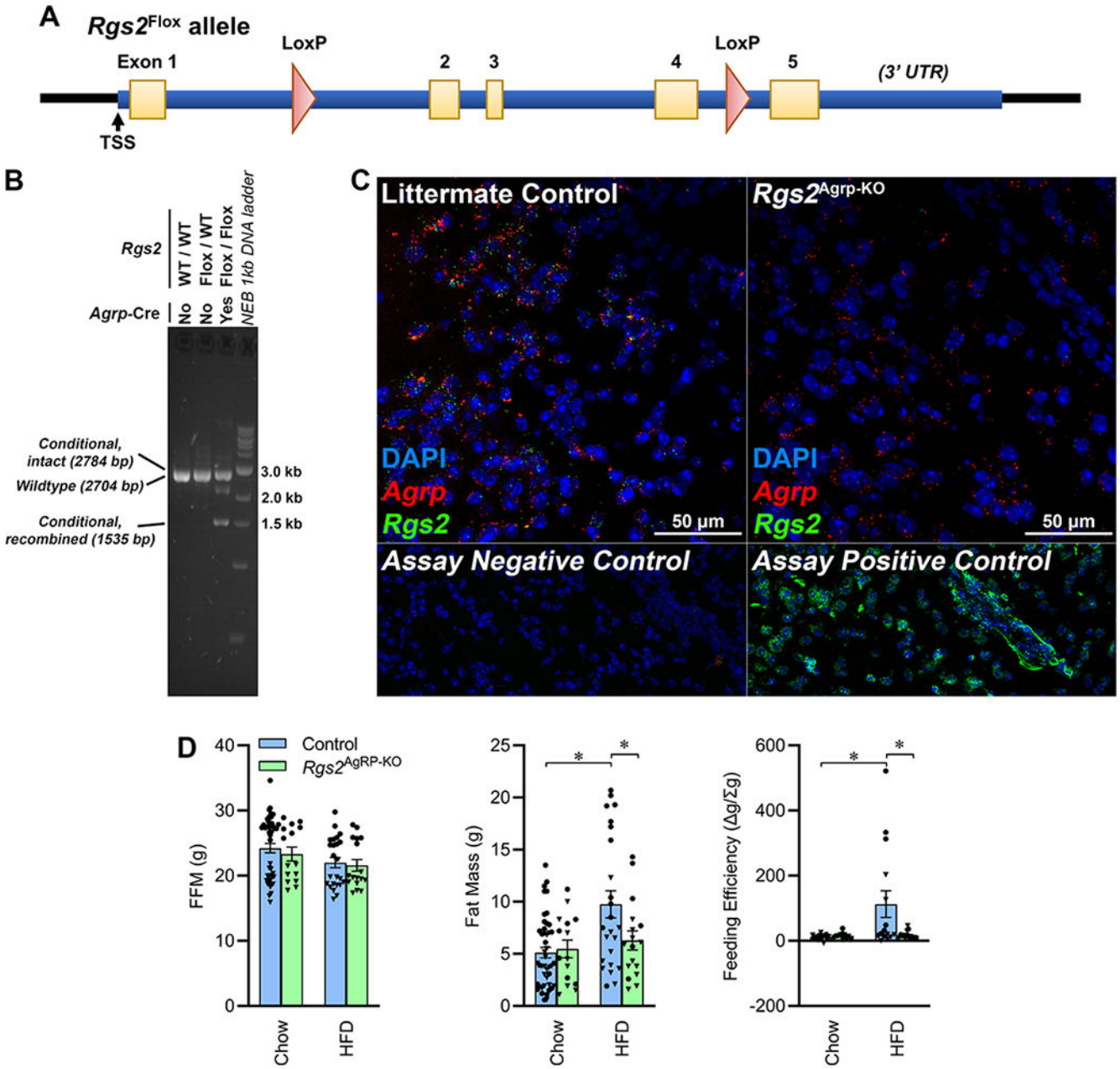

Figure 1. Deletion of *Rgs2* from Agrp-expressing cells in the *Rgs2*$^{\text{Agrp-KO}}$ model.

(A) Schematic illustrating design of novel *Rgs2*$^{\text{Flox}}$ allele. (B) Genomic DNA recombination within the hypothalamic ARC from adult male mice, demonstrating the appearance of the recombined conditional allele only in the presence of Cre-recombinase. (C) RNAscope in situ hybridization to detect mRNA transcripts for Agrp and Rgs2 in the hypothalamic ARC from adult male littermate control (*Agrp*-Cre$^{-/-}$, *Rgs2*$^{\text{Flox/Flox}}$) and *Rgs2*$^{\text{Agrp-KO}}$ mice, demonstrating colocalization of *Agrp* and *Rgs2* transcripts in control littermate mice, but loss of *Rgs2* transcript detection from Agrp-expressing cells in *Rgs2*$^{\text{Agrp-KO}}$ mice. (D) Fat-free and fat masses, and feeding efficiency at 18 weeks of age, after 10 weeks of HFD feeding. 3-way ANOVA results reported in Table S2; n=18-32 per group; males shown

as circles and females as triangles. *p<0.05 for indicated comparison by Sidak multiple comparisons procedure.

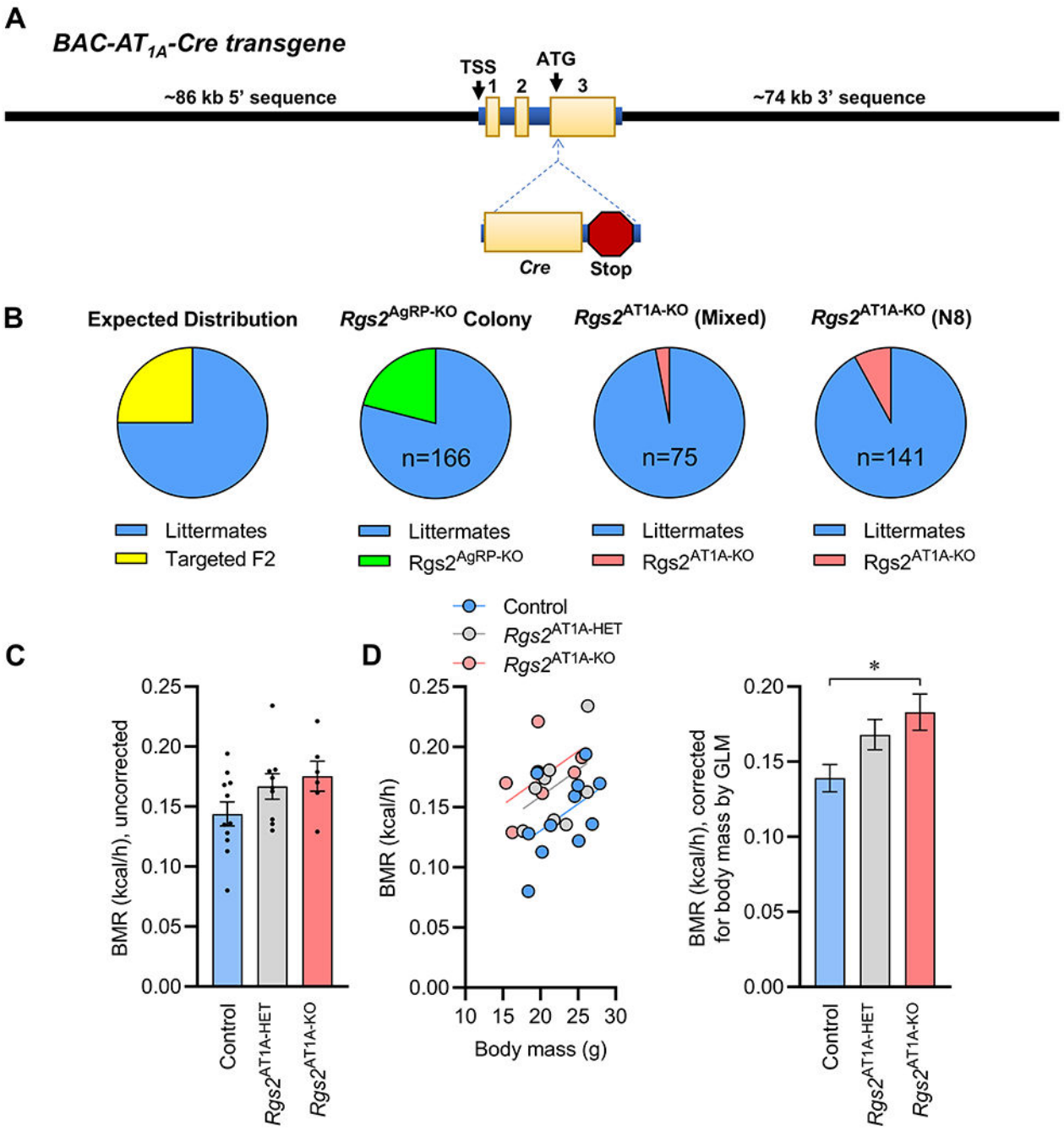

Figure 2. Increased BMR in the *Rgs2*$^{\text{AT1A-KO}}$ model.

(A) Schematic illustrating design of novel BAC-AT$_{1A}$-Cre transgene. (B) Comparison of expected F2 genotypes at weaning, versus the *Rgs2*$^{\text{Agrp-KO}}$ colony (p=0.36 vs expected) and the *Rgs2*$^{\text{AT1A-KO}}$ colonies on both mixed background (p=0.0001) and after eight generations of backcross (N8, p=0.0006) to the C57BL/6J background. (C) Uncorrected BMR values at 10-12 weeks of age. One-way ANOVA p=0.13. (D) Regression analysis of uncorrected BMR values versus body mass. Estimated marginal mean values for BMR, presented at body mass covariate value of 21.96 grams, determined by GLM analysis: model p=0.019,

intercept p=0.097, body mass p=0.017, genotype p=0.021. For panels A-C, control n=5 female + 6 male, *Rgs2*$^{\text{AT1A-HET}}$ n=5 female + 4 male, and *Rgs2*$^{\text{AT1A-KO}}$ n=4 female + 2 male. Summary data are presented as mean±SE. *p<0.05 for indicated comparison by Sidak multiple comparisons procedure.

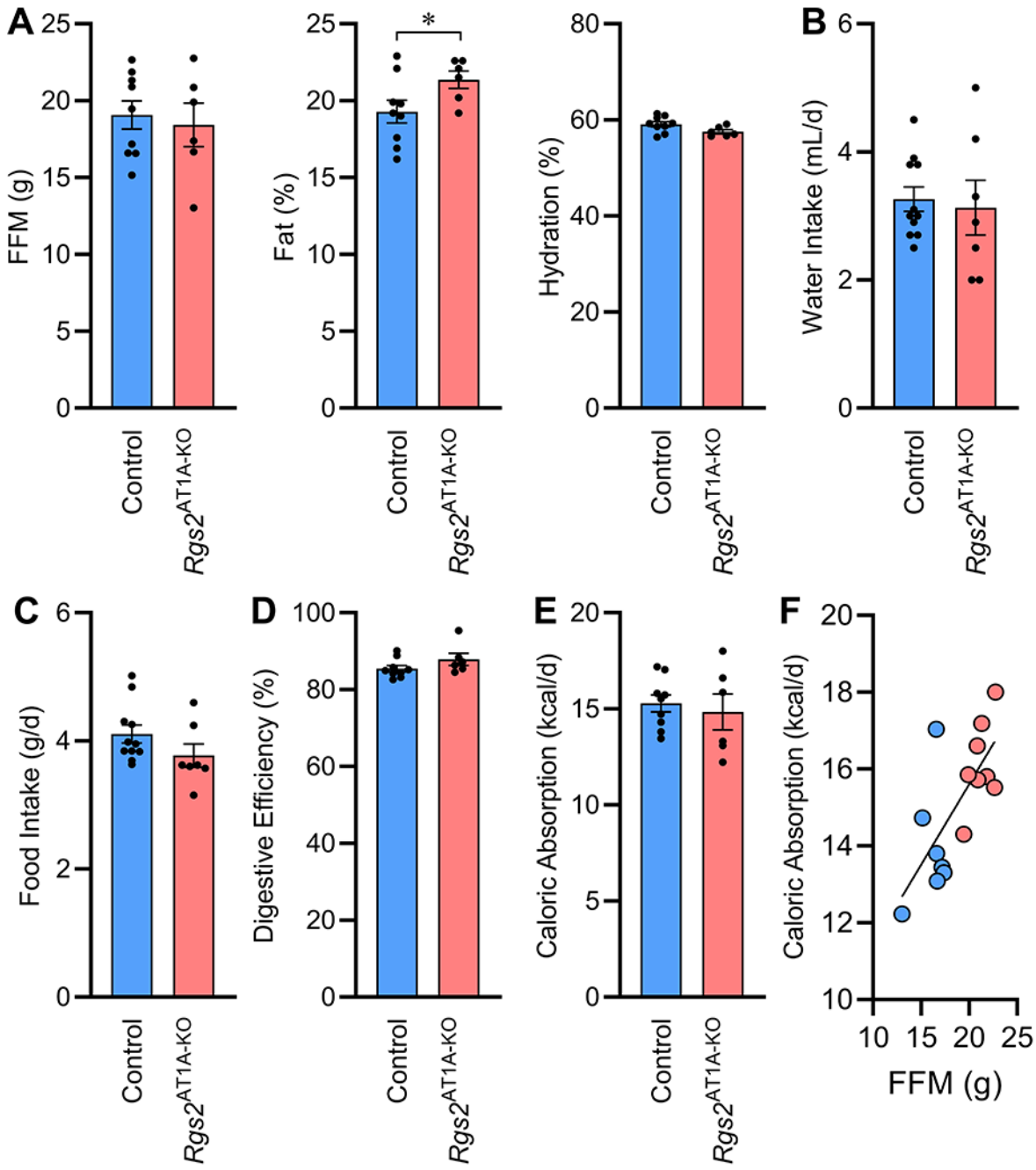

Figure 3. Ingestive behaviors in the *Rgs2*$^{AT1A-KO}$ model.

(A) Body composition. (B) Daily water intake. (C) Daily food intake. (D) Digestive efficiency, determined by bomb calorimetry. (E) Total daily caloric absorption. (F) Caloric absorption correlates with FFM. For all panels, Control n=4 male + 5 female; *Rgs2*$^{AT1A-KO}$ n=3 male + 3-4 female. All summary data presented as mean±SE. *p<0.05 by two-tailed t-test.

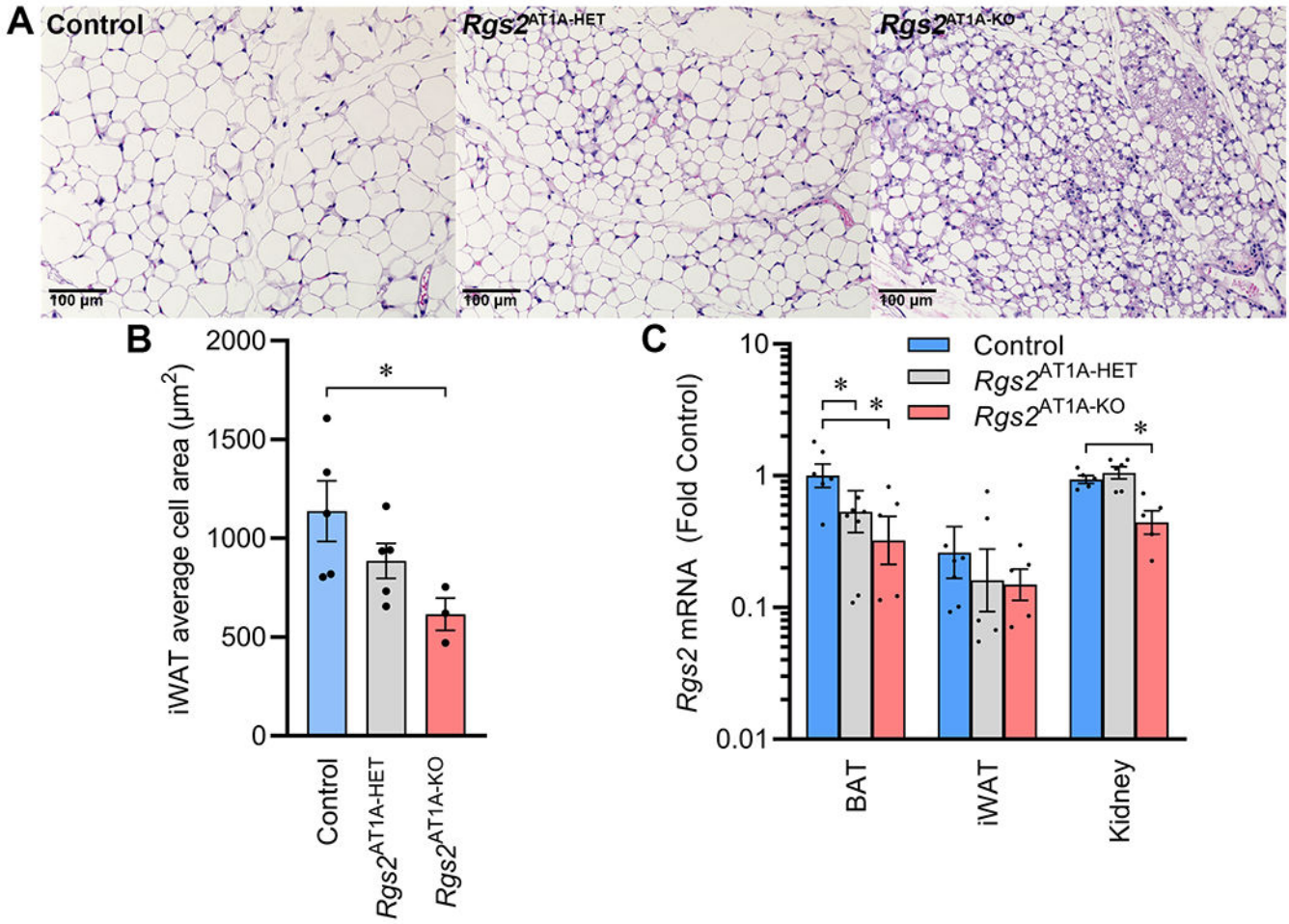

Figure 4. Inguinal adipocyte cell size and expression of *Rgs2* in selected tissues from male *Rgs2*$^{AT1A-KO}$ mice.

(A) Representative example iWAT tissue samples, stained with hematoxylin and eosin, from male control, *Rgs2*$^{AT1A-HET}$, and *Rgs2*$^{AT1A-KO}$ mice at 18 weeks of age, after 10 weeks of HFD feeding. (B) Average iWAT cell area; control (n=5), *Rgs2*$^{AT1A-HET}$ (n=5), and *Rgs2*$^{AT1A-KO}$ (n=3) mice. Each replicate represents the average of n=100 cells sampled from an individual animal. *p<0.05 for indicated comparison by Sidak multiple comparisons procedure. (C) Transcript levels for *Rgs2* mRNA in selected tissues, relative to expression in interscapular BAT from control littermate mice. One-way ANOVA indicates a significant effect of genotype for BAT (n=6, 6, 5; p=0.033) and kidney (n=5, 6, 5; p=0.001), but not inguinal WAT (iWAT, n=6, 5, 5; p=0.960). All summary data presented as mean±SE. *p<0.05 for indicated comparison by Sidak multiple comparisons procedure.

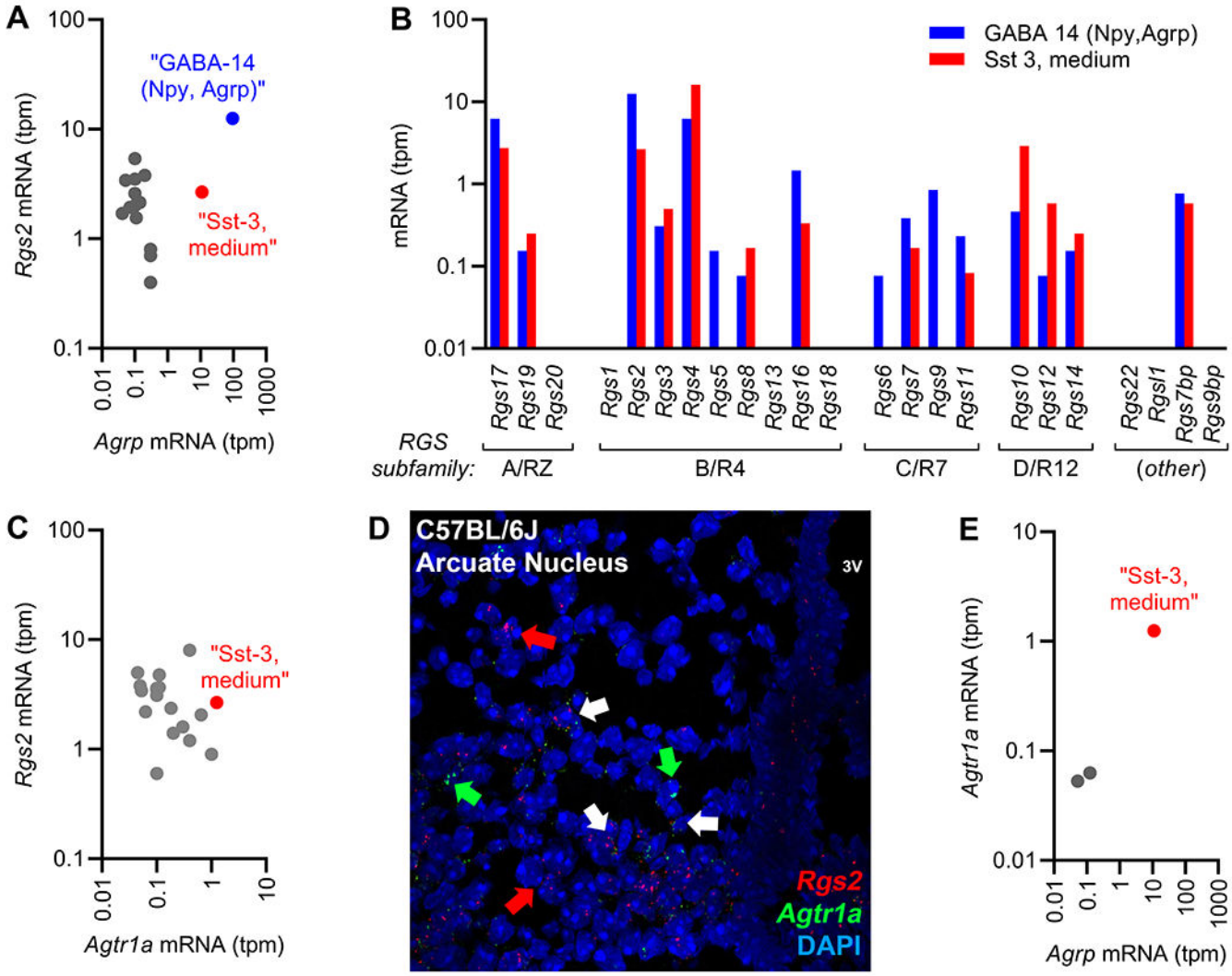

Figure 5. Cellular localization of *Agtr1a* and *Rgs2* expression within the ARC of the hypothalamus.

(A-C, E) In silico re-analyses of published single-cell RNAseq dataset describing the ARC of the mouse hypothalamus (GSE74672). For panels A, C, and E, dots represent average gene expression within distinct cell-type clusters identified by the original authors. (A) Correlation of *Rgs2* and *Agrp* mRNA transcripts within distinct cell types of the ARC. Two cell clusters ("GABA-14 (Npy, Agrp)" and "Sst-3, medium") express high levels of *Agrp*, and both express *Rgs2*. (B) Expression of RGS family members within the two dominant *Agrp*-expressing clusters. (C) Correlation of *Rgs2* expression with *Agtr1a* across cell type clusters. (D) Representative in situ hybridization (RNAscope) localizing *Rgs2* and *Agtr1a* mRNA transcripts within the hypothalamic ARC of an adult C57BL/6J mouse. Green arrows identify cells that only express *Agtr1a*; red arrows identify cells that only express *Rgs2*, and white arrows identify cells that co-express *Agtr1a* and *Rgs2*. (E) Correlation of *Agtr1a* expression with Agrp across cell type clusters.