Molecules and Cells

Makorin 1 Regulates Developmental Timing in Drosophila

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The central mechanisms coordinating growth and sexual maturation are well conserved across invertebrates and vertebrates. Although mutations in the gene encoding makorin RING finger protein 3 (*mkrn3*) are associated with central precocious puberty in humans, a causal relationship has not been elucidated. Here, we examined the role of *mkrn1*, a Drosophila ortholog of mammalian *makorin* genes, in the regulation of developmental timing. Loss of MKRN1 in *mkrn1*^{ex5} prolonged the 3rd instar stage and delayed the onset of pupariation, resulting in bigger size pupae. MKRN1 was expressed in the prothoracic gland, where the steroid hormone ecdysone is produced. Furthermore, *mkrn1*^{ex5} larvae exhibited reduced mRNA levels of *phantom,* which encodes ecdysone-synthesizing enzyme and E74, which is a downstream target of ecdysone. Collectively, these results indicate that MKRN1 fine-tunes developmental timing and sexual maturation by affecting ecdysone synthesis in Drosophila. Moreover, our study supports the notion that malfunction of *makorin* gene family member, *mkrn3* dysregulates the timing of puberty in mammals.

Keywords: Drosophila, growth, makorin1, makorin3, sexual maturation

INTRODUCTION

Puberty is the period during which sexual maturation occurs and is controlled by the hypothalamic-pituitary-gonadal axis. In mammals, puberty is initiated by a sustained increase in the pulsatile release of gonadotropin releasing hormone from the hypothalamus and is set by complex interplays between genetic, metabolic, and environmental factors (Gajdos et al., 2009; Palmert and Hirschhorn, 2003; Tena-Sempere, 2013). In humans, puberty usually begins between the ages of 8 and 12 years in girls and 9 and 14 years in boys. Initiation of puberty at younger or older ages outside of these limits is regarded as precocious or delayed puberty, respectively. Precocious puberty has physical and cognitive outcomes including short stature and increased risks of obesity, type 2 diabetes, breast cancer, and cardiovascular disease (Carel et al., 2004; Golub et al., 2008), but the precise mechanisms triggering the puberty initiation remain uncertain.

Recently, *mkrn3*, the gene encoding makorin RING finger protein 3, was reported associated with central precocious puberty in several ethnic populations (Abreu et al., 2013; Christoforidis et al., 2017; Lee et al., 2016b; Nishioka et al.,

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2017; Schreiner et al., 2014). *Makorin* gene family members encode zinc finger proteins with a RING domain responsible for ubiquitin ligase activity and are well conserved across invertebrate and vertebrate species (Gray et al., 2000). *mkrn1, 2,* and *3* have been identified in vertebrates (Gray et al., 2000; 2001; Jong et al., 1999). Although the molecular functions of MKRN1 as an ubiquitin ligase of several target proteins e.g. hTERT, p53, phosphatase and tensin homolog (PTEN), anaphase-promoting complex (APC), and AMP-activated protein kinase (AMPK) have been well-studied in mammalian cells, but sparse for MKRN3 (Kim et al., 2005; Lee et al., 2009; 2015; 2018a; 2018b). Given that *mkrn3* is expressed in the hypothalamus and begins to decline at the onset of puberty in mice, the inhibitory role of *mkrn3* in puberty initiation has been suggested (Abreu et al., 2013). However, a causal relationship of *mkrn3* in puberty regulation has not yet been elucidated.

Genetic studies of the fruit fly *Drosophila melanogaster* suggest that central mechanisms coordinating growth and sexual maturation are well conserved across invertebrates and vertebrates. The Drosophila life cycle, similar to that of other animals, consists of a juvenile growth phase (three larval instars; L1, L2, and L3), a sexual maturation phase called metamorphosis (pupae), and a reproductive adult stage. Notably, the transition from larval-to-pupal stages in Drosophila is primarily regulated by neuroendocrine mechanisms, similar to hypothalamic-pituitary-gonadal axis activation in mammals. Progression through each stage is controlled by surges of the steroid hormone 20-hydroxyecdysone. A single pulse of ecdysone triggers transition through L1 and L2, and three low pulses of ecdysone followed by a high level of ecdysone terminate L3 and larval growth, thus initiating metamorphosis (Rewitz et al., 2013).

Developmental and nutritional signals coordinate to fine-tune the timing and duration of ecdysone pulses. Most importantly, when larvae attain critical weight at L3, prothoracicotropic hormone (PTTH) released from the brain reaches the prothoracic gland (PG), which is a part of composite endocrine tissue called the ring gland and induces ecdysone production. The duration between critical weight attainment and pupariation is called the terminal growth period and is when most larval growth takes place, with the amount of growth during this period determining the final body size of adults. Loss of PTTH signaling prolongs larval development, resulting in larger body size (McBrayer et al., 2007). In addition, insulin/Tor signaling controls ecdysone synthesis by incorporating nutrient status (Caldwell et al., 2005; Colombani et al., 2005; Layalle et al., 2008; Mirth et al., 2005; Walkiewicz and Stern, 2009). Reduced insulin/Tor signaling specifically in the PG downregulates the ecdysone production, thereby delays the pupariation and increases animal size (Colombani et al., 2005; Layalle et al., 2008). On the other hand, increasing insulin signaling in the PG accelerates the ecdysone release and advances the metamorphosis (Caldwell et al., 2005). Ecdysone synthesis in the PG is catalyzed by a sequence of reactions mediated by enzymes encoded by the Halloween family of genes including *phantom*, *disembodied*, and *shadow* (Rewitz et al., 2006).

Here, we show that loss of *mkrn1*, an ortholog of vertebrate *mkrn* genes, lengthened the duration from 3rd instar-to-pupariation in Drosophila and produced bigger size pupae. MKRN1 protein is strongly expressed in the Drosophila endocrine tissue ring gland. Moreover, *phm* and *E74* mRNA levels were reduced in *mkrn1* null larvae, indicating down-regulation of ecdysone-mediated signaling. Taken together, our results demonstrate that MKRN1 controls larval developmental timing and body size by regulating steroid hormone ecdysone production. Furthermore, our study supports the notion that malfunction of *makorin* gene family member, *mkrn3* leads to puberty timing dysregulation in mammals.

MATERIALS AND METHODS

Generation of *mkrn1* mutants and fly strains

To create an *mkrn1*-null allele, we exploited P element excision mutagenesis. A fly line harboring a P element near the *mkrn1* gene, *P{EPgy}mkrn1*^{EY14602}, was mated to *Dr/TM3,p{Δ2-3}* flies. F1 males were then mated to MKRS/TM6B balancer females, and F2 progeny with TM6B were screened for white eye color. Approximately 100 white-eyed F2 flies were analyzed for deletion via polymerase chain reaction (PCR) analysis using genomic DNA as template. Finally, one fly line harboring a precisely excised chromosome (CTRL) and two fly lines harboring small and large deletions, named *mkrn1*^{exS} and *mkrn1*^{exL}, respectively, were mated to MKRS/TM6B balancer flies to establish a stock. The primer sequences used for genomic DNA PCR analysis were as follows: P1, forward, 5′-CCCGCCTTTTCCA TAATCGTGTCA-3′; P2, reverse, 5′-TCATTGCCGCGTCATTATT AGGAG-3′; P3, reverse, 5′-TTCATGCGCGGCTGGTCTATCG-3′; P4, forward, 5′-TACTGAGCCAAATATCGAATGCG-3′. The precision of deleted regions was confirmed by sequencing.

Other fly strains used included *w*^{1118} (BL5905), *UAS-dicer2;actin-Gal4/CyO* (BL25708), and *P{UAS-GFP.nls}* (BL4776) obtained from the Bloomington Drosophila stock center and *UAS-ptth* (McBrayer et al., 2007), *Tubulin-Gal4/TM6B,* and *phm22-Gal4* kindly provided by Michael B. O'Connor (University of Minnesota, USA), Jongkyeong Chung (Seoul University, Republic of Korea), and Seogang Hyun (Chung-Ang University, Republic of Korea), respectively. RNAi lines obtained from the Vienna Drosophila Resource Center (VDRC) were *CG12477* (VDRC 102882, VDRC 31944), *CG5334* (VDRC 102765), and *CG5347* (VDRC 22089 and 110427). Identification of trans-heterozygotes harboring both actin-Gal4 and UAS-RNAi was performed by selecting larvae without visible phenotypic bc marker after mating *UAS-dicer2;actin-Gal4/bc* to each line of UAS-RNAi flies.

Measurement of developmental timing

Virgin and male flies were crossed and transferred every 2 h at 25℃. 2nd instar larvae were collected 66 h after egg laying (AEL). Approximately 8-10 h later, 3rd instar larvae were collected and transferred to vials with 20 larvae per vial. Larval stage was determined by floating the larvae in chilled 25% sucrose solution and observing spiracle and mouth hook morphology. At this time, phenotypic marker selection (either bc- or TM6B-harboring depending on the cross) was

performed. Pupariation was scored at 2-h intervals in an incubator maintained at 25℃. Pupa length was measured 1 day after pupariation from images taken by a CCD camera using Optinity OptiView 3.7 software (Korea Lab Tech).

RT-PCR analysis

Total RNA was isolated from frozen larvae using QIAzol lysis reagent (QIAGEN). Total RNA (1 μg) was reverse-transcribed with oligo-dT primer using Prime Script reverse transcriptase (TAKARA), and PCR was performed using rTaq DNA polymerase (TAKARA). The following primers were used: *mkrn1*-forward, 5′-GCGTTCTGGATGGAGACCAA-3′; *mkrn1*-reverse, 5′-GTAACTTTCGGGTGCGCTTC-3′; *CG32442*-forward, 5′-TGCTGCTAATAATCACCGCCA-3′; *CG32442*-reverse, 5′-CGGTCGTGTTATTTCGCCAC-3′; *ppk5*-forward, 5′-CGGGAGTTGGAGTTGGTACC-3′; *ppk5*-reverse, 5′-AAAACTCGTCTGGTGCCCAA-3′; *gapdh*-forward, 5′-ACCGACTTCTTCAGCGACAC-3′; *gapdh*-reverse, 5′-GAGTTCGGTTACTCCAACCG-3′. *ptth*-forward, 5′-AAGGTTTGGACGAGATGGTG-3′; *ptth*-reverse, 5′-GCTGGGACTGAATCCGAATA-3′; *RpL32*-forward, 5′-TAAGCTGTCGCACAAATGG-3′; and *RpL32*-reverse, 5′-GTTCGATCCGTAACCGATGT-3′.

Quantitative real-time PCR was performed as previously described (Lee et al., 2016a). Real-time PCR was performed using a Rotor Gene 6000 (Qiagen) with SYBR® Premix Ex Taq™ (Tli RNaseH Plus; Takara). The following quantitative reverse-transcription (qRT)-PCR primers were used: *mkrn1*-forward, 5′-GACGTGCGGCATCTGCTTTG-3′; *mkrn1*-reverse, 5′-TGTTTGGCCTGACGCCATGT-3′; *CG12477*-forward, 5′-TTCGTCCGTCAGGTGTTGCC-3′, *CG12477*-reverse, 5′-TGCCGCACTTCTTGTCCTGG-3′; *CG5334*-forward 5′-CAATGGGCGCCAAGGATTGC-3′; *CG5334*-reverse 5′-ATGCGGTTATCACCCGCCAG-3′; *CG5347*-forward 5′-GCATTCGCACATGGCGTCAG-3′; *CG5347*-reverse, 5′-GCCCAAGCCCCCATTGAAGT-3′; *ptth*-forward, 5′-AAGGTTTGGACGAGATGGTG-3′; *ptth*-reverse, 5′-GCTGGGACTGAATCCGAATA-3′; *Phm*-forward, 5′-GCTTGCATTTCCGAGACGAT-3′; *Phm*-reverse, 5′-ACGATCATCGAACCACCCTT-3′; *E74*-forward, 5′-CAAACCGAAGCTGGAGATGG-3′; and *E74*-reverse, 5′-TCGTCCACTTGATGAAACGC-3′. mRNA encoding *cpb20* was used as a reference gene to normalize gene expression levels using the following primer sequence: *cbp20*-forward, 5′-GTATAAGAAGACGCCCTGC-3′; *cbp20*-reverse, 5′-TTCACAAATCTCATGGCCG-3′. Data were analyzed using Rotor Gene 6000 software, and relative mRNA levels were quantified using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Protein extracts from 3rd instar larvae were prepared using lysis buffer (10 mM HEPES, pH 7.5; 50 mM KCl; 10% glycerol; 5 mM Tris-HCl, pH 7.5) with freshly added 5 mM EDTA, 1 mM DTT, 0.1% Triton X-100, protease inhibitor (Sigma), 1 mM Na_3VO_4, and 0.25 mM NaF (final concentration). Protein extracts were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and blots were probed by the primary antibodies anti-MKRN1 (Rb-1, 1:3,000) or anti-ERK (Cell Signaling Technology, 1:2000).

Immunostaining

At the 3rd instar stage, brains attached with ring gland were dissected, fixed in 3.7% formaldehyde, and washed with PAXD buffer (1× PBS, 5% BSA, 0.03% sodium deoxycholate, 0.03% Triton X-100) (Gunawardhana and Hardin, 2017). Fixed larval brains were blocked in 10% horse serum in PAXD buffer (blocking solution) for 1 h. Anti-MKRN1 antibody (Rb-1, 1:1,000) was directly added and incubated overnight at 4℃. The next day, brains were washed and incubated with secondary antibody (Alexa Flour® 555 goat anti-rabbit IgG, 1:200) for 24 h at 4℃. Stained brain samples were washed with PAXD buffer, further stained with Hoechst 33342 (Sigma, 1:1,000), and mounted. Confocal images were obtained with an LSM 800 confocal microscope (Carl Zeiss) and processed with Zen software (Carl Zeiss). Representative images were selected from > 10 larval brains per genotype.

RESULTS

Loss of MKRN1 delayed development

In Drosophila, four members of the *mkrn* gene family have been annotated in the genome: *mkrn1*, CG12477, CG5347, and CG5334. Of these, CG12477, CG5347, and CG5334 are intronless and correspond to retrocopies of *mkrn1* (Bohne et al., 2010; Gray et al., 2000). Thus, we reasoned that *mkrn1* is a bona fide ortholog of vertebrate *mkrn* genes. To investigate the role of *mkrn1* in developmental timing and maturation, an *mkrn1* loss-of-function mutation was generated in flies by imprecise excision of the P element inserted in the line P{EY14602}. Two lines of deletion mutants were obtained and named *mkrn1*^{exS} and *mkrn1*^{exL}. Genomic DNA PCR and sequencing revealed the deleted regions of chromosome in two alleles (Figs. 1A and 1B). Of the two alleles, *mkrn1*^{exS} flies showed a specific disruption of *mkrn1* expression without affecting nearby CG32442 and *ppk5* gene expression (Fig. 1C). Thus, we used *mkrn1*^{exS} flies for further analysis, with precisely excised flies serving as control flies.

To determine whether MKRN1 regulates developmental timing, we compared pupariation timing between control and *mkrn1*^{exS} flies. We first validated our experimental setup by measuring the pupariation timing of *ptth*-overexpressing larvae. *ptth* expression was driven by *tubulin*-Gal4 throughout the body, with a significant increase in *ptth* expression confirmed by RT-PCR analysis (Fig. 2A). Given that PTTH released from the brain induces ecdysone synthesis in the PG, we expected advanced pupariation in *ptth*-overexpressing larvae. Consistent with a previous report (McBrayer et al., 2007) and our expectation, pupariation occurred 10-12 h earlier in *ptth*-overexpressing larvae than in two parental lines when measured from the 3rd instar transition (Fig. 2B). This premature pupariation resulted in shorter pupa length compared with control larvae (Fig. 2C).

Next, we performed developmental timing analysis of control and *mkrn1*^{exS} flies by measuring the duration of time AEL until puparium formation (Fig. 2D). The average total time AEL to pupariation increased from 121 h in controls to 125 h in *mkrn1*^{exS} larvae. To understand which developmental transition phase was affected by loss of MKRN1, we measured the duration of time from 3rd instar-to-pupariation

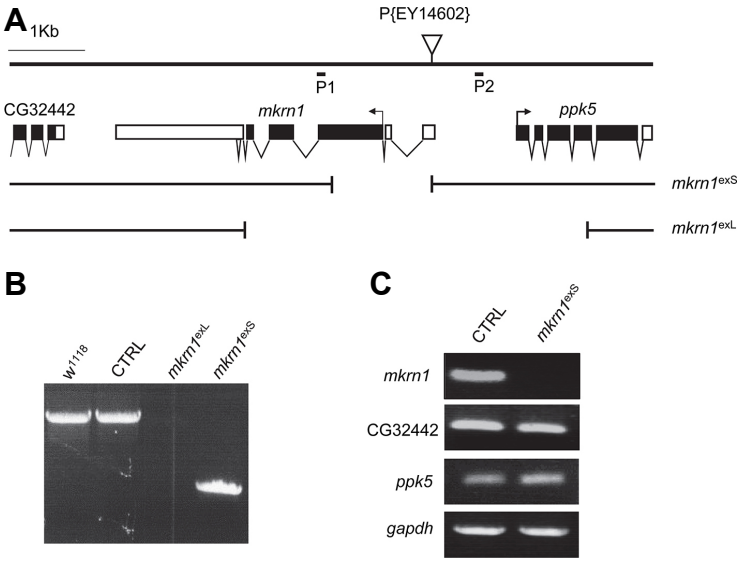

Fig.1. Generation of *mkrn1* deletion alleles. (A) Genomic organization of the *mkrn1* locus. The insertion site of P-element P{EY14602} is indicated by a white triangle. P1 and P2 denote primers used for genomic DNA PCR analysis in (B). Exons and introns are indicated by black and white rectangles, respectively. By mobilizing the P element, the two deletion alleles *mkrn1*^{exS} and *mkrn1*^{exL} were isolated. (B) Deletion was confirmed by genomic DNA PCR using primers P1 and P2. A precise excision line (CTRL) was isolated and used as a control. (C) RT-PCR analysis was performed with CTRL and *mkrn1*^{exS} alleles. In the *mkrn1*^{exS} allele, *mkrn1* was not expressed, whereas the expression of nearby genes *CG32442* and *ppk5* was similar to those in control flies.

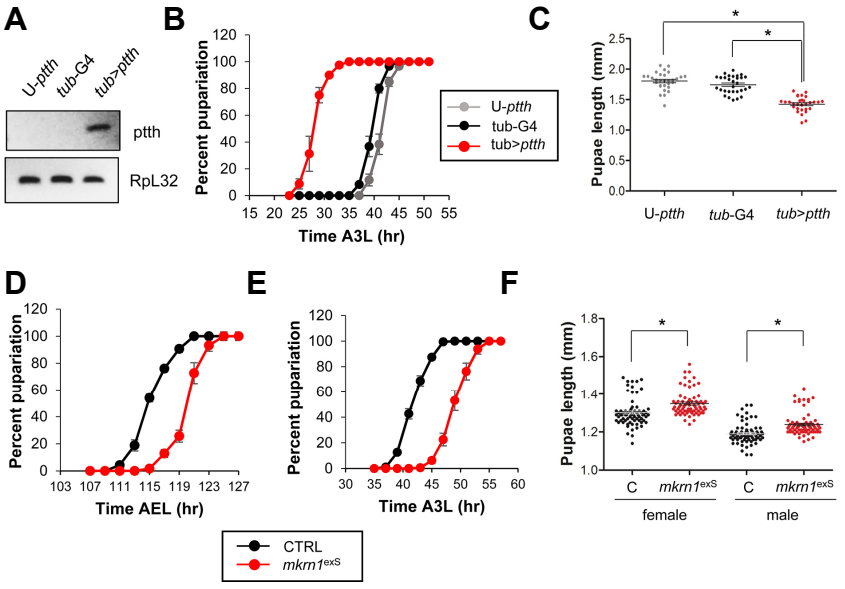

Fig. 2. Deletion of *mkrn1* delayed pupariation and increased pupa size. (A) RT-PCR analysis of larvae showed that *ptth* mRNA levels were markedly increased in *ptth*-overexpressing larvae (tub > *ptth*) compared with the parental lines UAS-*ptth* (U-ptth) and tub-Gal4 (tub-G4). *RpL32* was used as a reference. (B) The percentage of larvae of the indicated genotypes that formed pupa was plotted relative to time in hours after 3rd instar (A3L). Pupariation time was advanced in tub > *ptth* larvae compared with control U-*ptth* or tub-G4 larvae. (C) Pupa length in tub > *ptth* larvae was shorter than that in control U-*ptth* or tub-G4 larvae. Values are presented as mean ± standard error of the mean (SEM). n = 30 for all genotypes. * indicates statistically significant difference (Student's *t* test: $P < 0.0001$). (D) The percentage of larvae of the indicated genotypes that formed pupa was plotted relative to time in hours after egg laying (AEL). CTRL, n = 117; *mkrn1*^{exS}, n = 117. (E) The percentage of larvae of the indicated genotypes that formed pupa was plotted relative to time in hours A3L. CTRL, n = 163; *mkrn1*^{exS}, n = 160. (F) Pupa length was longer in *mkrn1*^{exS} larvae than in control larvae. Values are presented as mean ± SEM. Male CTRL, n = 72; Male *mkrn1*^{exS}, n = 73; Female CTRL, n = 71; Female *mkrn1*^{exS}, n = 74. * indicates statistically significant difference (Student's *t* test: $P < 0.001$).

(Fig. 2E). During this stage, widespread changes throughout larvae occur to signal the onset of maturation (Thummel, 2001). *mkrn1*^{exS} flies exhibited delayed pupariation by ~7 h compared with control flies, similar to the delay in timing AEL. This result indicates that MKRN1 mainly affects developmental timing from 3rd instar-to-pupariation. In Drosophila, the growth of larvae defines body size (Mirth and Shingleton, 2012). Thus, we measured pupa length and observed that both male and female *mkrn1*^{exS} pupae were slightly longer than control pupae (Fig. 2F). Taken together, our findings suggest that deletion of *mkrn1* lengthens the 3rd instar-to-pupariation duration, resulting in a slight increase in body size.

Knockdown of mkrn1 paralogs did not delay development

There are three *mkrn1* paralogs in the Drosophila genome: CG12477, CG5334, and CG5347. Although *mkrn1* seems to be the founder gene of *makorin* genes (Bohne et al., 2010), we tested whether these three genes affect developmental timing in Drosophila. We measured the pupariation timing

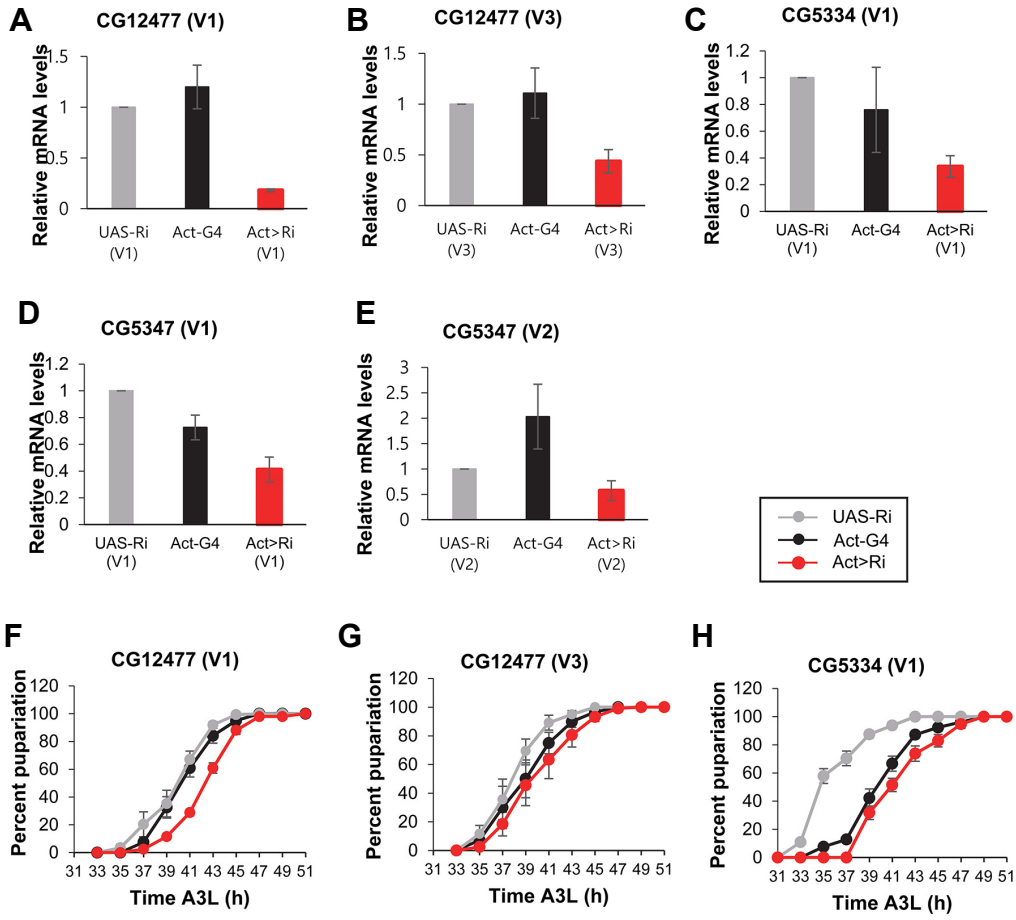

Fig. 3. Downregulation of *mkrn1* paralogs did not affect pupariation timing. (A to E) At 66 h AEL, larvae of each genotype were collected, and real-time RT-PCR analysis was performed. The following UAS-RNAi lines were used: *CG12477* (V1, VDRC 102882; V3, VDRC 31944), *CG5334* (V1, VDRC 102765), and *CG5347* (V1, VDRC 110427; V2, VDRC 22089). Values are presented as mean ± SEM from three independent experiments. (F to H) The percentage of larvae of the indicated genotypes that formed pupa was plotted relative to time in hours A3L. Values are presented as mean ± SEM. (F) Act-G4, n = 120; UAS-CG12477(V1), n = 121; Act > UAS-12477(V1), n = 119 (G) Act-G4, n = 121; UAS-CG12477(V1), n = 121; Act > UAS-12477(V1), n = 101. (H) Act-G4, n = 78; UAS-CG12477(V1), n = 64; Act > UAS-12477(V1), n = 76.

of the 3rd instar, wherein the expression of genes was downregulated by *actin*-Gal4 driving UAS-RNAi for each gene. First, the extent of knockdown was verified by real-time qRT-PCR analysis. For CG12477, two RNAi lines (V102882 and V31944) showed a 60-80% reduction compared with parental control larvae (Figs. 3A and 3B). For CG5334, one RNAi line (V102765) showed a 60% reduction compared with parental control larvae (Fig. 3C). For CG5347, two RNAi lines (V110427 and V22089) showed a 40-50% reduction (Figs. 3D and 3E). Thus, we examined the pupariation timing of CG12477 and CG5334 knockdown larvae as these lines showed the significant reduction in mRNA levels. One line of CG12477 knockdown larvae (V102882) formed pupae in a slightly delayed manner compared with two parental lines considering 50% pupariation, but all three genotypes of larvae eventually formed pupae about the same time (Fig. 3F). Two other larvae expressing CG12477 and CG5334 RNAi did not show developmental delays compared with two parental control lines (Figs. 3G and 3H). Thus, we conclude that knockdown of the three *mkrn1* paralogs does not exert strong effects on developmental timing. Nonetheless, the possibility that the knockdown was not sufficient to produce significant effects on developmental timing cannot be ruled out.

Ecdysone-synthesizing enzyme expression was reduced in MKRN1-null larvae

Developmental timing in Drosophila is regulated by concerted actions of the neuroendocrine system (Yamanaka et al., 2013). The PG in the ring gland is the principal organ producing the steroid hormone ecdysone, which controls all developmental transitions (Huang et al., 2008; Rewitz et al., 2006). Thus, we examined whether MKRN1 is expressed in the ring gland. To accomplish this, we raised MKRN1 antibody and confirmed its specificity by western blot analysis (Fig. 4A). In control larvae, the PG was marked by green

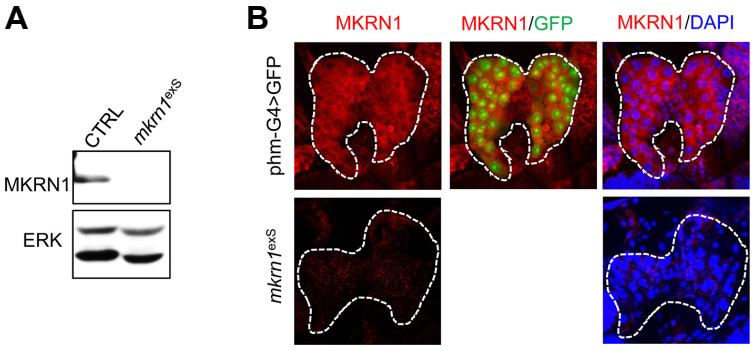

Fig. 4. MKRN1 was expressed in the PG. (A) Protein extracts were prepared from whole flies of the indicated genotypes, and western blot analysis was performed using anti-MKRN1 antibody. ERK served as a loading control. (B) The ring gland was dissected from control (*phantom*-Gal4 > GFP) and *mkrn1*^{exS} 3rd instar larvae and stained with anti-MKRN1 antibody (red). In control larvae, *phantom*-Gal4 driving GFP marked the PG of the ring gland. Stained images were obtained under a 40× objective, and confocal sections were combined. Note that MKRN1 staining was evident in the cytoplasm of cells in the ring gland of control larvae but was absent in *mkrn1*^{exS} larvae.

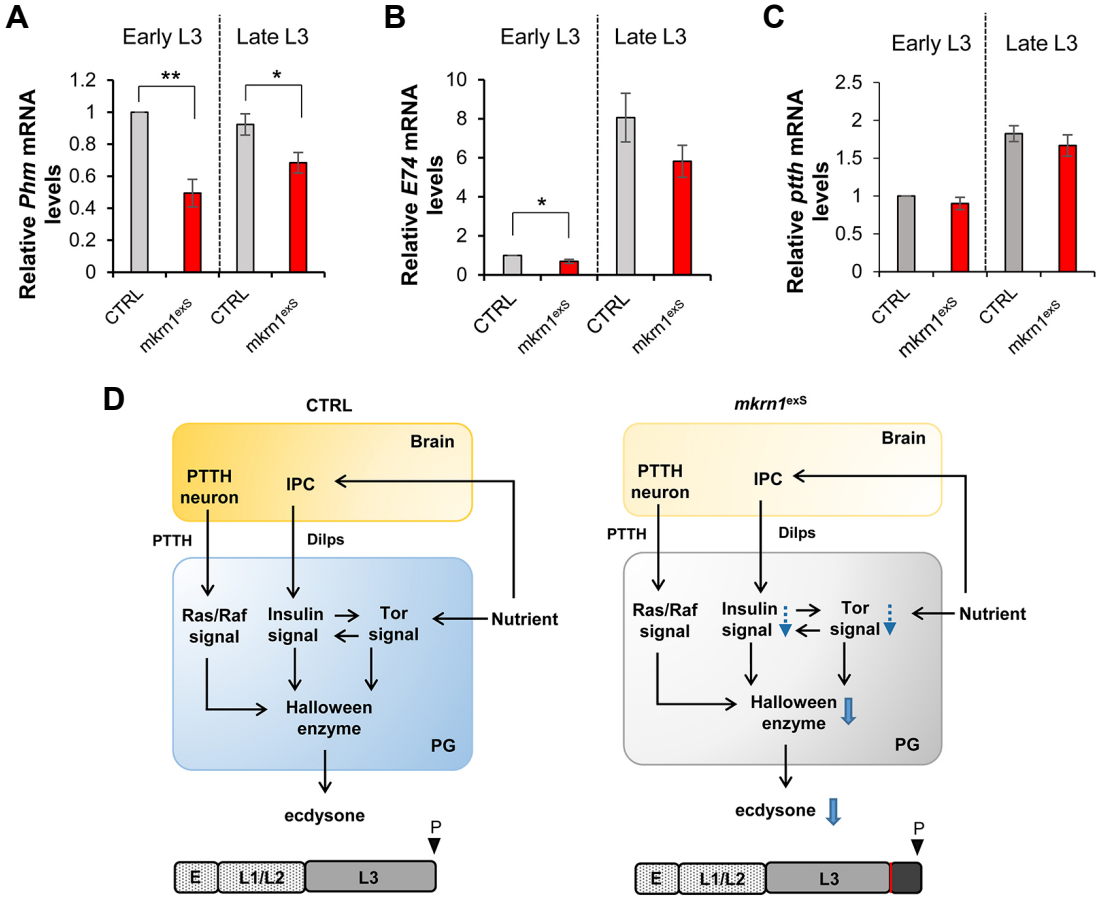

Fig. 5. Deletion of *mkrn1* reduced *phantom* and *E74* mRNA levels. (A to C) Larvae of the indicated genotypes were collected at the early (~74 h AEL) and late (~94 h AEL) 3rd instar stage. Real-time RT-PCR analysis was performed to measure the relative mRNA levels of *phm* (A), E74 (B), and *ptth* (C). Values are presented as mean ± SEM from three independent experiments. * indicates statistically significant difference (Student's t test: $*P < 0.05$; $**P < 0.001$). (D) Proposed model for delayed pupariation (P) in *mkrn1*^{exS}. Larval to pupal maturation is controlled by ecdysone hormone released from the prothoracic gland (PG). Ecdysone synthesis in the PG is catalyzed by a sequence of reactions mediated by enzymes encoded by the Halloween family of genes. The transcription of Halloween enzymes is controlled by upstreaming factors. In control larvae, PTTH regulate Halloween enzyme transcription through Ras/Raf signaling once the animal completes the enough larval growth. In addition, nutritional condition influences the Halloween enzyme transcription through insulin and Tor signaling. In *mkrn1*^{exS}, the loss of MKRN1 might reduce insulin/Tor signaling likely by stabilizing negative regulators of insulin/Tor signaling (dashed blue arrow) (Lee, Jeong et al., 2015; Lee, Han et al., 2018b, See Discussion), thereby downregulates the transcription of Halloween enzyme (straight blue arrow), which results in the lengthening of 3rd instar larval duration (dark grey rectangle). E, embryo; L1, 1st instar larvae; L2, 2nd instar larvae; L3, 3rd instar larvae; IPC, insulin producing cells.

fluorescent protein (GFP) expressed via the *phantom*-GAL4 driver (Fig. 4B). MKRN1 expression was evident in the cytoplasm of cells in the ring gland, including PG cells. By contrast, specific MKRN1 staining was not observed in the ring gland of *mkrn1*^{exS} 3rd instar larvae. These results indicate that MKRN1 is expressed in endocrine tissue in Drosophila, where it may control growth and maturation.

Given that surges of ecdysone at the 3rd instar are required for proper larval-to-pupal transition, we examined whether ecdysone signaling is perturbed in *mkrn1*^{exS} larvae. To test this possibility, we first measured mRNA levels of *phantom*, a Halloween family gene required for synthesis of ecdysone in the PG (Rewitz et al., 2006; Warren et al., 2004). *phantom* mRNA levels were significantly reduced in *mkrn1*^{exS} larvae compared with control larvae at the early (~74 h AEL) and late (~94 h AEL) 3rd instar (Fig. 5A). Next, we measured the transcription of E74, a downstream target gene whose transcription is induced by ecdysone pulses in various target tissues (Boyd et al., 1991). Control larvae showed an ~8-fold increase in E74 transcription from the early to the late 3rd instar stage, corresponding to the steep increase of ecdysone toward the end of the larval period (Fig. 5B). Consistent with our finding that ecdysone synthesis was reduced in *mkrn1*^{exS} larvae, E74 mRNA levels were reduced at the early and late 3rd instar stages, although the difference at the late 3rd instar stage was not statistically significant (Fig. 5B). As PTTH is required for proper production of ecdysone at metamorphosis onset (McBrayer et al., 2007; Rybczynski and Gilbert, 2003), we tested the possibility that a reduction in PTTH would downregulate ecdysone synthesis. No significant difference in *ptth* expression was observed in control and *mkrn1*^{exS} 3rd instar larvae at either early or late stages (Fig. 5C). Taken together, our findings suggest that the loss of MKRN1 reduces the expression of ecdysone-synthesizing enzyme and ecdysone downstream target gene expression in a PG cell-autonomous manner, thereby lengthening the duration from the 3rd instar stage to pupariation.

DISCUSSION

mkrn3 mutations are associated with precocious puberty in humans (Abreu et al., 2013; Christoforidis et al., 2017; Lee et al., 2016b; Nishioka et al., 2017; Schreiner et al., 2014). Although prolonged *mkrn3* expression before the onset of puberty in mice is consistent with an inhibitory role of *mkrn3* in puberty initiation (Abreu et al., 2013), a causal relationship between *makorin* genes and sexual maturation has not been elucidated. Our present results using Drosophila as a model system demonstrate that the loss of MKRN1, an ortholog of mammalian *makorin* genes, lengthened the 3rd instar larval growth period and delayed the onset of metamorphosis, resulting in larger body size. These results support the notion that *makorin* proteins play important roles in fine-tuning the timing of puberty in Drosophila and mammals.

Whereas deletion of *mkrn1* in Drosophila delayed pupariation in our study, *mkrn3* mutations in humans induce precocious sexual maturation (Abreu et al., 2013; Christoforidis et al., 2017; Lee et al., 2016b; Nishioka et al., 2017; Schreiner et al., 2014). The human *mkrn3* gene was originally identified as a Prader-Willi syndrome (PWS)-associated gene. The *mkrn3* gene resides in the maternally imprinted chromosome region 15q11.2-q13, which is deleted in a group of PWS patients (Jong et al., 1999; Butler, 2011). Clinical symptoms of PWS include hypogonadotropic hypogonadism with central adrenal insufficiency, general developmental delay, and obesity in children (Angulo et al., 2015). Several genes other than *mkrn3* are included in the deleted chromosomal region in PWS, with each gene possibly involved in different clinical symptoms (Butler, 2011). Nonetheless, it is intriguing to note the presence of phenotypic similarities between PWS and *mkrn1*-null flies, which exhibit hypogonadotropic hypogonadism and a developmental delay. Although speculative at this stage, complete deletion of *mkrn3* in PWS patients, similar to that in *mkrn1*^{exS} Drosophila, might produce a developmental delay, whereas site-substitutive mutations of *mkrn3* in children with precocious puberty might induce premature development via an antimorphic effect (Abreu et al., 2013; Christoforidis et al., 2017; Lee et al., 2016b; Nishioka et al., 2017; Schreiner et al., 2014).

mkrn1^{exS} larvae exhibited delayed pupariation and prolonged developmental duration at the 3rd instar stage. Moreover, pupa length was increased in *mkrn1*^{exS} larvae compared with control larvae, indicating that loss of MKRN1 did not change growth rate but prolonged larval development. What might have caused this pupariation delay in *mkrn1*^{exS} larvae? Similar to that juvenile growth concludes with a surge of steroid hormone production in vertebrates, 3rd instar-to-pupa metamorphosis is triggered by a surge of the steroid hormone ecdysone in Drosophila. Thus, we reasoned that delayed pupariation might be caused by reduced ecdysone signaling. Indeed, the induction of E74, a direct target of ecdysone signaling, was reduced in *mkrn1*^{exS} larvae as compared with control larvae at the 3rd instar, likely due to reduced ecdysone synthesis. As ecdysone synthesis in the PG is induced by PTTH released from small neuronal groups in the brain, we checked whether PTTH levels were reduced in *mkrn1*^{exS} larvae. There was no difference in PTTH mRNA levels between control and *mkrn1*^{exS} larvae, suggesting that loss of MKRN1 function does not affect PTTH release. During 3rd instar-to-pupa transition, the other crucial factor affecting development is nutrient signaling. Indeed, PTTH is released when larvae reach critical weight, the attainment of which is governed by larval nutrition. Nutrient status is conveyed via insulin/Tor signaling, and mutations that downregulate insulin/Tor signaling throughout the organism delay larval development and produce small adult flies, indicating that both growth rate and developmental timing is affected (Bohni et al., 1999; Oldham et al., 2000; Shingleton et al., 2005). Intriguingly, downregulation of insulin/Tor signaling in a PG-specific manner before the critical weight checkpoint delays metamorphosis and increases body size due mainly to a lengthening of the 3rd instar duration (Colombani et al., 2005; Layalle et al., 2008). Moreover, reduced Tor signaling in PG cells significantly delays transcriptional induction of ecdysone-synthesizing enzymes (Layalle et al., 2008). We are intrigued that these phenotypes are remarkably similar to those observed in *mkrn1*^{exS}

larvae. Thus, we proposed the model that in the *mkrn1*^{exS} 3rd instar stage, insulin/Tor signaling is compromised in PG tissue autonomously before the critical weight check-point, resulting in insufficient ecdysone synthesis and prolonged 3rd instar duration (Fig. 5D, See below). Immunostaining with our newly generated MKRN1 antibody revealed expression of MKRN1 in the cytoplasm of ring gland composite cells including PG, corpus allatum, and corpus cardiacum. This result supports the notion that MKRN1 functions in a PG cell-autonomous manner to control ecdysone synthesis.

makorin gene family members encode zinc finger proteins with a RING domain that have ubiquitin ligase activity and are well conserved across invertebrate and vertebrate species (Gray et al., 2000). Three functional genes, *mkrn1, 2,* and *3,* are identified in mammals, with the molecular functions of MKRN1 being extensively studied in mammals. Several substrates targeted by MKRN1 as an ubiquitin ligase have been identified including hTERT, p53, PTEN, APC, and AMPK. Downregulation of MKRN1 results in accumulation of p53, PTEN, APC, and AMPK in mammalian cancer cell lines or tissues and affects tumorigenesis (Kim et al., 2005; Lee et al., 2009; 2015; 2018a) and cellular energy metabolism (Lee et al., 2018b). Among substrates targeted by MKRN1, PTEN and AMPK are notable negative regulator of insulin/TOR signaling (Nakashima et al., 2000; Shaw et al., 2004). Although the direct target of MKRN1 is not known in Drosophila, it is possible that MKRN1 might function as ubiquitin ligase of PTEN and/or AMPK as in mammals, thereby loss of MKRN1 reduces insulin/Tor activity in *mkrn1*^{exS} larvae ultimately downregulating ecdysone synthesis (Fig. 5D). We cannot rule out the possibility of other substrates of MKRN1 that might function in a PG cell-specific manner. Indeed, a previous study reports that MKRN1 functions as a ribonucleoprotein in embryonic stem cells (Cassar et al., 2015), with MKRN1 being associated with mRNAs and RNA-binding proteins and controlling mRNA metabolism independently of its ubiquitin ligase activity. Further studies are needed to identify MKRN1 downstream targets in the PG. In addition, it would be interesting to test whether MKRN3 regulates developmental timing in mammals by affecting hypothalamic cell nutrient signaling.

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REFERENCES

Abreu, A.P., Dauber, A., Macedo, D.B., Noel, S.D., Brito, V.N., Gill, J.C., Cukier, P., Thompson, I.R., Navarro, V.M., Gagliardi, P.C., et al. (2013). Central precocious puberty caused by mutations in the imprinted gene MKRN3. N. Engl. J. Med. *368*, 2467-2475.

Angulo, M.A., Butler, M.G., and Cataletto, M.E. (2015). Prader-Willi syndrome: a review of clinical, genetic, and endocrine findings. J. Endocrinol. Invest. *38*, 1249-1263.

Bohne, A., Darras, A., D'Cotta, H., Baroiller, J.F., Galiana-Arnoux, D., and Volff, J.N. (2010). The vertebrate makorin ubiquitin ligase gene family has been shaped by large-scale duplication and retroposition from an ancestral gonad-specific, maternal-effect gene. BMC Genomics *11*, 721.

Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell *97*, 865-875.

Boyd, L., O'Toole, E., and Thummel, C.S. (1991). Patterns of E74A RNA and protein expression at the onset of metamorphosis in Drosophila. Development *112*, 981-995.

Butler, M.G. (2011). Prader-Willi Syndrome: obesity due to genomic imprinting. Curr. Genomics *12*, 204-215.

Caldwell, P.E., Walkiewicz, M., and Stern, M. (2005). Ras activity in the Drosophila prothoracic gland regulates body size and developmental rate via ecdysone release. Curr. Biol. *15*, 1785-1795.

Carel, J.C., Lahlou, N., Roger, M., and Chaussain, J.L. (2004). Precocious puberty and statural growth. Hum. Reprod Update *10*, 135-147.

Cassar, P.A., Carpenedo, R.L., Samavarchi-Tehrani, P., Olsen, J.B., Park, C.J., Chang, W.Y., Chen, Z., Choey, C., Delaney, S., Guo, H., et al. (2015). Integrative genomics positions MKRN1 as a novel ribonucleoprotein within the embryonic stem cell gene regulatory network. EMBO Rep. *16*, 1334-1357.

Christoforidis, A., Skordis, N., Fanis, P., Dimitriadou, M., Sevastidou, M., Phelan, M.M., Neocleous, V., and Phylactou, L.A. (2017). A novel MKRN3 nonsense mutation causing familial central precocious puberty. Endocrine *56*, 446-449.

Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carre, C., Noselli, S., and Leopold, P. (2005). Antagonistic actions of ecdysone and insulins determine final size in Drosophila. Science *310*, 667-670.

Gajdos, Z.K., Hirschhorn, J.N., and Palmert, M.R. (2009). What controls the timing of puberty? An update on progress from genetic investigation. Curr. Opin. Endocrinol. Diabetes Obes. *16*, 16-24.

Golub, M.S., Collman, G.W., Foster, P.M., Kimmel, C.A., Rajpert-De Meyts, E., Reiter, E.O., Sharpe, R.M., Skakkebaek, N.E., and Toppari, J. (2008). Public health implications of altered puberty timing. Pediatrics *121 Suppl. 3*, S218-230.

Gray, T.A., Azama, K., Whitmore, K., Min, A., Abe, S., and Nicholls, R.D. (2001). Phylogenetic conservation of the makorin-2 gene, encoding a multiple zinc-finger protein, antisense to the RAF1 proto-oncogene. Genomics *77*, 119-126.

Gray, T.A., Hernandez, L., Carey, A.H., Schaldach, M.A., Smithwick, M.J., Rus, K., Marshall Graves, J.A., Stewart, C.L., and Nicholls, R.D. (2000). The ancient source of a distinct gene family encoding proteins featuring RING and C(3)H zinc-finger motifs with abundant expression in developing brain and nervous system. Genomics *66*, 76-86.

Gunawardhana, K.L., and Hardin, P.E. (2017). VRILLE controls PDF neuropeptide accumulation and arborization rhythms in small ventrolateral neurons to drive rhythmic behavior in drosophila. Curr. Biol. *27*, 3442-3453 e3444.

Huang, X., Warren, J.T., and Gilbert, L.I. (2008). New players in the regulation of ecdysone biosynthesis. J. Genet. Genomics *35*, 1-10.

Jong, M.T., Carey, A.H., Caldwell, K.A., Lau, M.H., Handel, M.A.,

Driscoll, D.J., Stewart, C.L., Rinchik, E.M., and Nicholls, R.D. (1999). Imprinting of a RING zinc-finger encoding gene in the mouse chromosome region homologous to the Prader-Willi syndrome genetic region. Hum. Mol. Genet. *8*, 795-803.

Kim, J.H., Park, S.M., Kang, M.R., Oh, S.Y., Lee, T.H., Muller, M.T., and Chung, I.K. (2005). Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT. Genes. Dev. *19*, 776-781.

Layalle, S., Arquier, N., and Leopold, P. (2008). The TOR pathway couples nutrition and developmental timing in Drosophila. Dev. Cell *15*, 568-577.

Lee, E., Cho, E., Kang, D.H., Jeong, E.H., Chen, Z., Yoo, S.H., and Kim, E.Y. (2016a). Pacemaker-neuron-dependent disturbance of the molecular clockwork by a Drosophila CLOCK mutant homologous to the mouse Clock mutation. Proc. Natl. Acad. Sci. USA *113*, E4904-4913.

Lee, E.W., Lee, M.S., Camus, S., Ghim, J., Yang, M.R., Oh, W., Ha, N.C., Lane, D.P., and Song, J. (2009). Differential regulation of p53 and p21 by MKRN1 E3 ligase controls cell cycle arrest and apoptosis. EMBO J. *28*, 2100-2113.

Lee, H.K., Lee, E.W., Seo, J., Jeong, M., Lee, S.H., Kim, S.Y., Jho, E.H., Choi, C.H., Chung, J.Y., and Song, J. (2018a). Ubiquitylation and degradation of adenomatous polyposis coli by MKRN1 enhances Wnt/beta-catenin signaling. Oncogene *37*, 4273-4286.

Lee, H.S., Jin, H.S., Shim, Y.S., Jeong, H.R., Kwon, E., Choi, V., Kim, M.C., Chung, I.S., Jeong, S.Y., and Hwang, J.S. (2016b). Low frequency of MKRN3 mutations in central precocious puberty among Korean girls. Horm. Metab. Res. *48*, 118-122.

Lee, M.S., Han, H.J., Han, S.Y., Kim, I.Y., Chae, S., Lee, C.S., Kim, S.E., Yoon, S.G., Park, J.W., Kim, J.H., et al. (2018b). Loss of the E3 ubiquitin ligase MKRN1 represses diet-induced metabolic syndrome through AMPK activation. Nat. Commun. *9*, 3404.

Lee, M.S., Jeong, M.H., Lee, H.W., Han, H.J., Ko, A., Hewitt, S.M., Kim, J.H., Chun, K.H., Chung, J.Y., Lee, C., et al. (2015). PI3K/AKT activation induces PTEN ubiquitination and destabilization accelerating tumourigenesis. Nat. Commun. *6*, 7769.

McBrayer, Z., Ono, H., Shimell, M., Parvy, J.P., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-Villemant, C., Gilbert, L.I., and O'Connor, M.B. (2007). Prothoracicotropic hormone regulates developmental timing and body size in Drosophila. Dev. Cell *13*, 857-871.

Mirth, C., Truman, J.W., and Riddiford, L.M. (2005). The role of the prothoracic gland in determining critical weight for metamorphosis in Drosophila melanogaster. Curr. Biol. *15*, 1796-1807.

Mirth, C.K., and Shingleton, A.W. (2012). Integrating body and organ size in Drosophila: recent advances and outstanding problems. Front Endocrinol. (Lausanne) *3*, 49.

Nakashima, N., Sharma, P.M., Imamura, T., Bookstein, R., and Olefsky, J.M. (2000). The tumor suppressor PTEN negatively regulates insulin signaling in 3T3-L1 adipocytes. J. Biol. Chem. *275*, 12889-12895.

Nishioka, J., Shima, H., Fukami, M., Yatsuga, S., Matsumoto, T., Ushijima, K., Kitamura, M., and Koga, Y. (2017). The first Japanese case of central precocious puberty with a novel MKRN3 mutation. Hum. Genome Var. *4*, 17017.

Oldham, S., Montagne, J., Radimerski, T., Thomas, G., and Hafen, E. (2000). Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. Genes. Dev. *14*, 2689-2694.

Palmert, M.R., and Hirschhorn, J.N. (2003). Genetic approaches to stature, pubertal timing, and other complex traits. Mol. Genet. Metab. *80*, 1-10.

Rewitz, K.F., Rybczynski, R., Warren, J.T., and Gilbert, L.I. (2006). The Halloween genes code for cytochrome P450 enzymes mediating synthesis of the insect moulting hormone. Biochem. Soc. Trans. *34*, 1256-1260.

Rewitz, K.F., Yamanaka, N., and O'Connor, M.B. (2013). Developmental checkpoints and feedback circuits time insect maturation. Curr. Top. Dev. Biol. *103*, 1-33.

Rybczynski, R., and Gilbert, L.I. (2003). Prothoracicotropic hormone stimulated extracellular signal-regulated kinase (ERK) activity: the changing roles of Ca(2+)- and cAMP-dependent mechanisms in the insect prothoracic glands during metamorphosis. Mol. Cell Endocrinol. *205*, 159-168.

Schreiner, F., Gohlke, B., Hamm, M., Korsch, E., and Woelfle, J. (2014). MKRN3 mutations in familial central precocious puberty. Horm. Res. Paediatr. *82*, 122-126.

Shaw, R.J., Bardeesy, N., Manning, B.D., Lopez, L., Kosmatka, M., DePinho, R.A., and Cantley, L.C. (2004). The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell *6*, 91-99.

Shingleton, A.W., Das, J., Vinicius, L., and Stern, D.L. (2005). The temporal requirements for insulin signaling during development in Drosophila. PLoS Biol. *3*, e289.

Tena-Sempere, M. (2013). Keeping puberty on time: novel signals and mechanisms involved. Curr. Top. Dev. Biol. *105*, 299-329.

Thummel, C.S. (2001). Molecular mechanisms of developmental timing in C. elegans and Drosophila. Dev. Cell *1*, 453-465.

Walkiewicz, M.A., and Stern, M. (2009). Increased insulin/insulin growth factor signaling advances the onset of metamorphosis in Drosophila. PLoS One *4*, e5072.

Warren, J.T., Petryk, A., Marques, G., Parvy, J.P., Shinoda, T., Itoyama, K., Kobayashi, J., Jarcho, M., Li, Y., O'Connor, M.B., et al. (2004). Phantom encodes the 25-hydroxylase of Drosophila melanogaster and Bombyx mori: a P450 enzyme critical in ecdysone biosynthesis. Insect Biochem. Mol. Biol. *34*, 991-1010.

Yamanaka, N., Rewitz, K.F., and O'Connor, M.B. (2013). Ecdysone control of developmental transitions: lessons from Drosophila research. Annu. Rev. Entomol. *58*, 497-516.