# Sex-specific regulation of weight and puberty by the *Lin28/let-7* axis

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## Abstract

Growth and pubertal timing differ in boys and girls. Variants in/near *LIN28B* associate with age at menarche (AAM) in genome-wide association studies and some AAM-related variants associate with growth in a sex-specific manner. Sex-specific growth patterns in response to *Lin28b* perturbation have been detected in mice, and overexpression of *Lin28a* has been shown to alter pubertal timing in female mice. To investigate further how *Lin28a* and *Lin28b* affect growth and puberty in both males and females, we evaluated *Lin28b* loss-of-function (LOF) mice and *Lin28a* gain-of-function (GOF) mice. Because both *Lin28a* and *Lin28b* can act via the conserved microRNA *let-7*, we also examined *let-7* GOF mice. As reported previously, *Lin28b* LOF led to lighter body weights only in male mice while *Lin28a* GOF yielded heavier mice of both sexes. *Let-7* GOF mice weighed less than controls, and males were more affected than females. Timing of puberty was assessed by vaginal opening (VO) and preputial separation (PS). Male *Lin28b* LOF and male *let-7* GOF, but not female, mice displayed alteration of pubertal timing, with later PS than controls. In contrast, both male and female *Lin28a* GOF mice displayed late onset of puberty. Together, these data point toward a complex system of regulation by *Lin28a*, *Lin28b*, and *let-7*, in which *Lin28b* and *let-7* can impact both puberty and growth in a sex-specific manner, raising the possibility that this pathway may contribute to differential regulation of male and female growth and puberty in humans.

**Key Words**
- puberty
- *Lin28*
- *let-7*
- sex-specific

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## Introduction

Women tend to be shorter than men, and girls have their growth spurt at an earlier phase in puberty than boys (Styne & Grumbach 2012). Girls also experience puberty at younger ages than boys and are more likely to exhibit precocious puberty, while boys are more likely to have delayed puberty (Carel & Leger 2008, Walvoord 2010, Palmert & Dunkel 2012, Styne & Grumbach 2012). The factors that contribute to these differences are unknown but important because variations in the timing of puberty have been associated with higher risk for adult metabolic abnormalities, cancer, and osteoporosis (Weir *et al*. 1998, Golub *et al*. 2008, Lakshman *et al*. 2009,



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Joinson *et al.* 2011, Seselj *et al.* 2012, Widen *et al.* 2012, Ritte *et al.* 2013, Day *et al.* 2015).

Growth and the timing of puberty are highly heritable, and genome wide association studies (GWAS) have identified many loci that modulate both traits. Interestingly, variants in/near *LIN28B* affect both adult height and the age at menarche (AAM; Lettre *et al.* 2008, He *et al.* 2009, Ong *et al.* 2009, Perry *et al.* 2009, Sulem *et al.* 2009, Elks *et al.* 2010, Widen *et al.* 2010, Leinonen *et al.* 2012, Cousminer *et al.* 2013, Perry *et al.* 2014), and two of the puberty-related single nucleotide polymorphism (SNPs) associate with growth and/or adult BMI in a sex-specific manner (Lettre *et al.* 2008, Widen *et al.* 2010, Ong *et al.* 2011, Leinonen *et al.* 2012). That this axis regulates these traits in a sex-specific manner is further supported by the finding that male but not female *Lin28b* loss-of-function (LOF) transgenic mice are lighter than controls (Shinoda *et al.* 2013*b*).

*LIN28B* and its paralogue, *LIN28A*, function as pluripotency factors, and their dysregulation has been implicated in a number of cancers (Yu *et al.* 2007, Peng *et al.* 2010, King *et al.* 2011, Molenaar *et al.* 2012, Sakurai *et al.* 2012, Yuan *et al.* 2012). Both genes encode RNA-binding proteins that inhibit maturation of the evolutionarily conserved *let-7* microRNA (miRNA) family of developmental regulators (Heo *et al.* 2008). The *Lin28a/Lin28b/let-7* pathway is known to regulate the timing of larval development in *Caenorhabditis elegans*, germ layer specification in *Xenopus*, stem cell maintenance, glucose metabolism, and linear growth (Pasquinelli *et al.* 2000, Reinhart *et al.* 2000, Thomson *et al.* 2006, Heo *et al.* 2008, Newman *et al.* 2008, Rybak *et al.* 2008, Viswanathan *et al.* 2008, Melton *et al.* 2010, Zhu *et al.* 2011, Faas *et al.* 2013, Shinoda *et al.* 2013*b*). In rodents, expression patterns of *Lin28a*, *Lin28b*, and *let-7* in the hypothalamus-pituitary-gonad (HPG) tissues have been described by us and others (Zhu *et al.* 2010, Gaytan *et al.* 2013, Grieco *et al.* 2013, Sangiao-Alvarellos *et al.* 2013, Sangiao-Alvarellos *et al.* 2015). All genes are expressed in the hypothalamus, pituitary, and gonad around the age of puberty: while *Lin28b* is expressed at a higher level than *Lin28a* in the hypothalamus and testes, the relationship is reversed in the ovary (Gaytan *et al.* 2013, Grieco *et al.* 2013). In the pituitary *Lin28b* is only slightly more expressed than *Lin28a* (Grieco *et al.* 2013). *Lin28a* and *Lin28b* can downregulate *let-7*, but the expression patterns of *Lin28a* and *Lin28b* compared with *let-7* are not always completely reciprocal (Gaytan *et al.* 2013, Grieco *et al.* 2013, Sangiao-Alvarellos *et al.* 2013, 2015).

How this pathway regulates the timing of puberty is largely unknown. However, data from model systems indicate that the causative gene at the AAM-associated GWAS locus is indeed *LIN28B*. In *C. elegans*, *lin28* LOF as well as *let-7* gain-of-function (GOF) lead to precocious larval development (Ambros & Horvitz 1984, Reinhart *et al.* 2000). Analogously, *Lin28a* GOF female mice display delayed puberty (Zhu *et al.* 2010). This sex-specific regulation of body size by *LIN28B* in humans and mice and the involvement of this pathway in the regulation of pubertal timing lead us to examine whether *Lin28a* and *Lin28b* also regulate puberty in a sex-specific manner and whether *let-7* is mediating such effects. We first utilized *Lin28b* LOF, *Lin28a* GOF, and *let-7* GOF mice to validate the effects of perturbation of *Lin28a*, *Lin28b*, and *let-7* expression on body weight in male and female mice. We then extended our analysis to include investigation of the roles of *Lin28a*, *Lin28b*, and *let-7* in the timing of pubertal onset in male and female mice. Our results suggest that the regulation of these traits by *Lin28a* and *Lin28b* may differ and that some effects of *Lin28b* and *let-7* are sex-specific.

## Materials and methods

### Animals

Studies were approved by the Toronto Centre for Phenogenomics (TCP) Animal Care Committee (AUP 09-08-0097) in accordance with recommendations of the Canadian Council on Animal Care, the requirements under Animals for Research Act, RSO 1980, and the TCP Committee Policies and Guidelines. Mice were maintained under controlled conditions (25 °C and 10 h light:14 h darkness cycle) in sterile, individually ventilated cages, provided irradiated chow and sterile water, and allowed to feed *ad libitum* via an automated watering system.

In *Lin28b* LOF (*Lin28b*$^{-/-}$) mice, exon 2 of *Lin28b* has been excised by targeted mutagenesis resulting in global absence of the Lin28b protein (Shinoda *et al.* 2013*b*). In the *let-7* GOF strain, a M2-rtTA element has been inserted in the *Rosa26* locus and a tetracycline-responsive element, followed by a transgenic *let-7g* sequence, has been inserted in the *Col1A1* locus (Zhu *et al.* 2011, Shinoda *et al.* 2013*b*), leading to global upregulation of *let-7g* expression after administration of doxycycline (Beard *et al.* 2006). Normally, Lin28b binds to the stem loop of the *let-7* pre-miRNA and prevents its processing to mature *let-7* miRNA (Heo *et al.* 2008). To prevent such downregulation, the transgenic *let-7* element has been engineered so that it cannot be bound by Lin28a or Lin28b (Zhu *et al.* 2011). Both strains have been used previously, and alterations in

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expression of *Lin28b* and *let-7g* have been validated (Zhu *et al*. 2011, Shinoda *et al*. 2013*b*). In the *Lin28a* GOF mice, a tetracycline-responsive element, followed by a transgenic *Lin28a* sequence, has been inserted in the *Col1A1* locus. This transgene is 'leaky' and leads to global upregulation of *Lin28a* even in the absence of the M2-rtTA element and doxyclycline administation (Zhu *et al*. 2010). The *Lin28b* LOF and *let-7* GOF strains were created in the Daley laboratory and maintained on a mixed background of C57BL6/J, 129, and Balb/C; *Lin28a* GOF mice were maintained on a C57BL6/J background (Zhu *et al*. 2011, Shinoda *et al*. 2013*b*). For our studies, male mice were imported and mated with superovulated C57BL6/J females for strain rederivation at the TCP Transgenic Core. Embryos were transferred to an in-house C57BL6/J female and offspring were bred to each other to maintain the mixed genetic background. Prior to phenotyping the mice in our facility, LOF was confirmed by the absence of Lin28b protein in *Lin28b* LOF mice in the adult testes and *let-7* GOF was confirmed by upregulated *let-7g* levels in livers of doxycycline-fed *let-7* GOF mice. Successful rederivation of the *Lin28a* GOF mice was verified by recapitulation of the previously observed enlarged body sizes of *Lin28a* GOF mice.

Mice were fed a standard chow (Harlan, Teklad Global 18% Protein Rodent Diet, 2018, Madison, WI) or the equivalent diet containing 625 mg/kg doxycycline (Harlan, TD.01306). For *let-7* GOF experiments, mice were fed the doxycycline chow from birth, which induced transgenic *let-7g* expression in mice carrying the transgene, but not in littermate controls, by feeding the doxycycline chow to the lactating mother (beginning on the date of birth of her litter) and directly to the pups after weaning. Feeding doxycycline to lactating mothers is known to cause upregulation of doxycycline-inducible transgenes in pups (Sun *et al*. 2012). The *Lin28a* transgene is known to be leaky with increased expression that is sufficient to delay puberty in females and increase body weights in both males and females without administration of doxycycline (Zhu *et al*. 2010); thus, these mice were fed standard chow.

In every experiment mice were mated for one week, after which the male was removed. Females were transferred to an individual cage prior to giving birth. Date of birth was monitored daily between 0009 h and noon, and weaning was performed on postnatal day 21 with males and females then housed separately with a target of 3–4 weaned pups per cage. Littermate controls were used for all experiments. Phenotyping was done without knowledge of genotype, between 0009 h and noon every day. The timing of puberty was assessed by monitoring vaginal opening (VO) (Danilovich *et al*. 1999, Seminara *et al*. 2003, Krewson *et al*. 2004, Fernandez-Fernandez *et al*. 2006, Sanchez-Garrido *et al*. 2013) and preputial separation (PS) (Korenbrot *et al*. 1977, Nathan *et al*. 2006, Takashima-Sasaki *et al*. 2006, Deboer & Li 2011, Smith & Spencer 2012, Sanchez-Garrido *et al*. 2013, Novaira *et al*. 2014) every day post-weaning. VO and PS are estradiol- and testosterone-dependent processes respectively, and altered day of VO correlates with other measures of pubertal timing (Chehab *et al*. 1997, Divall *et al*. 2010, Zhu *et al*. 2010, Caron *et al*. 2012, Dicken *et al*. 2012, True *et al*. 2015). Body weights were measured to 0.1 g. Experiments were performed a minimum of two times to ensure that results were reproducible. Mouse genotypes from tail biopsies were determined using real-time PCR with specific probes designed for each gene (Transnetyx, Cordova, TN, USA).

### Tissue dissection

Tissues were harvested following decapitation under isoflurane anesthesia. For hypothalamic tissue, whole brains were dissected and flash frozen in liquid nitrogen. The hypothalamus was then microdissected while frozen, according to established coordinates and landmarks (Baker *et al*. 1983, Grieco *et al*. 2013, Sangiao-Alvarellos *et al*. 2013). All other tissues were kept in RNAlater (Ambion, AM7021, Foster City, CA), at −20 °C until RNA extraction.

### Gene expression

Tissues were homogenized in QIAsol solution (Qiagen, 1023537, Hilden, Germany) with a sonicator using a microtip, and RNA was extracted using the miRNeasy kit (Qiagen, 217004). DNAse treatment was performed during the RNA extraction using RNAse free DNAse (Qiagen, 79254).

RT-reactions were performed using the Taqman microRNA reverse transcription kit (ABI, 4366596, Foster City, CA) with specific RT-primers (ABI, 442795, assay id 377, 2282, and 1231, for *let-7a*, *let-7g*, and *sno142* (housekeeping gene for normalization) respectively). Twenty nanogram total RNA was used in each RT reaction. As a standard, ovarian RNA from several 20-day-old C57BL6/J females was pooled and used in every experiment. Following reverse transcription 60 µl of 10 mM Tris pH 8 was added to each 15 µl sample reaction and 6.65 µl of the diluted sample was added to each Q-PCR reaction. For all Q-PCRs, the PefeCta FastMix II (Quanta, 95120-012,

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Gaithersburg, MD, USA) was used along with the specific TM-primer for the Taqman microRNA assay.

Q-PCR was performed on a MX3 Pro Q-PCR system from Stratagene. Thermal cycling was performed with an initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Every reaction was run in duplicate. Quantification used the standard curve method.

### Dexa

Lean mass (g) and fat mass (g) were measured following euthanasia using peripheral dual-energy X-ray absorptiometry (pDXA, PIXImusII, GE Lunar Corp., Madison, WI, USA) at the Clinical Phenotyping Core at The Toronto Centre for Phenogenomics.

### Intraperitoneal glucose tolerance tests

Intraperitoneal glucose tolerance tests (IP-GTT) were performed after a 6-h fast. A baseline blood sample was obtained from the tail vein and glucose was measured with an Ascencia glucometer (Bayer). A 10% glucose solution (Sigma, G8644) was injected IP at a dose of 1 g glucose per kg bodyweight. Glucose was remeasured at 30, 60, and 120 min after the injection.

### Statistical analysis

Statistical analyses, except linear regressions, were performed using Graphpad (Prism, La Jolla, CA, USA). Although experimental and control groups displayed equal variances, puberty data were not invariably normally distributed; thus PS and VO data were analyzed with Mann–Whitney non-parametric *t*-tests. Body weights displayed normal distribution and were analyzed with the student's *t*-test. Body weights on postnatal day 80 were also analyzed with a two-way ANOVA to test for sex-specificity. Linear regression of weight and age at puberty data was done using glm package in R. The regression coefficients were estimated using the control data and the fit of the mutant data to the wild type regression was estimated with the Kolmogorov–Smirnov test. DEXA and GTT data displayed unequal variances between experimental and control groups, and therefore *t*-tests assuming unequal variances were employed. Gene expression data were analyzed using the student's *t*-test. Results are reported as mean ± S.D., with statistical significance attributed to $P < 0.05$.

## Results

### Growth

Assessing growth patterns is an important component of the assessment of pubertal timing; therefore we began our studies by re-phenotyping growth (Table 1) at our centre.

As demonstrated previously, both male and female mice globally overexpressing *Lin28a* weighed more than littermate controls (Fig. 1A and B) (Zhu *et al*. 2010), while global *Lin28b* LOF males, but not females, weighed significantly less than littermate controls from postnatal day 21 onward (Fig. 1C and D) (Shinoda *et al*. 2013*b*). The sex-specific effect on weight among adult *Lin28b* LOF mice (postnatal day 80) was statistically significant after assessment with two-way ANOVA ($P = 0.04$ after Bonferroni correction).

To begin to examine whether *Lin28a*/*Lin28b* regulate growth phenotypes via *let-7*, we phenotyped the *let-7g* GOF mice (Zhu *et al*. 2011) in which global overexpression was induced by feeding doxycycline chow to the pups from birth. Doxycycline administration was accomplished initially via transfer to the pups through the mother's milk (Sun *et al*. 2012) and subsequently by feeding directly to the pups after weaning. We chose to not induce *let-7* overexpression during embryogenesis because this could be detrimental, as seen in *Lin28a* LOF embryos that overexpress *let-7* and subsequently die as neonatal pups (Shinoda *et al*. 2013*b*). Because Lin28a and Lin28b can inhibit processing of *let-7* precursors into functional miRNA (Heo *et al*. 2008), *let-7* GOF mice were expected to phenocopy the *Lin28b* LOF mice if the effects of the *Lin28b* LOF model were mediated by increases in *let-7* miRNAs. Indeed, the *let-7* GOF mice weighed less than littermate controls (Fig. 1E and F) and males were more affected than females. Again, the sex-specificity of genotype effect on body weight on postnatal day 80 was found to be significant ($P = 0.0002$ after Bonferroni correction). Although the *Lin28b* LOF and *let-7* GOF mice were similar,

**Table 1** Summary of strains and numbers of mice per sex used in each experiment

| Perturbation | Genetic background | Puberty | Body weights |
|---|---|---|---|
| *Lin28a* GOF | C57BL6/J | 15–33 | 12–33 |
| *Lin28b* LOF | 129, Balb/C, C57BL6/J | 12–29 | 7–15 |
| let-7 GOF | 129, Balb/C, C57BL6/J | 12–24 | 7–24 |


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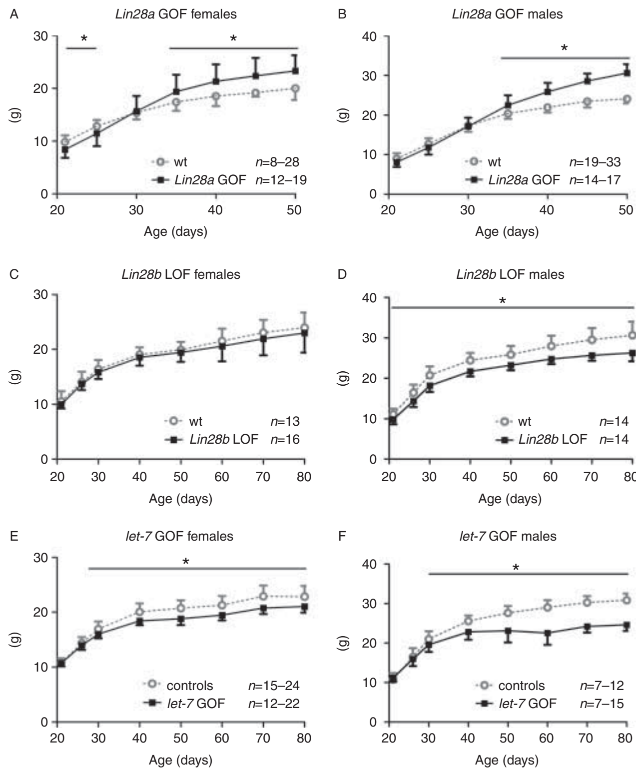


**Figure 1**
Growth patterns in *Lin28a* GOF (A and B), *Lin28b* LOF (C and D), and *let-7* GOF (E and F) mice. The number of animals per group is indicated in each chart. Error bars represent S.D. Growth data were analyzed with the student's *t*-test at each time point. $*P<0.05$.

differences were also apparent. For example, the lighter body weights in *let-7* GOF males did not become apparent until postnatal day 30, much later than the difference seen among the *Lin28b* LOF mice.

## Puberty

Having confirmed sex-specific effects on growth, we next examined whether pubertal timing was regulated in a sex-specific manner in the three mouse strains. Pubertal timing in female mice was determined by daily assessments of VO, and pubertal timing in male mice was determined by daily assessments of PS. It has previously been shown that *Lin28a* GOF females display delayed puberty, as measured by the day of VO, age at first estrus and time of first pregnancy (Zhu *et al*. 2010). To determine whether *Lin28a* exerts this effect in a sex-specific manner, we phenotyped male and female mice in parallel. Both *Lin28a* GOF females and males exhibited delayed puberty ($P\leq 0.0001$) (Fig. 2A and B) and were heavier at VO and PS than controls (Table 2).

Next we investigated pubertal timing in the *Lin28b* LOF mice. Female *Lin28b* LOF and WT littermates displayed VO at the same age (Fig. 2C). In contrast, the *Lin28b* LOF males displayed PS two days later than WT littermates ($P=0.004$) (Fig. 2D). The *let-7* GOF mice again phenocopied the *Lin28b* LOF mice: in *let-7* GOF females, despite the lighter weight, VO occurred at the same age as in littermate controls (Fig. 2E) while *let-7* GOF males reached PS later than controls ($P=0.004$) (Fig. 2E and F). The delay in PS resulted in mice that were older and hence heavier at PS than controls (Table 2), suggesting that, at least in this strain, delayed growth could not explain the delayed puberty.

## Age/weight relationship at puberty

Because the body weights at puberty were altered in different ways in the different strains the relationship between growth and age at puberty was examined further. A linear regression model was estimated for weight and age

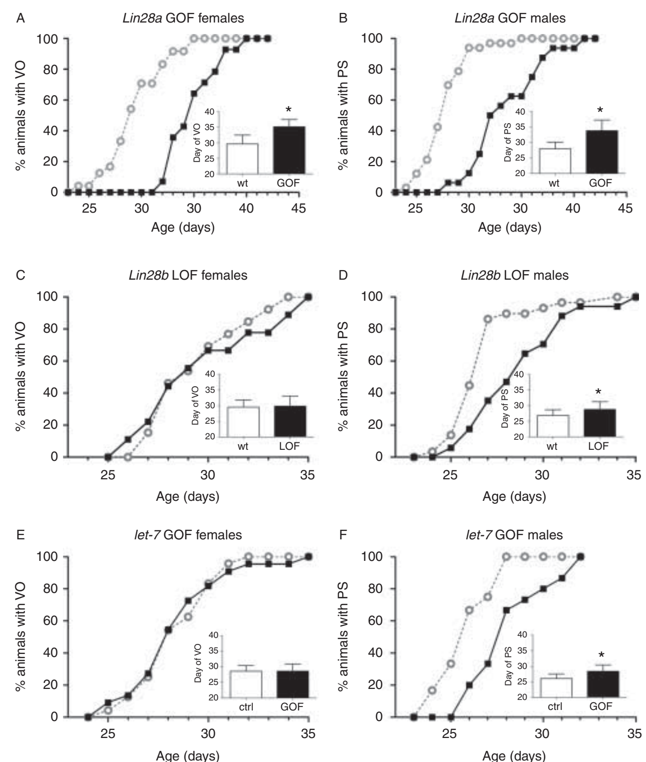


**Figure 2**
The onset of puberty, as assessed by VO and PS in *Lin28a* GOF, *Lin28b* LOF, and *let-7* GOF mice. In each chart black boxes represent the GOF or LOF and open circles represent the control group. The inserted bar graphs display the mean day of VO or PS. Error bars represent S.D. $n=9$–33, exact numbers per group are indicated in Table 2. Puberty data were analyzed with the Mann–Whitney non-parametric *t*-test. $*P<0.05$.



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**Table 2** Comparison of pubertal body weight at puberty in *Lin28a* GOF, *Lin28b* LOF, and *let-7* GOF male and female mice

| Genotype | Sex | Weight at VO/PS | *n* |
|---|---|---|---|
| Wt | M | 15.4±1.3 | 33 |
| *Lin28a* GOF | M | 20.8±2.1 | 16 |
| | | $P<0.0001$ | |
| Wt | F | 15.3±1.5 | 24 |
| *Lin28a* GOF | F | 21.1±2.0 | 15 |
| | | $P<0.0001$ | |
| Wt | M | 16.8±2.0 | 29 |
| *Lin28b* LOF | M | 16.6±2.1 | 17 |
| | | NS | |
| Wt | F | 15.5±0.8 | 13 |
| *Lin28b* LOF | F | 14.8±1.3 | 9 |
| | | NS | |
| Control | M | 16.5±1.9 | 12 |
| *let-7* GOF | M | 18.2±2.1 | 15 |
| | | $P=0.03$ | |
| Control | F | 15.9±0.8 | 24 |
| *let-7* GOF | F | 15.3±1.0 | 22 |
| | | $P=0.008$ | |

*P* values were calculated with the student's *t*-test.

at VO/PS for the littermate controls, and then we examined whether the data from the LOF/GOF mice showed the same relationship (that is, did the data from the transgenic mice fit on the line for the control mice). For both female and male mice, the *Lin28a* GOF weight/age relationship was significantly different from the weight/age relationship observed among controls ($P=2.5\times10^{-5}$ and $P=0.0015$ respectively, Fig. 3A and B), indicating that the alteration in *Lin28a* expression changed the relationship between growth and age of puberty in these mice. The relationship was changed similarly in female and male mice.

The *Lin28b* LOF females displayed body weights and VO similar to controls, and also exhibited a similar age/weight relationship at VO (Fig. 3C). The male *Lin28b* LOF mice displayed both slowed growth and delayed puberty, and the relationship between time of puberty and weight at PS was similar among the male *Lin28b* LOF mice and controls (Fig. 3D).

*let-7* GOF females displayed a different age/weight relationship at VO compared with controls ($P=2.4\times10^{-5}$) (Fig. 3E), perhaps indicating that the *let-7* GOF affected growth but not age at VO. As for *Lin28b* LOF males, the age/weight relationship at PS was not different among male *let-7* GOF mice and controls (Fig. 3F). These results provide further evidence that *let-7* exerts its function differently in males and females.

## Body composition and glucose metabolism in young *let-7* GOF mice

Because *let-7* GOF males weighed less than WT littermates, and because adult *let-7* GOF mice have less body-fat than controls (Brill & Moenter 2009, Frost & Olson 2011, Zhu *et al.* 2011), we hypothesized that the delay in puberty could be due to a lower fat content in the male GOF mice. However, DEXA scans of pre-pubertal *let-7* GOF mice showed no significant difference in body fat content or percent body fat compared with control littermates (Fig. 4A and B).

*let-7* also affects glucose metabolism in adult mice and glucose metabolism could affect pubertal onset (Brill & Moenter 2009, Frost & Olson 2011, Zhu *et al.* 2011);

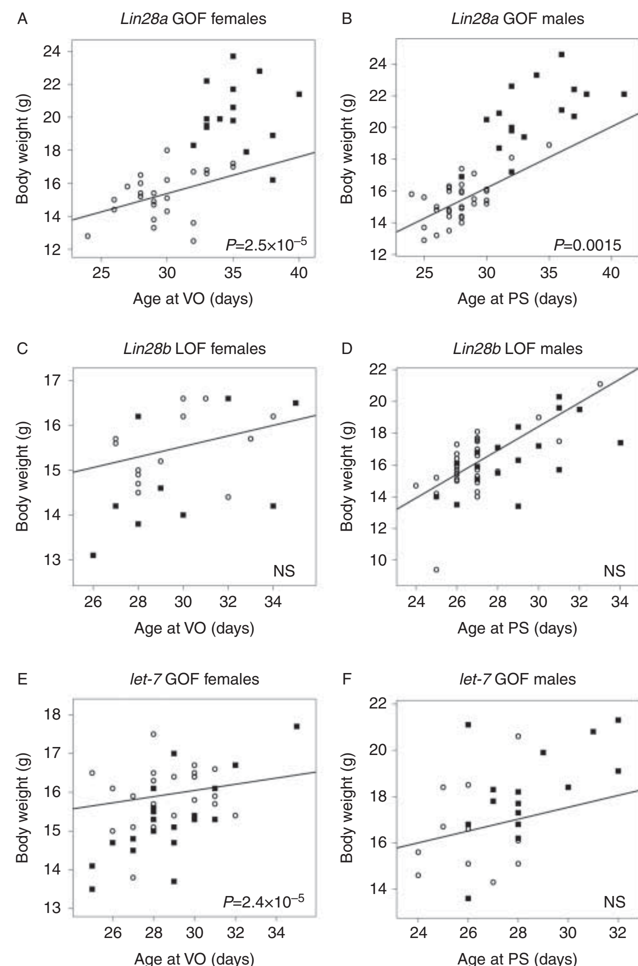


**Figure 3**
Weight and age at puberty for each control (open circles) and transgenic (black boxes) mouse. The line represents linear regression of weight and age at VO/PS for controls. Significant differences in the relationship of age and weight at puberty between controls and transgenic mice were detected in both male and female *Lin28a* GOF mice and among *let-7* GOF females.



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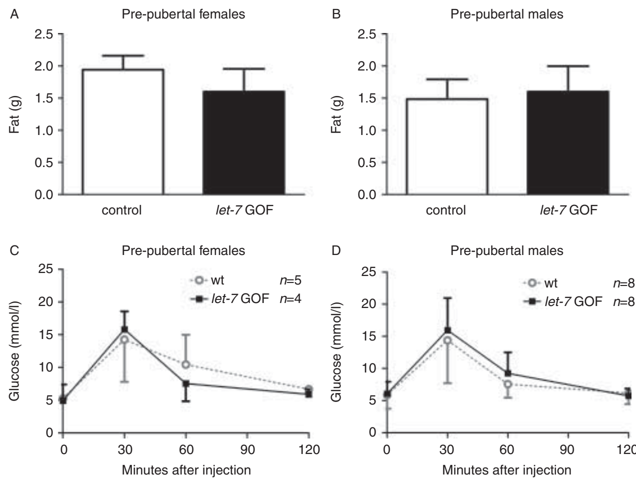


**Figure 4**

Body fat content and glucose metabolism are similar in *let-7* GOF mice and controls. (A and B) Body fat in 22-day-old *let-7* GOF and littermate control mice, $n=4$–8; similar results were obtained for percent body fat (data not shown). (C and D) IP-GTT in 21-day-old *let-7* GOF and littermate control mice, $n=4$–8. Error bars represent S.D. Data were analyzed with the student's *t*-test, assuming unequal variances. No data points reached $P<0.05$.

therefore we assessed glucose metabolism in pre-pubertal *let-7* GOF mice by an IP-GTT. At this young age, neither females nor males displayed abnormal glucose metabolism compared with controls (Fig. 4C and D). These data indicate that the decreased percent body fat and impaired glucose metabolism observed in adult *let-7* GOF mice (Frost & Olson 2011, Zhu *et al.* 2011) likely develop after puberty and are not a cause of the delayed puberty we observed.

### *let-7* levels in *Lin28b* LOF and *let-7* GOF mice

Others have shown that overexpression of *let-7* in the central nervous system leads to smaller body size (Frost & Olson 2011), and we hypothesized that the observed sex differences in growth and puberty in the *Lin28b* LOF and *let-7* GOF strains could derive from sex-specific effects on hypothalamic *let-7* expression. To test this hypothesis, *let-7* levels were assessed in the hypothalamus at the average age of VO or PS in the controls (see Fig. 2). In the *Lin28b* LOF strain, levels of *let-7a* and *let-7g* were evaluated as they have been shown to display expression levels representative of the whole *let-7* family (Newman *et al.* 2008, Shinoda *et al.* 2013*b*). In the *let-7* GOF strain, only *let-7g* was evaluated as this family member was the one being expressed by the transgene. No *let-7* upregulation was detected in the hypothalamus (Fig. 5A and B) in either the *Lin28b* LOF or the *let-7* GOF strain.

Because previous studies have demonstrated that *Lin28b* and *let-7* can regulate growth peripherally (Frost & Olson 2011, Shinoda *et al.* 2013*b*), we also investigated if the sex differences in puberty and body weight could derive from sexually dimorphic peripheral *let-7* expression. *let-7* levels were assessed in pituitary and gonads (as part of the HPG axis) and liver (as a non-HPG tissue) in *Lin28b* LOF mice. No differential regulation of *let-7a* or *let-7g* was detected in any tissue among *Lin28b* LOF female and male mice (Fig. 6A and B). We also assessed whether the lack of sufficient changes in peripheral *let-7* expression in female mice might explain why these mice were less affected than the males. However, contrary to this hypothesis, overexpression was either similar in both sexes or greater in females than in males (Fig. 6C and D).

## Discussion

Differences exist between boys and girls and men and women in many growth-related traits such as height, body mass index, waist to hip ratio, and the timing of puberty. The basis for these differences is not fully understood, but understanding the differences is important because they are linked to cardiovascular and other health outcomes (Weir *et al.* 1998, Golub *et al.* 2008, Lakshman *et al.* 2009, Joinson *et al.* 2011, Seselj *et al.* 2012, Widen *et al.* 2012, Ritte *et al.* 2013, Day *et al.* 2015). Certainly sex steroids play a role in this sex specificity and so do sex chromosomes as is evidenced by the height in women with complete androgen insensitivity and Turner syndrome. GWAS present a new opportunity to further our understanding of sex specificity among complex traits

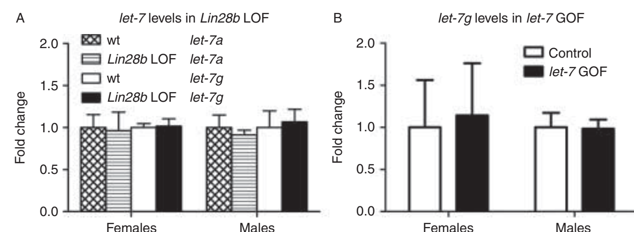


**Figure 5**

Hypothalamic *let-7* expression levels were unaltered in *Lin28b* LOF and in *let-7* GOF mice compared with controls. (A) *let-7a* and *let-7g* levels in hypothalamus on the mean day of PS (postnatal day 27) or VO (postnatal day 30) among control *Lin28b* LOF mice, $n=3$–4. (B) *let-7g* levels in hypothalamus on the mean day of PS (postnatal day 26) or VO (postnatal day 29) among controls and *let-7* GOF mice, $n=3$–4. Error bars represent S.D. Expression data were analyzed using the student's *t*-test. No data points reached $P<0.05$.


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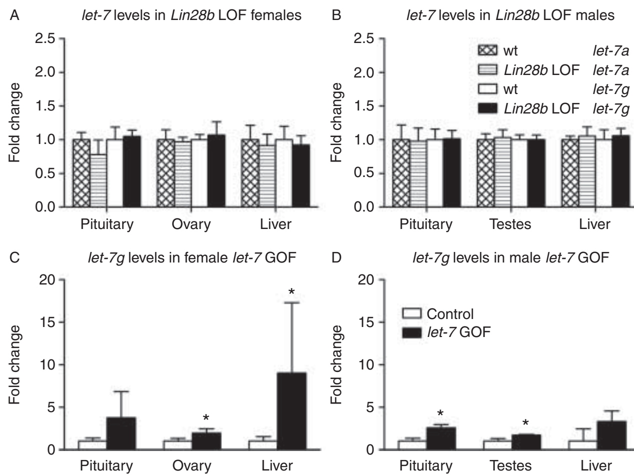


**Figure 6**

Peripheral *let-7* expression levels were unaltered in *Lin28b* LOF mice, but upregulated in in *let-7* GOF mice. (A and B) *let-7a* and *let-7g* levels in pituitary, gonads, and liver in *Lin28b* LOF mice on the mean day of VO (postnatal day 30) or PS (postnatal day 27) among control mice, $n=3$–4. (C and D) *let-7g* levels in pituitary, gonads, and liver on the mean day of VO (postnatal day 29) or PS (postnatal day 26) in *let-7* GOF mice, $n=3$–4. Error bars represent S.D. Expression data were analyzed using the student's *t*-test. $^*P<0.05$.

(Magi *et al.* 2010, Gilks *et al.* 2014). Although the vast majority of the identified loci are likely not to exert sex-specific effects, recent studies have identified sex-specific (Winkler *et al.* 2015) and sex-chromosome dependent loci (Lunetta *et al.* 2015) that affect body size and pubertal timing. Moreover, even in the absence of genetic variants with sex specific effects, the study of the genes and pathways identified by GWAS provides an opportunity to uncover new factors that contribute to male-female differences in traits such as growth and the timing of puberty since, for example, these genes and pathways may be modulated by differential sex-steroid exposures or epigenetic mechanisms.

Sex-specific effects of the *Lin28a/Lin28b/let-7* axis have already been identified in growth patterns in mice and humans (Lettre *et al.* 2008, Widen *et al.* 2010, Ong *et al.* 2011, Leinonen *et al.* 2012, Shinoda *et al.* 2013*b*, Cousminer *et al.* 2014). By verifying previously published sex differences in the regulation of body weight in a second animal facility and expanding these observations to include sex-specific effects on pubertal timing, we provide further evidence that the *Lin28a/Lin28b/let-7* axis can exert sex-specific effects in mice. The mechanism(s) for these sex-specific effects remains unclear, but recent evidence point towards neonatal sex hormone exposure as one regulator of the *Lin28a*, *Lin28b*, and *let-7* pathway (Sangiao-Alvarellos *et al.* 2013, Sangiao-Alvarellos *et al.* 2015). Our findings highlight the importance of understanding the sex-specific effects of this complex pathway and its contribution to differences in growth and puberty seen in boys and girls.

Following the association of *LIN28B* with AAM (He *et al.* 2009, Ong *et al.* 2009, Perry *et al.* 2009, Sulem *et al.* 2009, Elks *et al.* 2010), researchers investigated whether variants in *LIN28B* underlie central precocious puberty in girls (Silveira-Neto *et al.* 2012) or constitutional delay of growth and puberty in boys (Tommiska *et al.* 2010). The results indicate that such variation is not a common cause of these extreme phenotypes and suggest that the primary role of *LIN28B* may be in regulating the timing of puberty within the general population. In contrast to other studies using more severely affected animals (Good *et al.* 1997, Corradi *et al.* 2003, Seminara *et al.* 2003, Pask *et al.* 2005, Brill & Moenter 2009, DeBoer *et al.* 2010, Deboer & Li 2011, Elias & Purohit 2013), all three strains of our mice were healthy and fertile and were thus a good model for studying genes that affect timing of puberty in the general population. Similarly, the two-day delay detected in our transgenic male mice is of comparable magnitude to studies of litter-size and diet manipulations in male mice (DeBoer *et al.* 2010, Smith & Spencer 2012, Sanchez-Garrido *et al.* 2013). In contrast, when the HPG axis is severely perturbed with impaired fertility as a result, PS and VO can be delayed 7–11 and 9 days, respectively (Novaira *et al.* 2014). The five-day delay in puberty seen among our *Lin28a* GOF mice may indicate that *Lin28a* plays a larger role than *Lin28b* in regulating the reproductive endocrine axis in mice (Zhu *et al.* 2010).

Although the physiology of puberty in mice and humans is not identical, decades of experiments have demonstrated that the mouse is an informative model in the study of reproductive endocrinology. Mice, like humans, display sex-differences in the timing of puberty, although in mice the direction of this sex difference can be strain-specific (Krewson *et al.* 2004, Nathan *et al.* 2006, Tyl *et al.* 2008*a*,*b*, Divall *et al.* 2010). While the effects of SNPs and knockouts may also be species-specific, our data underscore that the *Lin28a/Lin28b/let-7* pathway is a key regulator of growth and pubertal timing and suggest that it may be responsible for some of the sex-specific differences seen in these traits among humans.

*Lin28a* and *Lin28b* are paralogues that appear to have overlapping as well as separate functions. Here we show that some aspects of *Lin28a* GOF are mirrored by *Lin28b* LOF mice (*Lin28a* GOF mice are larger and *Lin28b* LOF mice are smaller than controls). Other aspects differ, such



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as presence of sex-specificity and the direction of changes in pubertal timing. The age/weight relationship at puberty is also perturbed in the *Lin28a* GOF mice when compared with controls, whereas the relationship is unaltered in *Lin28b* LOF mice. Functions in humans may differ too. GWAS have not demonstrated that variants in *LIN28A* associate with AAM in humans, but this could stem from this gene having tolerated less sequence variation during evolution, thus lacking variants that could associate with AAM. Consistent with this idea is the observation that *Lin28a* LOF mice either die young or display reduced fertility as adults (Zhu *et al.* 2010, Shinoda *et al.* 2013*a*). In addition, a recent study by Sangiao-Alvarellos demonstrates that expression levels of *Lin28a* and *Lin28b* are regulated in opposite directions in the testes after hypophysectomy, supporting our findings that *Lin28a* and *Lin28b* act in different ways in the HPG axis (Sangiao-Alvarellos *et al.* 2015). That *Lin28a* and *Lin28b* may have separate functions is further supported by cancer studies that often identify either *Lin28a* or *Lin28b* as a regulator of cell fates (Zhou *et al.* 2013) and by different expression patterns that have been observed in mouse (Grieco *et al.* 2013) and rat tissues (Sangiao-Alvarellos *et al.* 2013). On a cellular level, *Lin28a* and *Lin28b* operate in separate compartments of the cell (Piskounova *et al.* 2011).

*Lin28a* and *Lin28b* exert most of their effects through inhibition of *let-7* levels but also have *let-7* independent functions (Polesskaya *et al.* 2007, Xu & Huang 2009, Xu *et al.* 2009, Balzer *et al.* 2010, Qiu *et al.* 2010, Peng *et al.* 2011, Wilbert *et al.* 2012). For example, it has been shown that *Lin28a* can bind mRNA directly and stimulate translation (Polesskaya *et al.* 2007, Xu *et al.* 2009, Xu & Huang 2009, Qiu *et al.* 2010) via motifs that are found in many genes (Peng *et al.* 2011, Wilbert *et al.* 2012). Our data are consistent with the concept of both *let-7* dependent and independent functions of *Lin28a/Lin28b*. For example, *let-7* GOF mice show similar weight and puberty phenotypes as the *Lin28b* LOF mice, including sex specificity, and the *let-7* GOF mice display body weight phenotypes in the opposite direction of *Lin28a* GOF mice. On the other hand, the *Lin28a GOF* mice display no sex specificity, and in the *Lin28b* LOF males, lighter body weights are apparent at younger ages than in the *let-7* GOF strain. In addition, the relationship between age/weight at puberty is not perturbed in the *Lin28b* LOF mice, but female *let-7* GOF mice display an altered relationship compared with controls, suggesting that some effects of *let-7* may be independent of *Lin28a* and *Lin28b*.

Because *Lin28b* can downregulate *let-7*, we expected elevated *let-7* levels in *Lin28b* LOF mice. However, as reported by several others using animals with modulated *Lin28a* or *Lin28b* expression, there were no apparent differences among *let-7* levels in the tissues examined in *Lin28b* LOF mice (Zhu *et al.* 2010, Faas *et al.* 2013, Shinoda *et al.* 2013*b*, Ouchi *et al.* 2014). It is possible that, as shown in recent studies in *Xenopus* and *C. elegans*, *Lin28a* and *Lin28b* may modulate *let-7* levels only during specific developmental stages (Vadla *et al.* 2012, Faas *et al.* 2013), and we may not have assessed the specific timepoint(s) at which this modulation occurs. This possibility is further supported by the observation that the sex-specificity in the body weight reduction is detected when the *let-7* GOF is induced from birth, but not when induced from weaning (Zhu *et al.* 2011).

The concept of age/developmental stage-specific regulation is supported by the finding that changes in *Lin28b* expression in the fetus have greater effects on growth than changes later in life (Shinoda *et al.* 2013*b*). Thus, elevated *let-7* levels during a short, but critical, window during development may be sufficient to induce phenotypic effects later in life. In WT animals, gene expression of *Lin28a* and *Lin28b* decrease and *let-7* levels increase from birth to adulthood, but the gene expression levels are relatively stable between juvenile and pubertal stages, raising the possibility that the puberty-modulating phase may occur earlier during development (Grieco *et al.* 2013, Sangiao-Alvarellos *et al.* 2013). Further studies of the *Lin28a/Lin28b/let-7* axis at different times during development are needed to inform us of critical windows during which this axis may program future phenotypes. A time-course of *Lin28a/Lin28b/let-7* expression throughout postnatal development and across the pubertal transition in mice with perturbed *Lin28a/Lin28b/let-7* pathways might be particularly informative.

Despite the limitations related to critical windows, we examined levels of *let-7* gene expression in several tissues to explore the origin of the male-specific responses to *let-7* GOF. Although males exhibited more and stronger phenotypes than females, overall males displayed a lower degree of *let-7* overexpression than females, raising the possibility that regulation of growth and puberty may be more *let-7* sensitive in males than in females. Interestingly, the degree of *let-7g* upregulation in the *let-7* GOF mice varied among tissues, and was absent in the CNS as has been reported by others (Frost & Olson 2011, Zhu *et al.* 2011), suggesting that either we assessed expression at a time point at which *let-7* is resistant to upregulation, or that weight and puberty phenotypes may not be mediated by *let-7* action in the hypothalamus. Indeed, others have previously demonstrated that phenotypes detected in



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CNS-restricted *let-7* GOF mice are not always detected in global *let-7* GOF mice, and *vice versa*, perhaps suggesting a combination of central and peripheral effects (Frost & Olson 2011). Further studies using tissue-specific GOF or LOF of *let-7* miRNAs are needed to determine the precise site(s) of regulation of growth and puberty by *let-7*.

In summary, the data from our LOF and GOF strains demonstrate complex and sex-specific regulation of growth and puberty by *Lin28a*, *Lin28b*, and *let-7*. The data also point to possible critical windows and peripheral vs central regulation. This complexity consistent with human data showing that the dominant allele at rs314276 (the SNP that displays the strongest association with AAM) is associated with shorter height and heavier weight in women but not men (Ong *et al*. 2011). In addition, two other *LIN28B* SNPs that associate with AAM and adult height, rs314277 and rs7759938, contribute differentially to adult height in females and males (Widen *et al*. 2010). These findings in humans and our data in mice suggest that further study of the *Lin28b* LOF and *let-7* GOF strains will provide an important opportunity to expand our understanding of how puberty and growth are modulated in males and females, including the basis of some sex-specific effects. In addition, our models have no apparent underlying illness, in keeping with humans with genetic variants in *LIN28B*, indicating that these strains also provide an opportunity to study a pathway identified through GWAS that regulates the onset of puberty within the 'normal' population.

### Declaration of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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### Author contribution statement

C C carried out experiments, performed data analysis, and wrote the manuscript. D L C, C C, C C, and C B carried out experiments. G S, H Z, G Q D, A G, and M R P conceived the study, assisted in interpretation of results, and edited the manuscript. A G performed linear regression analysis.

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