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A new pathway in the control of the initiation of puberty: the *MKRN3* gene

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Abstract

Pubertal timing is influenced by complex interactions among genetic, nutritional, environmental, and socioeconomic factors. The role of *MKRN3*, an imprinted gene located in the Prader-Willi syndrome critical region (chromosome 15q11-q13), in pubertal initiation was first described in 2013 after the identification of deleterious *MKRN3* mutations in five families with central precocious puberty (CPP) using whole-exome sequencing analysis. Since then, additional loss-of-function mutations of *MKRN3* have been associated with the inherited premature sexual development phenotype in girls and boys from different ethnic groups. In all of these families, segregation analysis clearly demonstrated autosomal dominant inheritance with complete penetrance, but with exclusive paternal transmission, consistent with the monoallelic expression of *MKRN3* (a maternally imprinted gene). Interestingly, the hypothalamic *Mkrn3* mRNA expression pattern in mice correlated with a putative inhibitory input on puberty initiation. Indeed, the

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initiation of puberty depends on a decrease in factors that inhibit the release of GnRH combined with an increase in stimulatory factors. These recent human and animal findings suggest that *MKRN3* has an inhibitory role in the reproductive axis to represent a new pathway in pubertal regulation.

Introduction

Puberty, a complex biologic process involving sexual maturation and accelerated linear growth, is initiated when the pulsatile secretion of gonadotropin-releasing hormone (GnRH) increases after a quiescent period during childhood. The regulation of puberty initiation remains one of the great mysteries of human biology and it is thought that a conjunction of factors play a role to initiate puberty.

Environmental and metabolic factors are important regulators of pubertal development, but these influences are superimposed upon substantial genetic control. Similar timing of puberty shared by mothers and her children, and within racial groups, suggests a genetic component to the onset of puberty (Herman-Giddens, et al. 1997; Zacharias and Wurtman 1969). Substantial efforts have been made to identify genes that are responsible for the initiation of puberty (Ojeda and Lomniczi 2014a). The identification of these genes is critical for advancing the understanding of the neuroendocrine regulation of puberty initiation.

In an attempt to identify genes operating within the neuroendocrine brain that ultimately regulate the reproductive axis, researchers have been studying patients with pubertal disorders. The hypothalamic-pituitary-gonadal axis regulates puberty initiation and reproduction. GnRH is produced and secreted by the hypothalamus in a pulsatile fashion during the embryonic and neonatal phases of life. GnRH secretion is actively inhibited during infancy and puberty begins with the reactivation of its secretion. GnRH deficiency results in hypogonadotropic hypogonadism, in which patients fail to develop puberty and are usually infertile. Conversely, early reactivation of GnRH secretion results in central precocious puberty (CPP). Whilst several genes have been detected in association with GnRH deficiency and have contributed to the current knowledge of GnRH regulation (Bianco and Kaiser 2009; Semple and Topaloglu 2010), genes linked to CPP have until recently only been identified subsequent to their association with hypogonadotropic hypogonadism, such as *KISS1* and *KISS1R.* However, only rare genetic defects in KISS1 and its receptor have been identified in patients with CPP (Silveira, et al. 2010; Teles, et al. 2008).

The advances in sequencing methods permitted the detection of new genes implicated in the neuroendocrine regulation of puberty. Using exome sequencing analysis and studying familial cases of CPP, genetic defects in a gene with no previous link to the hypothalamic-pituitary-gonadal axis, *MKRN3*, were identified as the cause of premature sexual development in one-third of the families (Abreu, et al. 2013). *MKRN3* is located on the long arm of chromosome 15 in the Prader-Willi syndrome critical region and is maternally imprinted (discussed below). Subsequently, we showed that mutations in *MKRN3* are also the cause of CPP in apparently sporadic cases (Macedo, et al. 2014). These findings were

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significant contributions to the field and will advance the understanding of the pubertal process. We will review the genetic defects identified in patients with CPP and their clinical implications, and discuss here the possible role of MKRN3 within the reproductive axis.

Loss-of-function mutations of MKRN3 cause familial CPP

The role of *MKRN3* in pubertal initiation was first described in 2013 after a comprehensive genetic study of several families with CPP (Abreu et al. 2013). In this study, the authors investigated 40 members of 15 families with CPP from different ethnic groups (12 Brazilian, 2 American and 1 Belgian families), and applying whole exome sequencing four deleterious *MKRN3* mutations - three frameshift and a missense mutation (Figure 1) - were detected in five of these families (33%). Both sexes were equally affected by *MKRN3* mutations (8 girls and 7 boys) (Table 1).

More recently, Macedo et *al.* (Macedo, et al. 2014) studied 215 unrelated children (207 girls and 8 boys) with CPP from three different University Medical Centers and identified five novel heterozygous mutations in 8 unrelated Brazilian girls, including 4 frameshift variants and one missense variant (Figure 1, Table 1). No family history of premature sexual development was reported in the majority of these studied patients. However, segregation analysis was performed for five of these 8 girls, and in all cases demonstrated that the mutant allele was paternally inherited in all families with *MKRN3* mutations.

In order to investigate if the CPP phenotype could arise from loss of *MKRN3* expression by the paternal allele due to a *de novo* deletion, maternal uniparental disomy, or an imprinting defect, Macedo *et al.* (Macedo et al. 2014) investigated 52 patients with familial and sporadic CPP, without known *MKRN3* sequence defects, by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) of chromosome 15q11. No copy number changes or methylation abnormalities were detected in the 15q11 locus, suggesting that epigenetic defects involving this *locus* are likely rare mechanisms in this disorder (Macedo et al. 2014).

Recently, other investigators have also reported *MKRN3* defects associated with familial CPP. Settas et *al.* (Settas, et al. 2014) described a novel heterozygous missense mutation (p.Cys340Gly) in *MKRN3* in two Greek siblings, a girl with CPP and a boy with early puberty. Soon thereafter, Schreiner *et al.* (Schreiner, et al. 2014) identified two heterozygous *MKRN3* mutations (p.Glu111* and p.Ala162Glyfs*14) in affected members of 2 German families. Lastly, de Vries et al (de Vries, et al. 2014) identified a novel missense mutation (p.His420Gln) in four siblings, including a boy, from an Ashkenazi-Sephardic Jewish family (Figure 1, Table 1). These studies further expanded the *MKRN3* mutational spectrum.

Clinical Features of CPP associated with *MKRN3* mutations

All patients with CPP carrying loss-of-function mutations in *MKRN3* exhibited typical clinical and hormonal features of premature activation of the reproductive axis, including early pubertal signs, such as breast, testes, and pubic hair development, accelerated linear growth, advanced bone age, and elevated basal and/or GnRH-stimulated LH levels (Table 1). Except for two related patients (one girl and her brother) who presented with esotropia

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(Abreu et al. 2013), which is a minor criterion for Prader-Willi syndrome (PWS), no other signs of PWS were detected in any patient with CPP caused by *MKRN3* defects. Another female patient had mild nonspecific syndromic features, including a high-arched palate, dental abnormalities, clinodactyly and hyperlordosis (Macedo et al. 2014). Regarding the therapeutic response of CPP patients with *MKRN3* defects, six of eight patients have demonstrated adequate responses to conventional treatment with depot GnRH agonists to date (Macedo et al. 2014).

In patients with CPP due to *MKRN3* defects, the median age of puberty onset was 6.0 years in girls (ranging from 3.0 to 7.5 years) and 8.25 years in boys (ranging from 5.9 to 9.0 years) (Abreu et al. 2013; de Vries et al. 2014; Schreiner et al. 2014; Settas et al. 2014), suggesting that the *MKRN3* mutations may affect girls more severely than boys (Table 1). Given the median age of pubertal onset of affected patients with *MKRN3* mutations, it is speculated that the prepubertal inhibitory tonus on GnRH secretion took place normally, but was lost prematurely in patients with *MKRN3* mutations. This clinical observation suggests that MKRN3 may not be crucial for GnRH suppression after the mini-puberty of early infancy, but that the downregulation of MKRN3 plays a relevant role for the re-emergence of GnRH pulses in the pubertal onset (Macedo et al. 2014).

MKRN3 mutations and polymorphisms

Currently, 12 distinct loss-of-function mutations of *MKRN3* have been described in 30 patients (22 girls and 8 boys) with CPP from 17 families from different ethnicities (Figure 1, Table 1). Remarkably, eight of these mutations encode either premature stop codons or frameshift mutations (Table 1). The four missense mutations (p.Cys340Gly, p.Arg365Ser, p.Phe417Ile and p.His420Gln) were located within a zinc finger motif or a RING finger motif (Figure 1), regions predicted to be involved in RNA binding and ubiquitin ligase activity of the protein respectively, and essential for protein function. All missense mutations are predicted to be pathogenic by *in silico* analysis. Additionally, *ab initio* modeling of the mutations p.Arg365Ser and p.Cys340Gly predicts that these mutations lead to significant structural perturbations in the 3D structure of the RING finger motif (Settas et al. 2014). The substitution of histidine 420 with glutamine in the MKRN3 protein is predicted reduce the affinity between the Zn ion binding site and the relevant Zn, disrupting the binding pocket leading to unfolding of the finger (de Vries et al. 2014). Interestingly, most of the *MKRN3* mutations (64%) were located in the amino-terminal region of the protein, which is encoded by a poly C rich sequence, suggesting that this area may be a potential hotspot.

The important role of MKRN3 in human puberty initiation was reinforced recently by large genome-wide and custom-genotyping arrays in up to 182,416 women of European descent (57 studies) (Perry, et al. 2014). There was evidence of 123 signals at 106 genomic *loci* associated with age at menarche. Three of these *loci* were located in imprinted regions, including the *MKRN3 locus*, demonstrating parent-of-origin-specific associations concordant with known parental expression patterns (Perry et al. 2014). This study suggests that not only are rare variants in *MKRN3* associated with CPP, but that more common variants/polymorphisms may be associated with changes in the timing of puberty (as

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reflected by age of menarche) within the normal range and within the general population. This was a remarkable extension of the findings of rare variants in a disease such as CPP to more common variants in a polygenic trait such as age of menarche. The lack of parent-of-origin-specific analysis in previous GWAS studies may explain why *MKRN3* has not been previously associated with age of menarche (Elks, et al. 2010; He, et al. 2009).

MKRN3 Gene and Protein Structure and Expression

Makorin ring finger 3 (*MKRN3*) was first cloned in 1999 by Jong *et al.* (Jong, et al. 1999b) during a study of the Prader-Willi/Angelman syndrome (PW/AS) critical region. They identified a cDNA in the PW/AS region encoding a zinc finger protein, initially named *ZNF127* (zinc finger protein 127) and later renamed *MKRN3*. The functional and physiological relevance of MKRN3 is not known and despite its location in the PWS critical region, its role in this syndrome is also unclear. An antisense transcript was concomitantly identified and named *ZNF127AS*. The antisense gene is not translated and is expressed weakly during fetal development and at very low levels in adult brain regions (Jong et al. 1999b). Similar to other antisense genes, *ZNF127AS* may regulate the expression of the "sense" gene (*MKRN3*).

MKRN3 is located on human chromosome 15q11–13 (chromosome 7C in mouse) in a region that contains a cluster of imprinted genes associated with two different neurobehavioral disorders (Lalande 1996; Nicholls, et al. 1998). Mutations or loss of expression of the maternally expressed gene *UBE3A* lead to Angelman syndrome (AS), while PWS is thought to be a contiguous gene syndrome arising from the loss of expression of multiple paternally expressed genes, including *MKRN3.* The report of two patients with PWS with a deletion of the 15q11–13 *locus* that did not include *MKRN3* suggested that this gene is not required for the development of the syndrome, but we still cannot rule out a role for *MKRN3* in some of the clinical features of PWS (Kanber, et al. 2009).

MKRN3 differential allele expression occurs through silencing of the *MKRN3* maternal allele, which is associated with 5' CpG island methylation. (Hershko, et al. 1999; Jong, et al. 1999a). Methylation of *MKRN3* and the other imprinted genes located within the PW/AS region of chromosome 15 is coordinately regulated by a bipartite imprinting control center (IC), composed of a sequence around the *SNRPN* promoter, the PWS-IC, and an 880 bp sequence located 35 kb upstream, the AS-IC (Rabinovitz, et al. 2012). The molecular mechanisms of IC function are not well understood, but it has been postulated, based on studies in humans and mouse models, that the PWS-IC is a positive regulatory element and activates paternal-specific gene expression (Bielinska, et al. 2000; Bressler, et al. 2001), whereas the AS-IC functions in the maternal imprint by allele-specific repression of the PWS-IC to prevent the paternal imprinting program (Ohta, et al. 1999; Rodriguez-Jato, et al. 2005; Shemer, et al. 2000) (Jong et al. 1999a).

MKRN3 is an intronless gene, and likely arose by germline retrotransposition from the *Makorin ring finger protein 1* gene (*MKRN1*), a highly transcribed, intron-containing gene, 180-90 million years ago (Rapkins, et al. 2006). *MKRN1* is the ancestral founder of the Makorin gene family (Gray, et al. 2000). Several other retrocopies of *MKRN1* have been

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identified in mammalian genomes, most of them probably corresponding to pseudogenes. Nine *MKRN* family *loci* distributed throughout the human genome have been identified so far, with only three functional genes - *MKRN1*, *MKRN2* and *MKRN3* - identified in vertebrates (Bohne, et al. 2010; Gray et al. 2000).

The MAKORIN family

The *MKRN* gene family encodes putative ribonucleoproteins with a distinctive array of zinc-finger motifs including several C3H zinc fingers, a makorin-specific Cys-His arrangement, and a RING zinc finger (Jong *et al.*, 1999a; b). In particular, MKRN3 has a centrally located RING finger motif (C3HC4), two amino-terminal C3H zinc finger motifs followed by the unique pattern of conserved Cys-His residues called a Makorin zinc finger motif, and a carboxy-terminal C3H zinc finger motif (Jong et al. 1999b) (Figure 1). *MKRN3* is highly conserved among species, and the mouse and human MKRN3 amino acid sequences share 69% identity and 82% similarity (Jong et al. 1999b). Mice and humans usually do not have conserved untranslated regions (UTRs), yet the MKRN3 3'-UTR has 90% identity between these two species, implying a functional significance to this region of *MKRN3*. *MKRN3* is ubiquitously expressed in adult human tissue, with highest expression levels in the testis. In the fetus, it is highly expressed in the central nervous system and is expressed in post-meiotic sperm germ cells, particularly in round spermatids (Jong et al. 1999b).

The characteristic arrangement of cysteine (Cys or C) and histidine (His or H) residues in the zinc finger proteins in MKRN3 can allow some predictions of its function. C3H zinc fingers have been identified in RNA-binding proteins (Barabino, et al. 1997; Murray, et al. 1997). RING zinc fingers have been shown to mediate protein:protein interactions (Schwabe and Klug 1994). More recent evidence suggests that the RING zinc finger is a signature domain for E3 ligases, a category of enzymes mediating the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to target protein substrates. (Deshaies and Joazeiro 2009). Ubiquitination can have diverse effects on the protein substrate, varying from proteasome-dependent proteolysis to modulation of protein function and/or localization (Behrends and Harper 2011; Deshaies and Joazeiro 2009). The multiple types and number of zinc finger motifs in makorin proteins suggest possible multiple cellular functions for this protein.

Makorin proteins share a highly homologous amino acid sequence, particularly in the zinc finger domains (Figure 2), suggesting that they may share similar functions or regulatory mechanisms. Although there are not many studies on MKRN3 function to date, MKRN1 function has been better explored. A possible important cellular function for MKRN1 is supported by the high identity (92%) between the human and murine orthologs and the ubiquitous protein expression in human and mouse tissues (Gray et al. 2000). MKRN1 acts as an E3 ubiquitin ligase, inducing degradation of human telomerase reverse transcriptase (hTERT), viral capsid proteins, p53 and p21 cell cycle regulators, and peroxisome proliferator activated receptor gamma (PPARγ) (Kim, et al. 2014; Kim, et al. 2005; Lee, et al. 2009).

An alternative mechanism of action for MKRN1 was described when it was identified as a repressor of c-Jun transcriptional activity using a yeast assay. Through regulation of RNA

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polymerase II-dependent transcription, MKRN1 can have either negative or positive effects on gene expression (Omwancha, et al. 2006). Interestingly, it was shown that the disruption of ubiquitin ligase activity of MKRN1 did not affect its inhibitory transcriptional activity, suggesting that this function is independent of protein ubiquitination (Omwancha et al. 2006).

MKRN2 is also ubiquitously expressed (Gray et al. 2000). Like other members of this family, MKRN2 is highly conserved throughout evolution. It has been shown that mkrn2 negatively regulates neurogenesis via PI3K/Akt signaling in *Xenopus* embryos; however, the detailed molecular mechanisms of this effect and the potential functions of mammalian MKRN2 remain to be studied (Yang, et al. 2008).

MKRN3 presumptively possesses ubiquitin-protein isopeptide ligase (E3) activity, intimated by the presence of the highly conserved C3HC4 RING finger domain. The tandem repeat of C3H zinc fingers may provide high specificity RNA binding (Hudson, et al. 2004), and the unique Cys-His makorin motif has been suggested to be a DNA binding domain. Taken together, the studies of MKRN1 and MKRN2 imply that MKRN3 can likewise act as an E3 ligase, based on the high homology of the RING finger domain (Figure 2). A previous mouse study showed that *Mkrn3* is highly expressed in the hypothalamic arcuate nucleus during the infantile and early juvenile periods, with a reduction in expression at postnatal days 12 to 15, prior to puberty initiation (Abreu et al. 2013). The arcuate nucleus is the site of expression of critical regulators of GnRH secretion, such as kisspeptin, neurokinin B and dynorphin (Navarro, et al. 2011). Puberty is initiated with a loss of inhibitory inputs and a gain in excitatory inputs (Ojeda and Lomniczi 2014b). The *Mkrn3* expression pattern in the hypothalamic arcuate nucleus suggests that this peptide plays a role in the inhibition of GnRH secretion during the prepubertal quiescent period (Figure 3). The decrease in *Mkrn3* expression is hypothesized to be associated with an increase in GnRH stimulatory factors and/or GnRH expression, and it can be postulated that MKRN3 may be acting at the hypothalamic level as an E3 ligase to inhibit stimulatory inputs, so that loss-of-function mutations of MKRN3 result in early activation of the hypothalamic-pituitary-gonadal axis, expressed phenotypically as central precocious puberty. It is also possible that MKRN3 can act as a transcriptional regulator, as has been demonstrated for MKRN1. Data from the Human Protein Atlas shows that MKRN3 is located primarily in the plasma membrane and cytoplasm, but is also located in the nucleus (www.proteinatlas.org). Based on its location in the plasma membrane, we can speculate that MKRN3 may also be involved in endocytosis and downregulation of receptors, as has been demonstrated for some other E3 ligases (Hershko and Ciechanover 1998). The genetic findings from patients with CPP are in agreement with the hypothesis that MKRN3 may act as a ‘brake’ or inhibitor of GnRH secretion during childhood (Hughes 2013). Further studies are needed to elucidate the precise mechanism(s) of action of MKRN3.

Conclusion

Age of puberty initiation is associated with causes of substantial morbidity and mortality. Early age of menarche is associated with breast cancer and cardiovascular disease (Kvale 1992; Lakshman, et al. 2009). Therefore it is important to understand the mechanisms

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controlling puberty initiation. In an attempt to identify genes that will broaden the knowledge on GnRH regulation to bring new genetic screening, and diagnostic and treatment tools, researchers have been trying to identify genes associated with CPP for several years. By studying individuals with hypogonadotropic hypogonadism, a genetic disorder caused by absence or deficiency of GnRH secretion, we learned about important stimulators of GnRH secretion (Bianco and Kaiser 2009; Semple and Topaloglu 2010). Because of the complexity of the mechanisms involved in puberty initiation and the initial failure to identify genes associated with CPP, it has been hypothesized that CPP was not caused by mutations in a single gene, but rather was a consequence of complex interactions among environment, metabolic factors and polygenic defects. However, with the development of new sequencing methodologies, the combination of whole exome sequencing analysis with detailed phenotypic characterization and the careful selection of the correct cohort of patients led to the identification of *MKRN3* as an important regulator of pubertal development. *MKRN3* is the first gene with a probable inhibitory effect on GnRH secretion, with mutations identified with in humans. To date, *MKRN3* is the most common genetic defect associated with CPP. The identification of loss-of-function mutations in this gene can contribute to the diagnosis of CPP, especially in boys in whom the signs of puberty initiation are not easily detectable, thereby helping to make the diagnosis earlier and facilitating treatment decisions. In addition, the presence of *MKRN3* mutations can contribute to early diagnosis of CPP in familial cases and guide genetic counseling. It is not clear if there is a gender difference in the effect of *MKRN3* mutations, but it is evident that mutations in this gene accelerate puberty initiation in both sexes. Although the precise mechanism of regulation of GnRH secretion by MKRN3 is not yet understood, its importance in the hypothalamic-pituitary-axis is indisputable. The studies of *Mkrn3* expression in the hypothalamic arcuate nucleus of mice support the findings that loss-of-function mutations in humans lead to early puberty initiation and strengthen the hypothesis that MKRN3 acts as an inhibitor of GnRH secretion during childhood (Figure 3). Ultimately, the recent GWAS findings linking *MKRN3* with age of menarche have consolidated the involvement of this gene in pubertal timing.

The identification of a putative inhibitory factor in the hypothalamic-pituitary-gonadal axis has opened an exciting new arena in the neuroendocrine field. Further studies will elucidate the precise mechanism of action of this important regulator of GnRH secretion.

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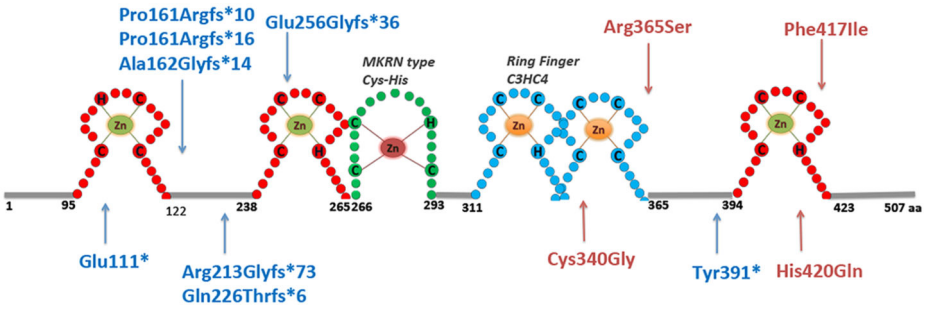

Figure 1. MKRN3 protein structure and mutations identified in patients with central precocious puberty

(Zn) zinc; (H) histidine; (C) cysteine. The three C3H zinc finger motifs are shown in red, the C3HC4 RING finger motif is in blue, and the MKRN-specific Cys–His domain is shown in green. The numbers correspond to the amino acid positions in the protein. Blue mutation labels and arrows indicate the location of frameshift mutations; pink mutation labels and arrows indicate the location of the missense mutations.

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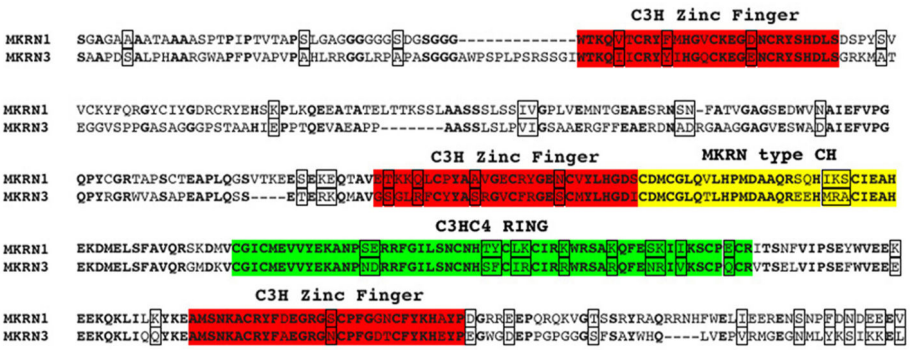

Figure 2. MKRN1 and MKRN3 protein sequence alignment

The two protein sequences share high homology and similarity, especially in the RING finger domains. Bold letters represent conserved amino acids, and squares similar amino acids. Sections highlighted in red represent the three zinc fingers C3H motifs, in yellow the MKRN-specific Cys-His motif, and in green the RING finger domain. MKRN1 NCBI Reference Sequence NP_038474, UniProtKB/Swiss-Prot: Q9UHC7.3. MKRN3 NP_005655.1 and UniProtKB/Swiss-Prot Q13064.1.

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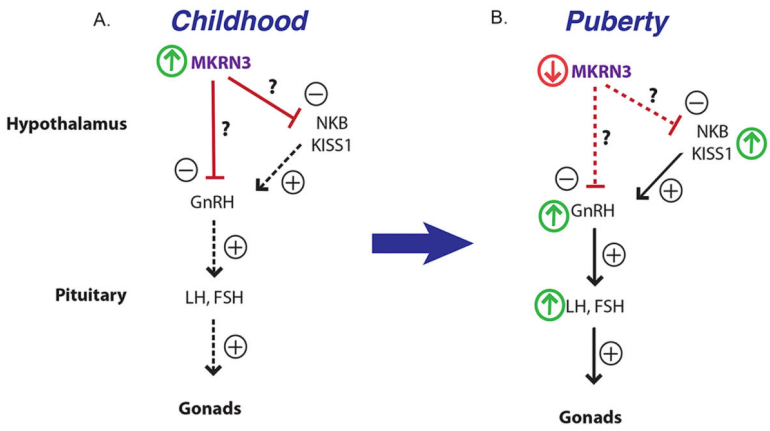

Figure 3. Schematic representation of MKRN3 mechanism of action

Human and mouse studies suggest that MKRN3 acts as an inhibitor of GnRH secretion during childhood (diagram on A), and that a decrease in *MKRN3* expression is associated with an increase in GnRH stimulatory factors and/or GnRH resulting in puberty initiation (diagram on B). NKB, neurokinin B; − inhibition, + stimulation. ↑ increase ↓ decrease.

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Table 1

MKRN3 mutations described in children with CPP from different origins.

Family number	Origin	Patient number	Initial clinical manifestation (Age in Years)		Diagnosis		*MKRN3* mutation		References
				Age (Years)	Breast/ Genital development Tanner stage	Bone age (Years)	DNA	Protein	
1	USA	I	Thelarche (5.7)	6.5	2	7.7	637delC	Arg213Glyfs*73	Abreu AP *et al*, 2013
		II	Gonadarche (8.0)	8.7	3	11	637delC	Arg213Glyfs*73	
		III	Thelarche (6.5)	6.7	2	7.8	637delC	Arg213Glyfs*73	
2	Brazil	I	Thelarche (6.2)	7.0	3	7.0	1171_1172insA	Tyr391*	Abreu AP *et al*, 2013
		II	Thelarche (5.7)	6.0	3	6.0	1171_1172insA	Tyr391*	
3	Brazil	I	Gonadarche and pubarche (5.9)	8.1	3	10	475_476insC	Ala162Glyfs*14	Abreu AP *et al*, 2013
		II	Gonadarche and pubarche unknown	9.7	3	9.7	475_476insC	Ala162Glyfs*14	
4	USA	I	Gonadarche and pubarche (8.5)	8.8	3	11	475_476insC	Ala162Glyfs*14	Abreu AP *et al*, 2013
		II	Thelarche (5.0)	6.5	2	8.3	475_476insC	Ala162Glyfs*14	
5	Brazil	I	Thelarche and pubarche (6.4)	7.8	3	11	482delC	Pro161Argfs*10	Macedo DB *et al*, 2014
6	Brazil	I	Thelarche (5.4)	6.1	3	8.8	482_483insC	Pro161Argfs*16	Macedo DB *et al*, 2014
7	Brazil	I	Thelarche (6.0)	7.9	4	11	482_483insC	Pro161Argfs*16	Macedo DB *et al*, 2014
8	Brazil	I	Thelarche (3.0) and pubarche (6.0)	6.7	3	8.8	482delC	Pro161Argfs*10	Macedo DB *et al*, 2014
9	Brazil	I	Thelarche (4.0)	6.8	4	12	675_676insA	Gln226Thrfs*6	Macedo DB *et al*, 2014
10	Brazil	I	Thelarche and pubarche (6.0)	6.6	3	7.8	766_767delA	Glu256Glyfs*36	Macedo DB *et al*, 2014
11	Brazil	I	Thelarche (6.0) and pubarche (6.3)	6.9	3	9.3	482_483insC	Pro161Argfs*16	Macedo DB *et al*, 2014
12	Belgium	I	Gonadarche and pubarche unknown	9.7	3	12	1095G>T	Arg365Ser	Abreu AP *et al*, 2013
		II	Thelarche (6.2)	6.4	2	9.4*	1095G>T	Arg365Ser	

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Family number	Origin	Patient number	Initial clinical manifestation (Age in Years)		Diagnosis		*MKRN3* mutation		References
				Age (Years)	Breast/ Genital development Tanner stage	Bone age (Years)	DNA	Protein	
		III	Thelarche and pubarche (5.4)	5.7	2	8.5	1095G>T	Arg365Ser	
13	Brazil	I	Thelarche (6.0)	7.1	3	8.8	1249T>A	Phe417Ile	Macedo DB *et al*, 2014
14	Greece	I	Thelarche (6.0)	7.1	3	10	1018T>G	Cys340Gly	Settas N et al, 2014
		II	Gonadarche unknown	9.2	2	10	1018T>G	Cys340Gly	
15	Germany	I	Gonadarche and pubarche (9.0)	10	3	-	331G>T	Glu111*	Schreiner F et al, 2014
		II	Thelarche (6.1)	7.5	3	-	331G>T	Glu111*	
16	Germany	I	Thelarche (7.5)	7.5	-	-	475_476insC	Ala162Glyfs*14	Schreiner F et al, 2014
		II	Thelarche (6.8)	7.1	2	-	475_476insC	Ala162Glyfs*14	
17	Israel	I	Thelarche and pubarche (5.5)	5.8	3	6.8	1260T>G	His420Gln	De Vries L et al, 2014
		II	Gonadarche and pubarche unknown	9.8	3	11.5	1260T>G	His420Gln	
		III	Thelarche and pubarche (5.5)	6.3	3	8.8	1260T>G	His420Gln	
		IV	Thelarche (4.5)	4.5	2	6.0	1260T>G	His420Gln	

E2: estradiol; T: testosterone

Gonadarche or testicular enlargement. Breast/Genital development Tanner stage and bone age assessed at time of diagnosis.

*Bone age at 7.4yr.