The imprinted gene *Magel2* regulates normal circadian output

Kozlov et al Supplementary Fig 1

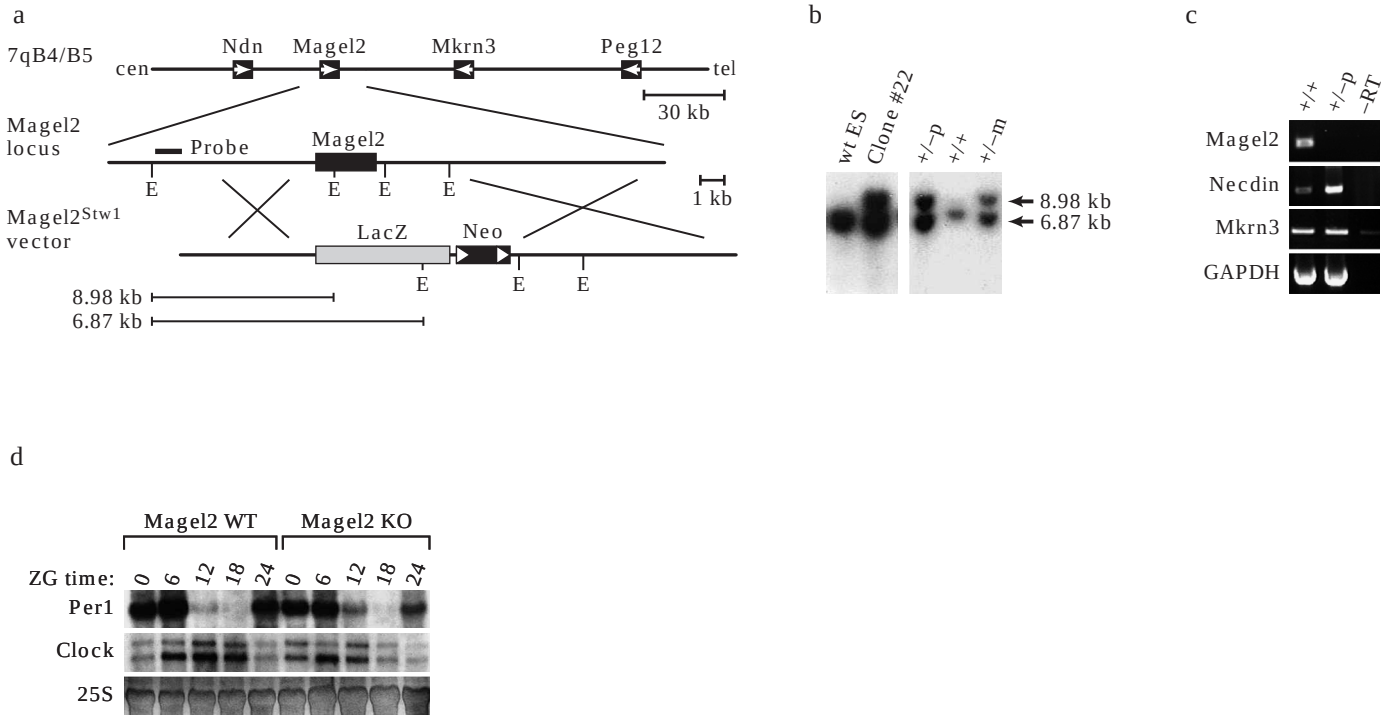
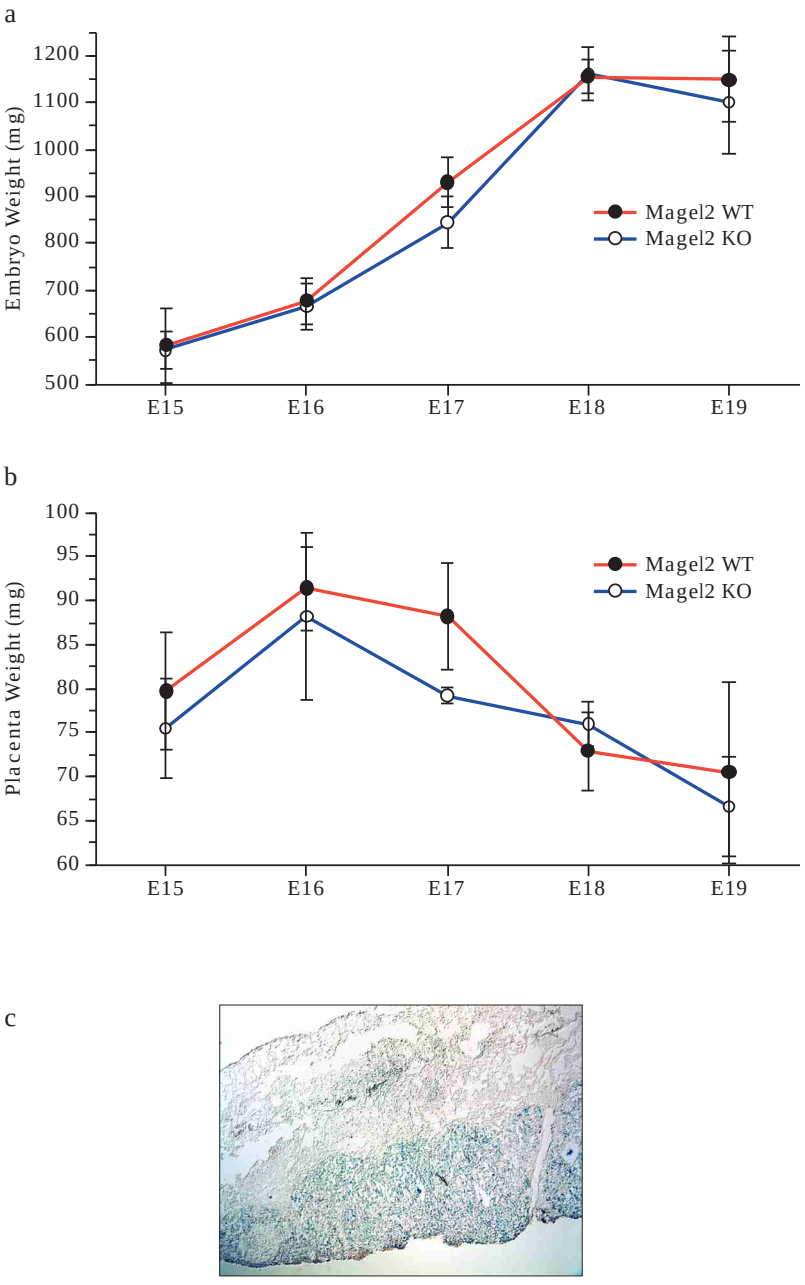


Fig. 1 **(a)** Inactivation and derivation of a Lac-Z reporter allele of *Magel2*. A. Outline of the mouse locus 7qB4/B5 (syntenic with the Prader-Willi region at chromosome position 15q11-q13 in humans) encompassing the cluster of paternally expressed imprinted genes Necdin (*Ndn*), *Magel2*, *Mkrn3*, and Paternally Expressed Gene 12 (*Peg12*). The LacZ knock-in (KI) targeting vector contained a *Neo* minigene flanked by loxP sites (white arrows). Positions of EcoRI restriction sites (E), diagnostic DNA fragments for Southern blot analysis, and restriction length fragment polymorphism resulting from homologous recombination are indicated. (**b**). Southern blot analysis on EcoRI-digested genomic DNAs isolated from wild-type ES cells, ES clone #22 was used to generate the *Magel2* deficient mouse line, and tail biopsies from either wild-type (+/+) animal, or heterozygous mice by either paternal (pat) or maternal (mat) transmission of the mutant allele. (**c**). RT-PCR analysis of tota RNA isolated from adult hypothalamuses of either wild-type (+/+) or paternal heterozygous (+/-(pat)) animals with primer pairs specific for *Magel2*, *Necdin*, *Mkrn3*, or *Gapdh* (used as control) cDNAs. RT- denotes samples with omitted reverse transcription step. Loss of *Magel2* results in an increase in *Ndn* expression. (**d)** Circadian expression of *Per1* and *Clock* genes in liver samples isolated at different Zeitgeber (ZT) times from wild-type and *Magel2*$^{m+/p-}$ mice. No major difference is detected in the rhythmicity of *Per1* and *Clock* transcripts between *Magel2*$^{m+/p-}$ and wt-control mice.

Kozlov et al Supplementary Fig 2



Figs 2 a and b Fetal and placental growth rates are unaffected by loss of *Magel2*.

c. *Magel2* transcripts are expressed in the spongiotrophblast of an E16 placenta

Kozlov et al Supplementary Fig 3

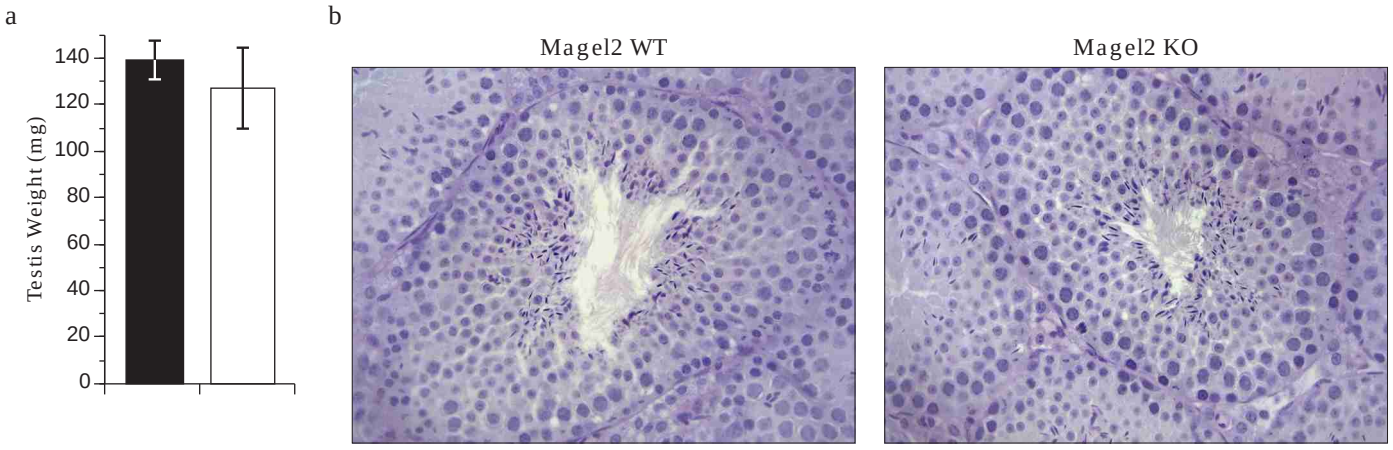


Fig 3 (a) Testis weights in from *Magel2*$^{m+/p-}$ males a slight decline in mean weight, **(b)** although spermatogenesis is overtly normal

KOZLOV ET AL SUPPLEMENTARY TABLES

Kozlov Supp Table 1.

Nos of Offspring produced by *Magel2*$^{m+/p-}$ males mated to +/+ females revealing an ~10% reduction in the numbers of expected *Magel2*$^{m+/p-}$ offspring

Number of Litters	Total number of pups	Expected Number of +/-pat	Observed Number of +/-pat	Standard Deviation	z-value	p-value
56	402	201	178	3.742	6.147	<0.001

Kozlov Supp Table 2. *Magel2* deficiency results in male specific decline in fertility

Breeding efficiencies of *Magel2*$^{m+/p-}$ males

	Plug Frequency	Average litter size	% of heterozygotes
8 week-old males (n = 8)			
Magel2$^{m+/p-}$	0.33	8.7 ± 2.1 (n = 13)	46.7 ± 24.4 (n = 13)
Magel2$^{m+/p+}$	0.40	8.2 ± 2.3 (n = 15)	N/A
16 week-old males (n = 8)			
Magel2$^{m+/p-}$	0.06	8.5 ± 0.7 (n = 2)	46.5 ± 12.8 (n = 2)
Magel2$^{m+/p+}$	0.43	8.0 ± 3.7 (n = 17)	N/A

Breeding efficiencies of *Magel2*$^{m+/p-}$ females

Nos Females	Nos Litters		Litter Size		Age of last litter (months)	
	Average	Range	Average	Range	Average	Range
N = 7	6.42	3-11	7.92	2-14	9.86	6-15

Reproductive capacity of *Magel2*$^{m+/p-}$ male mice rapidly declines with age. Breeding capability was assessed at 8 and 16 weeks of age by average plug frequency (plugs per mouse per day over the 14-day observation period), average litter size, and genotype distribution in litters sired. In contrast the reproduction of *Magel2*$^{m+/p-}$ females (n=7) revealed by the number and size of litters

produced over an 9-15 month lifespan, as well as the age at time when the last litter was unaffected by loss of *Magel2* expression.

Kozlov Supp Table 3. The numbers of Orexin expressing neurons are decreased in *Magel2*$^{m+/p-}$ mice. However orexin mRNA levels are unaffected

Parameter	*Magel2*$^{m+/p+}$	*Magel2*$^{m+/p-}$	Wt/ *Magel2*$^{m+/p-}$
(1)			
Orexin B +ve neurons	53 ± 11	33±9 ($p<0.01$)	8/12
(2)			
Prepro-orexin RNA (a.u.)	1.00 ± 1.04	0.98 ± 1.09	4/4
Orexin R1 RNA (a.u.)	1.00 ± 1.55	1.43 ± 1.29	4/4
Orexin R2 RNA (a.u.)	1.00 ± 1.33	0.46 ± 1.22	4/4

(1). Statistical analysis the numbers of orexin B positive neurons and the levels of orexin A/B peptides in brain samples from the *Magel2*$^{m+/p-}$ animals compared to their wild-type littermates. At least eight sections obtained from three individual mice were scored for each genotype to determine the average number of orexin expressing neurons. Four samples per genotype were analyzed by enzymimmunoassay to determine the relative amounts of orexin A and orexin B neuropeptides per mg of total extracted brain tissue protein.

(2) Prepro-orexin and orexin A and B mRNA levels were quantified by Real-time PCR and did not show any statistically significant differences between *Magel2*$^{m+/p-}$ and wt mice.

Kozlov Supp Table 4.

Name	Sequence	Purpose
LacZ1924.for	GTCTCGTTGCTGCATAAACC	Genotyping for Magel2 KO allele
LacZ2406.rev	TCGTCTGCTCATCCATGACC	Genotyping for Magel2 KO allele
Nlg2F	CAGTCCCCATCCTCACTAATAGA	Genotyping for Magel2 WT allele
Nlg9R	TCTCCAGACAGTATTTTACCGATG	Genotyping for Magel2 WT allele
NdnRT.for	CATCCTCGCCAGAGTGTTCG	RT-PCR for Necdin mRNA
NdnRT.rev	GGCCTGGGCAGCAAGATTAG	RT-PCR for Necdin mRNA
Mkrn3RT.for	TGTTCCTGGACAGCCTTACC	RT-PCR for Mkrn3 mRNA
Mkrn3RT.for	ACTTCTCCACCTGCGGATAC	RT-PCR for Mkrn3 mRNA

Primer sequences for genotyping RT-PCR