Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Abreu AP, Dauber A, Macedo DB, et al. Central precocious puberty caused by mutations in the imprinted gene *MKRN3*. N Engl J Med 2013;368:2467-75. DOI: 10.1056/NEJMoa1302160

Supplementary Appendix

Supplementary Methods:

Genetic studies

Whole exome sequencing of the selected individuals was performed at the Broad Institute. Hybrid selection was performed using Agilent's SureSelect Human All Exon Kit v2 (Agilent Technologies, Santa Clara, CA). We sequenced the samples using the Illumina HiSeq 2000 platform (Illumina Inc, San Diego, CA) and aligned the resulting reads to the human genome version 19 reference genome with Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/).[1] We then applied the Genome Analysis Toolkit (http://www.broadinstitute.org/gatk),[2] base quality score recalibration, and indel realignment, and performed single nucleotide polymorphism and indel discovery and genotyping across all samples simultaneously using variant quality score recalibration.[3] Variants were annotated for functional effect using SnpEff 2.0.5 (http://snpeff.sourceforge.net/).[4]

For PCR amplification of the *MKRN3* the following primers pairs were used: 1F: 5'-GGGGAAGGAAAAAGAGATGC-3', 1R: 5'-AGCCATCTGCTTCCTCTCAG-3'; 2F: 5'-CCAATTGCAACCATTCCTTC-3', 2R: 5'-CACCATAATCCTAGGGGGAAA-3'. Amplification reactions were performed in a final volume of 50 µl containing 20 pmol of each primer, 150 ng template DNA, 200 µmol/L dNTPs, 2.5 U *Taq polymerase,* 2.5 µl

10x buffer containing 1.5 mM MgCl2 and carried out for 30 cycles: denaturation at 94^{o}C for 30 sec, annealing at 59-60^{o}C for 30 sec, extension at 72^{o}C for 1 min, followed by a final extension for 10 min at 72^{o}C. All PCR products were separated on 1% agarose gel electrophoresis and sequenced at the Dana-Farber/Harvard Cancer Center DNA Resource Core (Boston, MA).

Quantitative real-time PCR

One microgram of total RNA was reverse transcribed using the Superscript III cDNA synthesis kit (Invitrogen), followed by quantitative real-time PCR analysis of murine *Mkrn3* on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). SYBR green mix (Bio-Rad, Hercules, CA) was used according to the manufacturer's instructions. Levels of mRNA were normalized to ribosomal protein L19 as an internal control. The following primer pairs were used to amplify *Mkrn3*: F: 5'-AAGCGCATACTGGCATCAAG-3'; R: 5'-AGCCAACGGTCATCAGAGAA-3' and *L19*: F: 5'-CTGAAGGTCAAAGGGAATGTG-3'; R: 5'-GGACAGAGTCTTGATGATCTC-3'. The results were analyzed using ABI Prism 7000 SDS software (Applied Biosystems), and data presented as fold change compared to P10.

Supplementary Results:

Genotype-Phenotype Correlation

Family A

The frameshift heterozygous variant in *MKRN3*, c.637delC, p.Arg213Glyfs*73, was identified in the three affected siblings of this family from the United States of European ancestry and in the unaffected father (Figure 1). DNA from the paternal grandmother was not available for genetic studies but her history of early age of menarche suggests that individual II.1, the father of the affected siblings, inherited this variant from his mother but does not manifest the CPP phenotype because only the paternally inherited *MKRN3* allele is expressed. The unaffected family members III.4, III.5, and II.2 are wild-type for *MKRN3*. The proband presented with thelarche at age 5 years 9 months and pubic hair Tanner stage 2 at 6 years and 6 months (III.1). Her sister also presented with early thelarche at age 6 years 6 months (III.3). Her brother had increased testicular volume at age 8 (III.2). All siblings had pubertal basal and stimulated luteinizing hormone (LH) levels and advanced bone age upon diagnosis of CPP (Table 1). The deletion of cytosine 637 in the *MKRN3* results in a frameshift mutation in the amino acid 213 of the protein and in a premature stop codon generating a truncated protein of 286 instead of 507 amino acids (Figure 2).

Family B

The heterozygous insertion of an adenosine at position 1171 of *MKRN3*, c.1171_1172insA, p.Tyr391*, was identified in the two affected siblings of a Brazilian family (III.1 and III.2) and in their affected father, aunt and grandfather (I.1, II.2 and II.3)

(Figure 1). The two affected siblings presented with early thelarche at age 6 years and 3 months (III.1) and 5 years and 9 months (III.2) and pubertal levels of stimulated LH (Table 1). The insertion of an adenosine at position 1171 of *MKRN3* results in a stop codon at this position and generates a protein of 391 amino acids instead of 507, lacking the last zinc finger motif (Figure 2).

Family C

A novel missense mutation, c.1095G>T, p.Arg365Ser, was identified in the *MKRN3* in all three affected siblings from a Caucasian family in Belgium (Figure 1). The proband presented with thelarche at age 6 years and 3 months (III.1) and her sister with thelarche and pubarche at age 5 years and 5 months (III.3). Upon diagnosis patient III.1 was 6 years and 5 months and patient III.3 was 5 years and 9 months, both had advanced bone age, and pubertal stimulated LH levels (Table 1). The brother presented at 9 years 9 months with advanced bone age, testicular enlargement and Tanner stage 3 pubic hair (III.2). It was not entirely clear at what age puberty began but his first clinical presentation suggested the diagnosis of precocious puberty. The mutation was inherited from their father (II.1), who carries the mutation but is not affected; their mother (II.2) is wild type for *MKRN3*. Both parents had a history of normal pubertal development. Arginine 365 is located in the RING-type zinc finger and is highly conserved among species (Figure 2). The p.Arg365Ser substitution is predicted to affect protein function with a Polyphen2 score of 1.0 and Panther $P_{deleterious}$ of 0.95.

Family D

A heterozygous insertion of a cytosine, c.C475_476insC, p.Ala162Glyfs*14, was identified in two siblings with CPP in a Brazilian family (Figure 1). Patient III.2 has a history of penile and pubic hair maturation at age 5 years 11 months. He sought medical assistance at age 8 years 1 month, presenting with a bone age of 10 years, testicular size of 10 ml bilaterally, and penile length of 9.5 cm. His hormonal profile corroborated the diagnosis of CPP with pubertal basal and stimulated LH levels and high testosterone levels (Table 1). His brother (II.3) also has a history of early penile and pubic hair maturation and sought medical assistance at age 9 years and eight months with Tanner stage 3 pubic hair and testis. Following the pattern of paternal imprinting, both siblings inherited the mutation from their father (II.1), who carries the mutation but is not affected and likely inherited the variant from his mother. However we have neither clinical history nor DNA from his mother (Figure 1). This insertion results in a truncated protein of 176 amino acids (Figure 2).

Family E

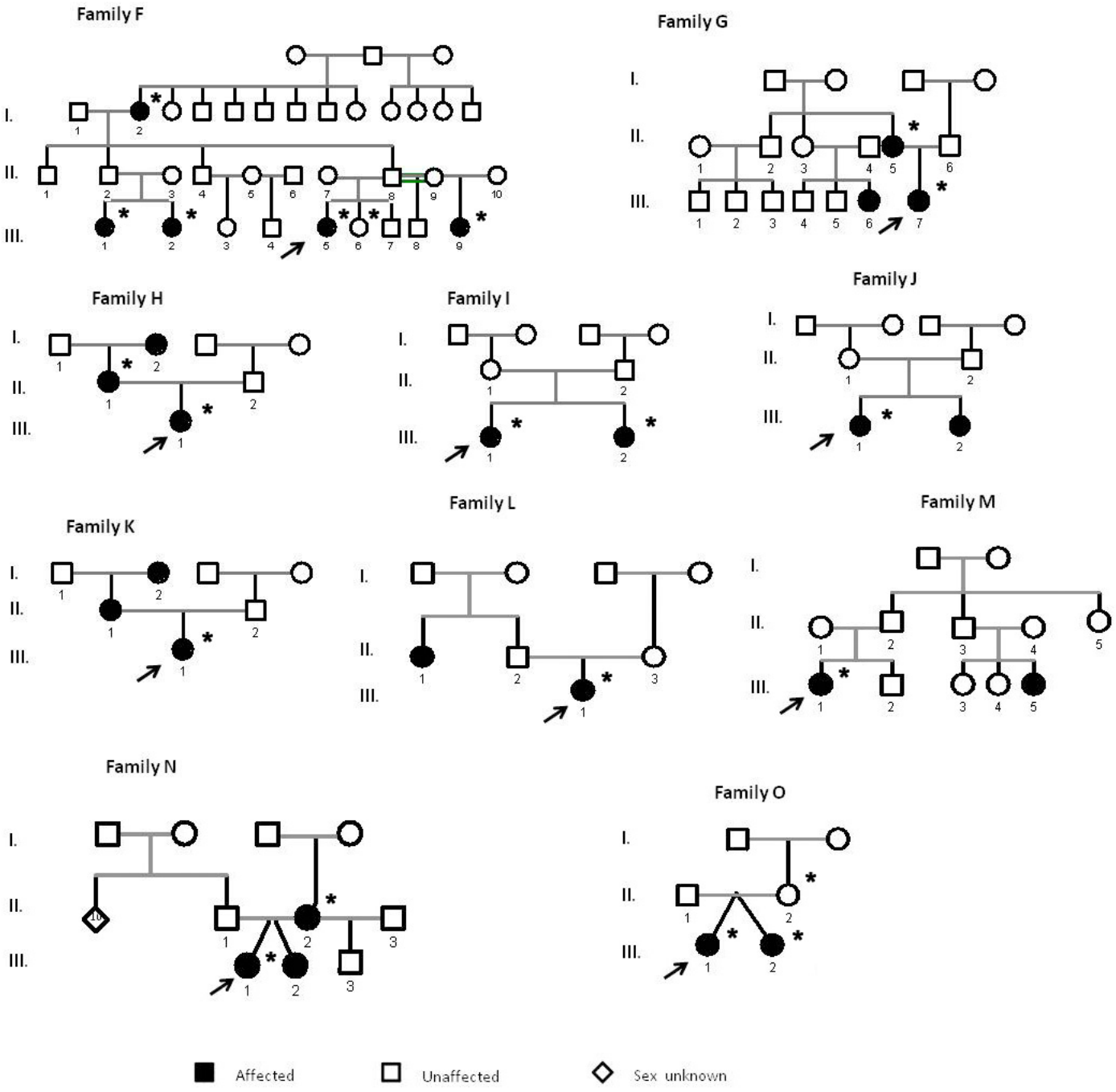
The two affected siblings from this family from the United States of European ancestry, also harbor the variant c.475_476insC, p.Ala162Glyfs*14, identified in family D (Figure 1). The proband (III.1) had increased testicular volume and penile enlargement at age 8 years 6 months. His sister had thelarche at age 5 years (III.2). Upon diagnosis the boy was 8 years and 10 months and the girl 6 years and 6 months, both had advanced bone

age and pubertal GnRH stimulated LH levels (Table 1). The unaffected father of Italian ancestry also has the variant in the heterozygous state, corroborating the paternal imprinting pattern of segregation. The unaffected mother is homozygous wild-type for *MKRN3* gene. It is possible that families D and E are distantly related. We have not confirmed nor excluded this possibility.

SUPPLEMENTARY REFERENCES

1. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25:1754-60.
2. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20:1297-303.
3. DePristo MA, Banks E, Poplin R, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011;43:491-8.
4. Cingolani P, Platts A, Wang le L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin) 2012;6:80-92.
5. Brito VN, Batista MC, Borges MF, et al. Diagnostic value of fluorometric assays in the evaluation of precocious puberty. J Clin Endocrinol Metab 1999;84:3539-44.

Supplementary Figure S1.



Supplementary Figure S1: Pedigrees of families without *MKRN3* mutations. Black symbols are affected subjects and white symbols are unaffected subjects. Arrows indicate the proband in each family. Individuals with asterisks were screened with exome sequencing.

Supplementary Table S1. Clinical and hormonal features of patients with familial CPP selected for whole exome sequencing, without *MKRN3* defects.

Family	Patient Number	Initial Clinical Presentation (Age in Years)	Diagnosis			LH IU/L		FSH IU/L		E2 pg/mL	Additional Cases [£] (Age in Years)
			Age (Years)	Breast Tanner Stage	Bone Age (Years)	Basal	Post GnRH	Basal	Post GnRH		
F	III.1	Thelarche (4.5)	6.4	3	7.8	<0.6	11.4	1.6	12.7	<13	I.2 Menarche (10.0)
	III.2	Thelarche (5)	8.0	4	11.0	1.7	-	3.9	-	34.6	
	III.5*	Thelarche (5.8)	6.7	4	11.0	1.9	31.5	6.2	12.8	53.3	
	III.9	Thelarche and pubarche (5.9)	6.9	4	11.0	1.6	-	4.7	-	29	
G	III.7*	Thelarche (7.7)	8.2	3	11.0	<0.6	17.6	4.2	-	31	II.5 Menarche (8.0) III.6 Menarche (9.0)
H	III.1*	Thelarche and pubarche (5.0)	5.5	3	11.0	1.0	8.8	1.9	-	17.4	I.2 Menarche (8.0) II.1 Menarche (9.0)
I	III.1*	Thelarche (6.5) and menarche (10.0)	10.2	4	11.0	4.2	39.5	2.6	13.7	99	-
	III.2	Thelarche (6.8) and menarche (9.7)	9.8	4	11.5	2.2	-	4.6	-	<13	
J	III.1*	Thelarche and pubarche (7.3)	8.0	3	11.0	1.5	46	2.7	-	<13	-
	III.2	Thelarche and pubarche (7.5)	7.9	3	12.0	<0.6	6.9	1.2	-	13	
K	III.1*	Thelarche and pubarche (6.6)	7.3	4	9.5	0.1	16	3.7	-	13	I.2 Menarche (10.0) II.1 Menarche (9.0) and short stature
L	III.1*	Thelarche and pubarche (5.5)	7.2	3	7.8	<0.6	6.5	1.5	20.0	<13	II.1 Menarche (10.0)
M	III.1*	Thelarche (3.0)	6.0	4	8.8	<0.6	9.8	3.7	16.1	<13	III.5 Thelarche (3.0)
N	III.1*	Thelarche (1.8)	8.0	4	10.0	0.1	6.9	2.2	13.0	<13	II.2 Menarche (9.0)
	III.2	Thelarche (7.0)	6.4	3	11.5	2.5	6.5	6.9	9.2	25	
O	III.1*	Thelarche (7.4)	7.8	2	10.0	0.2†	18.6†	1.6 †	11.0 †	24	-
	III.2	Thelarche (6.9)	7.8	2	11.0	<0.07 †	5.0 †	1.4 †	9.3†	29	

E2: estradiol; T: testosterone

Normal values: T (FIA): prepubertal levels < 19 ng/dl; E2 (FIA): prepubertal levels < 21 pg/ml; LH (IFMA)[5]: prepubertal basal levels < 0.6 (IU/L), peak < 6.9 (IU/L) in girls and < 9.6 (IU/L) in boys. † LH (ICMA): prepubertal basal levels < 0.15 (IU/L), peak <5.0 (IU/L) in both girls and boys. Normal pubertal levels of FSH have not been established because the normal ranges for pubertal and prepubertal FSH overlap.

Breast Tanner stage and bone age assessed at time of diagnosis. *Index case; [£] Relatives with clinical history suggestive of precocious puberty.