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Inactivation of the mouse *Magel2* gene results in growth abnormalities similar to Prader-Willi syndrome

Jocelyn M. Bischof[1], Colin L. Stewart[2] and Rachel Wevrick[1,*]

[1]Department of Medical Genetics, University of Alberta, 8–16 Medical Sciences Building, Edmonton, AB, Canada T6G 2H7 and [2]Cancer and Developmental Biology Laboratory, National Cancer Institute, Frederick, MD 21702, USA

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Prader-Willi syndrome (PWS) is an imprinted genetic obesity disorder characterized by abnormalities of growth and metabolism. Multiple mouse models with deficiency of one or more PWS candidate genes have partially correlated individual genes with aspects of the PWS phenotype, although the genetic origin of defects in growth and metabolism has not been elucidated. Gene-targeted mutation of the PWS candidate gene *Magel2* in mice causes altered circadian rhythm output and reduced motor activity. We now report that *Magel2*-null mice exhibit neonatal growth retardation, excessive weight gain after weaning, and increased adiposity with altered metabolism in adulthood, recapitulating fundamental aspects of the PWS phenotype. *Magel2*-null mice provide an important opportunity to examine the physiological basis for PWS neonatal failure to thrive and post-weaning weight gain and for the relationships among circadian rhythm, feeding behavior, and metabolism.

INTRODUCTION

Prader-Willi syndrome (PWS) is a contiguous gene deletion disorder caused by loss of imprinted genes on chromosome 15q11–q13. Clinical findings include neonatal hypotonia and failure to thrive, low metabolic rate, disordered sleep, growth hormone deficiency, childhood-onset severe obesity, hypogonadotrophic hypogonadism and developmental delay, all strongly suggestive of hypothalamic dysfunction. Mouse strains with deficiencies of one or more of the PWS genes have been constructed, but do not recapitulate the PWS obesity phenotype (1–11). MAGEL2 and necdin are related proteins that are both inactivated in PWS. Necdin and a third related protein, MAGED1, have roles in cell survival, neurotrophin signaling, cytoskeletal rearrangement in neurons, and neurite outgrowth (6,7,12–17). The murine *MAGEL2* ortholog, *Magel2*, is highly expressed in a circadian fashion in the suprachiasmatic nucleus of the hypothalamus (18). Mice with a targeted deletion of *Magel2* have a circadian rhythm defects; while they do entrain to light cycles, they have reduced total activity and increased daytime activity when released into constant darkness (18). *Magel2*-null mice also have significantly reduced levels of the neuropeptide hormones orexinA/B and fewer neurons expressing orexin in the lateral hypothalamus (18). This suggests that some of the consequences of *Magel2* loss may be mediated through altered levels of orexins, which are implicated in the regulation of sleep and of food intake. *Magel2* thus appears to be important for translating the endogenous central circadian rhythm into behavioral output. The role of the hypothalamus in the coordinated regulation of appetite and body weight prompted us to examine whether *Magel2* is also required for additional hypothalamic functions that are relevant to abnormal growth and metabolism in PWS.

RESULTS

Inactivation of *Magel2* was achieved using gene-targeting to replace the open reading frame with an in frame LacZ knock-in expression cassette (18), then backcrossing to C57Bl/6. We then bred C57Bl/6 female mice to *Magel2* − / + male mice carrying a maternally inherited *Magel2-lacZ* knock-in allele, to generate *Magel2* + /− mice carrying a paternally inherited *lacZ* knock-in allele (hereafter referred to as *Magel2*-null) and Magel2 + /+ (wild-type control littermate) offspring. Expression of *Magel2* is ablated in all tissues of the *Magel2*-null mice because of deletion of the paternal

*To whom correspondence should be addressed. Tel: +1 7804927908; Fax: +1 7804921998; Email: rachel.wevrick@ualberta.ca

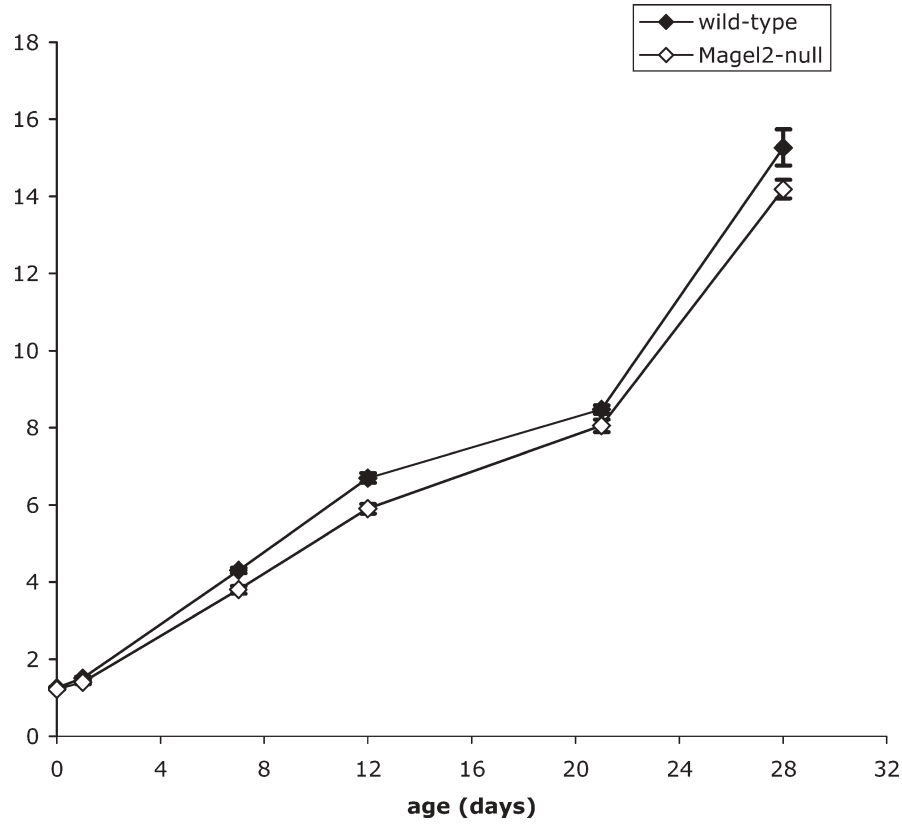

Figure 1. Mean weight ± SEM (standard error of the mean) of *Magel2*-null mice and control littermates pre-weaning ($n = 12–15$ mice of each genotype in each age category). Both sexes were included for time points up to P12. Only female mice are included at P21 and P28, but the mean weight of *Magel2*-null male mice was similarly reduced compared to wild-type ($n = 15–25$ mice of each genotype in each age category).

allele of *Magel2* and imprinting that silences the maternal allele. Consistent with our previous observations (18), we noted that *Magel2*-null mice are under-represented among embryos and pups genotyped from 42 litters (102 *Magel2*-null versus 145 wild-type, $P = 0.009$). Reduced viability was evident by embryonic day 12.5 (E12.5), both male and female *Magel2*-null pups are under-represented, and no additional lethality was evident after mid-gestation. There was no evidence of malformed or reabsorbed embryos, suggesting an early embryonic lethal event in a subset of *Magel2*-null embryos. In human pregnancies, increased early miscarriages of congenitally *MAGEL2*-null PWS fetuses would not be detected because almost all cases of PWS are sporadic and not identified until birth.

Mice with mutations that cause a loss of expression of all PWS-equivalent genes (2,5,10), and mice deficient for necdin alone (14), are 15–20% underweight at birth. In contrast, there was no difference in the weight of *Magel2*-null versus wild-type embryos at E18.5 or postnatal day 1 (P1) (Fig. 1). However, growth retardation was evident soon after birth. *Magel2*-null pups were significantly lighter than wild-type littermates from P7 until weaning between P21 and P28 ($P < 0.0001$ by two-way analysis of variance (ANOVA) with repeated measures, both sexes). By 5–6 weeks of age, catch-up growth in the *Magel2*-null mice equalized their mean weight to wild-type levels for both sexes. Subsequently, *Magel2*-null mice gained significantly more weight than their wild-type littermates from 5 to 12 weeks of age ($P < 0.0001$ for females and $P < 0.01$ for males by repeated-measures ANOVA) (Fig. 2A). Increased weight gain was most pronounced between 4 and 6 weeks of age, and the weight difference became less apparent in older adults. We noted that ~30% of older *Magel2*-null mice developed weight loss and scruffiness, confounding weight measurements after 20 weeks of age. The crown-to-rump lengths of the two genotypes of mice were comparable at all time points. These results identify reduced weight gain in *Magel2*-null pups that reverses after weaning to produce adult *Magel2*-null mice that are overweight compared to their littermates.

Children and adults with PWS have altered body composition, with increased fat mass and decreased lean mass (19,20). To determine whether abnormal weight gain in *Magel2*-null mice is accompanied by altered body composition or metabolic imbalance, we performed additional measurements on male mice at 16 weeks of age. The percentage lean mass was reduced (mean standard deviation score (SDS) −3.2 (range −0.5 to −6.6)) and the percentage fat mass was increased (mean SDS 2.8 (range 0–5.8)) as measured by quantitative nuclear magnetic resonance body composition analysis (21) (Table 1). Plasma leptin was elevated in the *Magel2*-null mice in proportion to their increased adiposity. On necropsy, the weights of the internal organs (brain, heart, liver and kidneys) were not different between genotypes, but the epididymal and retroperitoneal fat pads

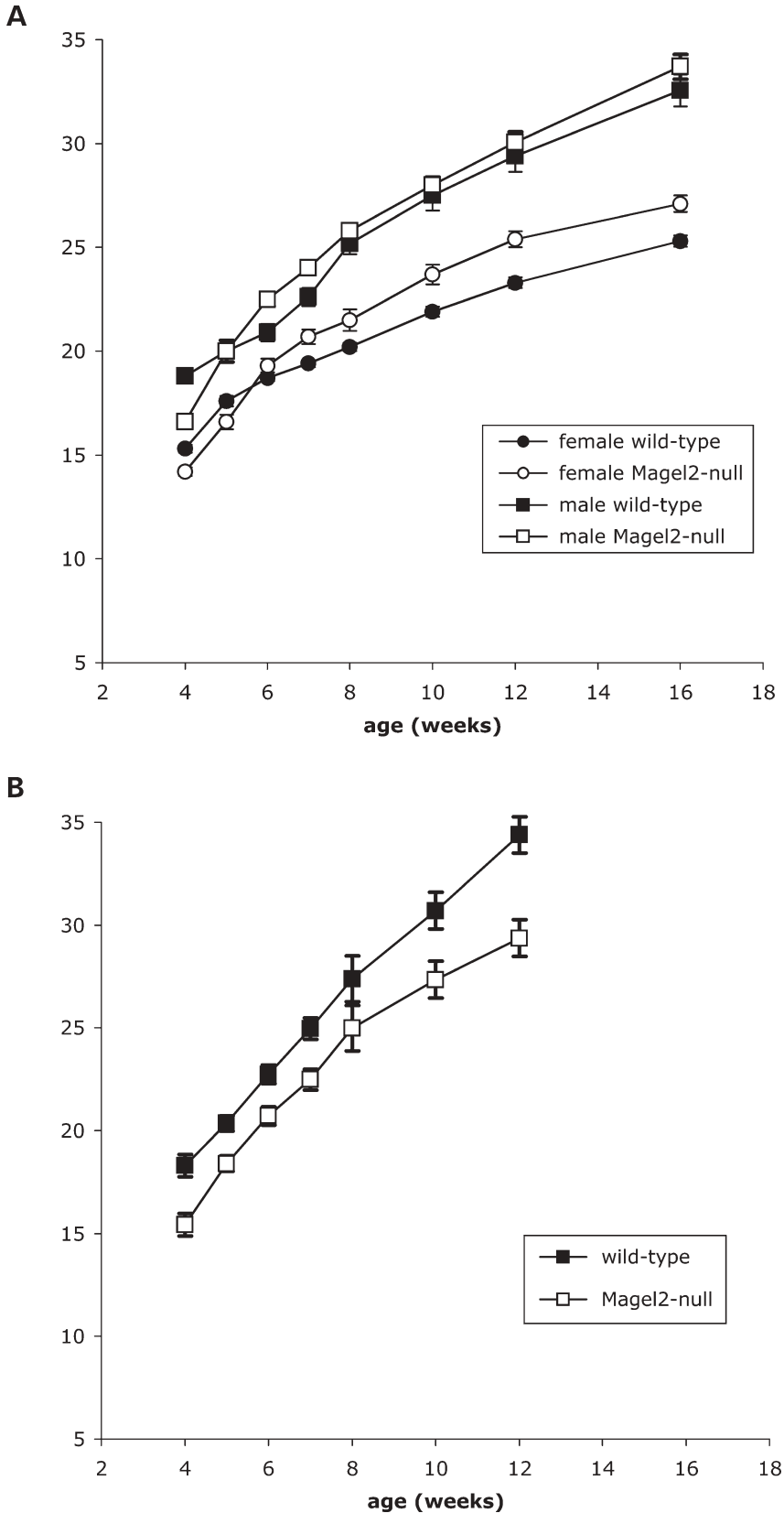

Figure 2. (A) Mean weight ± SEM of *Magel2*-null mice and control littermates fed a standard chow diet from weaning to 16 weeks of age. (B) Mean weight ± SEM of *Magel2*-null male mice and control littermates fed a high-fat diet from weaning to 12 weeks of age ($n = 8–12$ mice of each genotype in each age category).

Table 1. Metabolic indices of 16 week-old wild-type ($n = 8$) and *Magel2*-null ($n = 7$) male mice

Measurement	Wild-type (mean ± SEM)	*Magel2*-null (mean ± SEM)	
Body weight (g)	31 ± 1	33 ± 1	n.s.
Body fat (% of total mass)	9 ± 1	18 ± 2	$P < 0.001$
Lean mass (% of total mass)	83 ± 1	75 ± 2	$P < 0.001$
Fat pad mass (g)	1.6 ± 0.2	3.7 ± 0.4	$P < 0.005$
Daily food consumption (g)	4.59 ± 0.05	4.05 ± 0.08	$P < 0.002$
Triglycerides (mg/dl)	55 ± 3	45 ± 3	n.s.
Phospholipids (mg/dl)	172 ± 8	178 ± 6	n.s.
Cholesterol (mg/dl)	138 ± 5	161 ± 8	$P < 0.05$
Nonesterified fatty acids (mg/dl)	0.62 ± 0.02	0.68 ± 0.02	n.s.
Glucose (mg/dl)	171 ± 6	185 ± 4	n.s.
Insulin (pM)	115 ± 19	162 ± 22	$P < 0.05$
Leptin (pM)	131 ± 33	296 ± 73	$P < 0.05$

n.s., not significant; *P*-values calculated by one-way ANOVA.

were over twice as heavy in the mutant mice (Table 1). Two other indices of altered metabolism, increased fasting insulin and elevated cholesterol, were observed in the *Magel2*-null mice, while fasting glucose, triglycerides, phospholipids and non-esterified fatty acids were not significantly different between genotypes (Table 1).

Some strains of mice, including C57Bl6/J, are susceptible to diet-induced obesity when fed a high-fat diet (22). To determine whether high-fat feeding exacerbated weight gain in *Magel2*-null mice, we fed male mice a high-fat diet from weaning until 12 weeks of age. Wild-type mice responded to the high-fat diet with increased weight gain compared to the standard chow-fed wild-type mice (Fig. 2B compared to male mice in Fig. 2A). Surprisingly, weight gain in *Magel2*-null mice fed with a high-fat diet attenuated compared to wild-type. The mutant mice never attained comparable body weights to their high-fat diet-fed wild type littermates, but instead closely followed the weights of the *Magel2*-null mice fed a standard diet. We then fed adult male mice a high fat diet for 5 weeks, and measured baseline fasting blood glucose levels. As expected, the wild-type mice developed hyperglycemia, with a mean fasting blood glucose of 220 ± 45 mg/dl, a mean increase of 15 mg/dl over their pre-diet fasting blood glucose. In contrast, the mean fasting blood glucose of *Magel2*-null mice fell from a hyperglycemic state of 231 ± 29 mg/dl to a mean of 171 ± 21 mg/dl at the end of the high fat diet period.

Magel2-null mice have ~10% reduced food intake compared to wild-type littermates, but are also less active (Table 1, (18)). They also have a reduced number of orexin-producing neurons (18). Orexins are a vital link between arousal from sleep and the regulation of energy metabolism, and orexin-null mice are hypophagic (23,24). To determine whether the circadian timing of food intake is perturbed by the loss of *Magel2*, we measured food consumption hourly for 3 days, following a 2-day habituation period. The pattern of food intake in both genotypes of mice is clearly circadian, consistent with our previously reported activity patterns in normal lighting (Fig. 3). However, *Magel2*-null mice consistently fed less in the latter half of the dark cycle, and had abnormally early feeding in anticipation of the dark cycle compared to wild-type ($P < 0.03$ by repeated measures ANOVA), and reduced total food intake (Table 1, Fig. 3). Overall, our results indicate that loss of *Magel2* causes increased susceptibility to obesity with increased abdominal fat deposition, reduced lean muscle mass, altered insulin homeostasis and abnormalities in the circadian pattern of feeding behavior.

DISCUSSION

The discovery that loss of the *Magel2* gene in mice causes altered activity patterns consistent with a defect in circadian rhythm output prompted us to investigate weight gain and metabolism in *Magel2*-null mice. *Magel2*-null mice have reduced embryonic viability but otherwise normal embryonic growth in survivors, followed by post-natal growth retardation. In contrast, mice mutant for *Clock*, a core component of the circadian oscillator in the suprachiasmatic nucleus of the hypothalamus, have normal pre-weaning growth, suggesting that loss of *Magel2* impairs neonatal growth independent of its effect on circadian rhythm. After weaning, weight gain with increased fat deposition and altered metabolism are apparent. Circadian feeding patterns are also altered, with a normal period length but with lights out feeding onset and lights on feeding offset both 1 h early compared to wild-type. By comparison, feeding problems and poor weight gain in infancy with excessive weight gain after 1 year of age are found in over 93% of PWS infants (25). Circadian dysfunction *per se* is not included in the diagnostic criteria for PWS; sleep disturbance is one of the minor criteria, and excessive daytime sleepiness is a common finding in PWS (26), and disruption of the orexin system has previously been postulated to contribute to these findings (27). Our findings of reduced orexin and advanced phasing of feeding behavior in *Magel2*-null mice strengthened the hypothesis that MAGEL2 deficiency is responsible for sleep anomalies in PWS. Furthermore, *Magel2*-null mice display abnormalities of body composition that closely recapitulate the reduced lean mass and almost two-fold increased adiposity in PWS, and strengthen our hypothesis that multiple hypothalamic functions are perturbed by loss of *Magel2*.

The complex phenotype of PWS is likely due to the additive effect of the loss of multiple paternally expressed, imprinted genes, but the relationships between the loss of individual PWS genes and aspects of the PWS phenotype are poorly defined. Mouse strains carrying a loss of paternally-derived expression of all PWS genes (2–5) gain weight poorly and typically succumb to neonatal lethality within a week. Deficiency of the PWS candidate gene necdin is associated with a strain-dependent neonatal lethality that causes a congenital deficiency of the central respiratory drive (14,28). *Necdin*-null mice that survive into adulthood are not obese (7,9,29), and have altered pain sensitivity (7,30). Excess weight gain and increased fat deposition in *Magel2*-null mice may become apparent because they do not suffer from reduced birth weight and severe neonatal growth retardation that occurs in mice with combined deficiency of *Magel2* and other PWS genes.

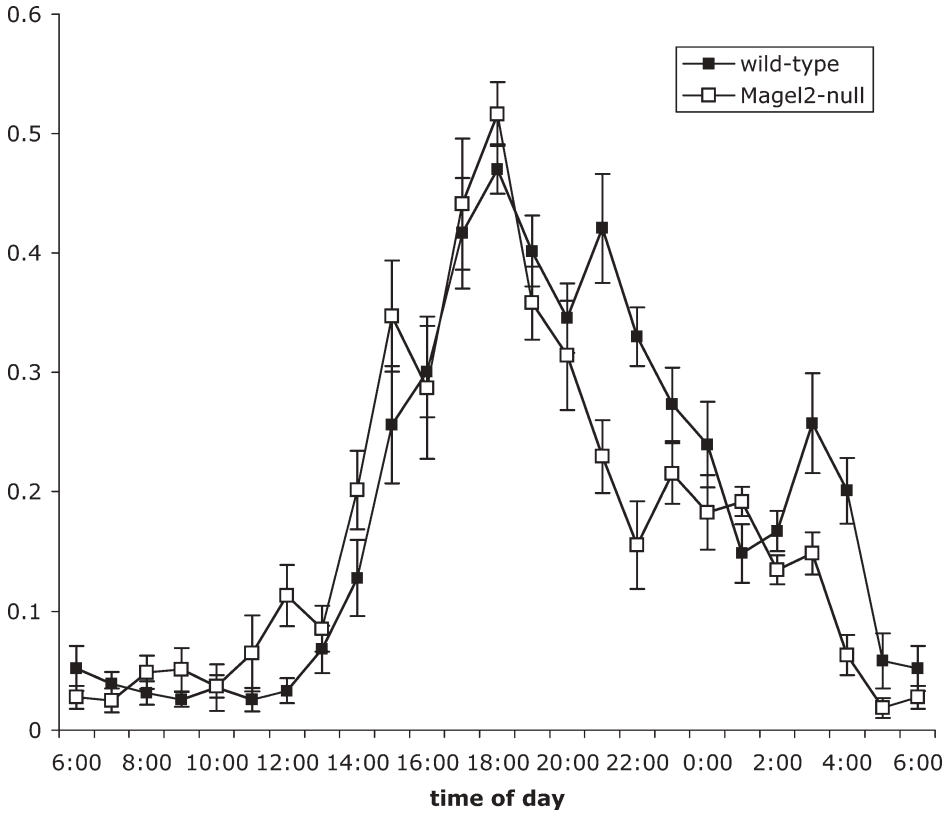

Figure 3. Mean hourly food intake ± SEM of *Magel2*-null male 16 week-old mice ($n = 8$) and control littermates ($n = 7$) averaged over 3 days. The dark period is from 18:00 p.m. to 6:00 a.m.

In the absence of intervention, people with PWS become morbidly obese. In contrast, mice deficient for *Magel2* have twice the adiposity of their wild-type littermates, a difference that is comparable to the increased adiposity found in diet-induced obesity models. However, *Magel2*-null are not morbidly obese to the extent seen in monogenic forms of murine obesity such as mice with mutations in the leptin pathway. Moreover, loss of *Magel2* causes hypophagia in mice, in contrast to the extreme hyperphagia that is a hallmark of untreated PWS. Children with PWS are typically relatively growth hormone deficient, and have delayed bone maturation with short stature, low insulin and IGF-1 levels, and reduced lean body mass. It is possible that the timing of growth hormone release is perturbed by the altered circadian rhythm and could contribute to the small but significant reduction in lean mass. However, the normal linear growth and high insulin levels argue against a significant growth hormone deficiency as being central to altered body composition in *Magel2*-null mice. The relative resistance of the *Magel2*-null mice to the effects of diet-induced obesity (weight gain, increased blood glucose, impaired glucose tolerance) seems contradictory to the increased fat mass found in these mice. However, a similar profile of altered glucose metabolism and diabetes resistance has been observed in the PWS population, with obese PWS subjects tending toward resistance to type II diabetes and relatively normal insulin sensitivity (31–33), and it has been suggested that reduced visceral fat could partially account for this paradox (34,35). The *Magel2*-null mice present an opportunity for further investigation of the metabolic state induced by Magel2-deficiency in the context of PWS. With respect to the lack of hyperphagia in the mutant mice, we speculate that concurrent loss of contiguous genes could provoke hyperphagia in humans but not in mice, or that breeding onto different mouse strain backgrounds could exacerbate deficits in the various mouse models of PWS.

Our results strongly suggest that *Magel2* is important to the coordination of circadian rhythms with the regulation of food intake and metabolism, just as the genetic disruption of master regulators of circadian rhythm can lead to obesity (36,37). Further studies are necessary to determine whether strategies that reinforce the circadian rhythm, such as sleep aids or phototherapy, could be useful adjuvant therapy in PWS. Although hypotonia is proposed to be a major component of failure to thrive in PWS infants, other nutritional or metabolic deficits could also be present. In conclusion, our findings unite aspects of the PWS phenotype that were not previously related to loss of a single gene, and support the hypothesis that hypothalamic dysfunction from loss of *MAGEL2* underlies many of the major features of this multi-faceted disorder.

MATERIALS AND METHODS

Mouse breeding and handling

Animal procedures were approved by the University of Alberta Animal Policy and Welfare Committee. The *Magel2*-null mice

were originally on a mixed genetic background (W9.5 (129S1) / C57Bl/6) (18), and subsequently, back-crossed to C57Bl/6 for at least 14 generations. The *Magel2* mouse colony was maintained by breeding Magel2 − /+ female mice carrying a maternally inherited *Magel2-lacZ* knock-in allele with C57Bl/6 male mice to generate heterozygous, functionally wild-type offspring. C57Bl/6 female mice were then bred to Magel2 − /+ male mice carrying a maternally inherited *Magel2-lacZ* knock-in allele, to generate Magel2 + /− mice carrying a paternally inherited *lacZ* knock-in allele (hereafter referred to as *Magel2*-null) and Magel2 + /+ (control littermate) offspring. Because of imprinting that silences the maternally inherited allele, *Magel2*-null mice with a paternally inherited *lacZ* knock-in allele retain expression only of this mutant allele and have no expression of *Magel2*. Mice were genotyped from tissue samples or ear notch biopsies as described in reference (28). Mice were weaned between 3 and 4 weeks of age then housed 2–3 per cage with food (PicoLab Mouse Diet 20, LabDiet) and water *ad libitum*, and maintained under 12 : 12 light dark conditions. One set of mice was fed a high fat diet (Basal purified diet w/60% energy from fat, LabDiet) for 8 weeks after weaning. A second set of male mice was fed the high fat diet at 10 months of age. Male mice used in food consumption and metabolic analyses were singly housed.

Metabolic analyses

Measurements of body composition by NMR, serum leptin, and fasting glucose, insulin, triglycerides and cholesterol, and hourly food intake were performed at the University of Cincinnati Mouse Metabolic Phenotyping Center. SDS were calculated as the difference between the measurement and the wild-type mean, divided by the wild-type standard deviation. Statistical analyses of differences between genotypes were performed using the repeated measures two-way ANOVA function of the GraphPad Prism 4 software package. Differences with $P < 0.05$ were considered significant.

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Conflict of Interest statement. The authors declare no conflict of interest related to this study.

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