Supplementary Subjects and Methods

Subjects

A total of 216 IHH patients (nIHH, n = 181 and KS, n = 35) from 178 independent families were included in the study. IHH patients had absent pubertal development by age 13 and 14 in girls and boys respectively and low or normal basal gonadotropin levels in the face of low estradiol/testosterone levels. The KS patients also had anosmia/hyposmia. The levels of olfactory function were determined based on self-reporting and physical examination. All individuals and/or their legal guardians provided written informed consent, and the study was approved by the Ethics Committee of the Cukurova University Faculty of Medicine and by the institutional review board of the University of Mississippi Medical Center.

DNA Sequencing

The genomic DNA samples for exome sequencing (ES) were prepared as an Illumina sequencing library, and in the second step, the sequencing libraries were enriched for the desired target using the Illumina Exome Enrichment protocol. The captured libraries were sequenced using Illumina HiSeq 2000 Sequencer (Macrogen, Seoul, Korea). The reads were mapped against UCSC (https://genome.ucsc.edu/cgi-bin/hgGateway) hg19.

The ES data from the 216 IHH cohort patients were screened for rare sequence variants (RSVs) in the *SEMA3F* (NM_004186) and *PLXNA3* (NM_017514). A total of 53 genes (*AMH*, *AMHR*, *ANOS1*, *AXL*, *CCDC141*, *CHD7*, *DMXL2*, *DUSP6*, *FEZF1*, *FGF17*, *FGFR1*, *FGFR8*, *FLRT3*, *FSHB*, *GNRH1*, *GNRHR*, *HESX1*, *HS6ST1*, *IGSF10*, *IL17RD*, *KISS1*, *KISS1R*, *KLB*, *LEP*, *LEPR*, *LHB*, *NDNF*, *NR0B1*, *NSMF*, *OTUD4*, *PCSK1*, *PLXNA1*, *PNPLA6*, *POLR3A*, *POLR3B*, *PROK2*, *PROKR2*,

RAB18, *RAB3GAP1*, *RAB3GAP2*, *RNF216*, *SEMA3A*, *SEMA3E*, *SMCHD1*, *SOX10*, *SPRY4*, *SRA1*, *STUB1*, *TAC3*, *TACR3*, *TBC1D20*, *TUBB3*, and *WDR11*) known to be associated with nIHH/KS were also screened with added scrutiny for additional variants. Moreover, these data were also analyzed for new candidate gene detection in the etiology of IHH. The presence of significant variants was verified by Sanger sequencing on an Applied Biosystems PRISM 3130 auto sequencer.

The NHLBI Trans-Omics for Precision Medicine Whole Genome Sequencing Program, TOPMed (https://bravo.sph.umich.edu/freeze5/hg38/), the Genome Aggregation Consortium, gnomAD (http://gnomad.broadinstitute.org/), and the Greater Middle East Variom Project, GME (http://igm.ucsd.edu/gme/data-browser.php) were used to determine the allelic frequencies. Variants with minor allele frequency (MAF) <0.01% in each of these database were considered rare.

The Combined Annotation Dependent Depletion, CADD (https://cadd.gs.washington.edu/snv) scores were used for *in silico* prediction. Additionally, variants were evaluated by four different *in slico* pathogenicity prediction methods: MutationTaster; SIFT, Sorting Intolerant From Tolerant; LRT, Likelihood Ratio Test; PolyPhen-2, Polymorphism Phenotyping v2. The pathogenicity prediction scores 0-4 out of 4 were obtained for each variant. InterVar (http://wintervar.wglab.org/) was used to determine variant classification based on the 2015 American College of Medical Genetics and Genomics and Association for Molecular Pathology guidelines (ACMG/AMP) [1]. To determine if putative pathogenic variants were more enriched in the disease cohort than in the control population, gene-based burden tests were performed by Fisher exact test. We retrieved all the protein altering missense variants as well as the protein truncating ones that are rarer than 1:10.000 in the reference population database, TOPMed. We further filtered missense variants by the CADD score greater than 20, as used in a similar study to better discriminate the damaging variants [2]. As the TOPMed database

features allele frequency and variant-specific CADD scores for each variant, we were readily able to collect and compare the selected allele counts between our patient cohort and TOPMed database using Fisher exact test.

Collection and Processing of Human Fetuses

Tissues were made available in accordance with French bylaws (Good Practice Concerning the Conservation, Transformation, and Transportation of Human Tissue to Be Used Therapeutically, published on December 29, 1998). The studies on human fetal tissue were approved by the French agency for biomedical research (Agence de la Biomédecine, Saint-Denis la Plaine, France, protocol n: PFS16–002). Non-pathological fetuses (7.5 and 10.5 gestational weeks (GW), n = 2 per each developmental stage) were obtained from voluntarily terminated pregnancies after written informed consent was obtained from the parents (Gynaecology Department, Jeanne de Flandre Hospital, Lille, France) and were fixed by immersion in 4% PFA at 4°C for 5 days. The tissues were then cryoprotected in PBS containing 30% sucrose at 4°C overnight, embedded in Tissue-Tek OCT compound (Sakura Finetek), frozen in dry ice, and stored at -80°C until sectioning. Frozen samples were cut serially at 20 μm with a Leica CM 3050S cryostat (Leica Biosystems Nussloch GmbH) and immunolabeled, as described below and as previously described [3; 4].

Immunohistochemistry

Immunohistochemistry for GnRH was performed as previously reported [3; 4] using the following antibodies: Guinea pig anti-GnRH (EH#1018; 1:10000), produced by Dr. Erik Hrabovszky (Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary) ; Rabbit anti-peripherin (Millipore AB1530, 1:2000) ; Goat anti-Plexin A1 (R&D AF4309, 1:50) ; Rat anti-Plexin A2 (R&D Systems MAB5486, 1:100) ;

Goat anti-Plexin A3 (R&D AF4075, 1:200) ; Rabbit anti-Plexin A4 (Abcam ab39350, 1:250) ; Goat anti-Neuropilin2 (R&D AF2215, 1:50) ; Sheep anti-Semaphorin 3F (R&D AF3237, 1:500) ; Goat anti-TAG1 1:500 (R&D AF4439, 1:500).

Sections were first subjected to antigen retrieval in homemade citrate buffer for 20 minutes at 90°C, then rinsed in PBS and blocked for 2 h at room temperature in blocking solution: PBS, 0.3% Triton X-100 (T8787, Sigma), 0.25% Bovine Serum Albumin (Euromedex), 10% normal donkey serum (D9663; Sigma). Sections were incubated in a cocktail of primary antibodies diluted in blocking solution for 48 h at 4°C. Sections were then rinsed in PBS and incubated in a cocktail of fluorochrome-conjugated secondary antibodies (all raised in donkey; Alexa-Fluor 488-, 568-, 647-conjugated secondary antibodies; Molecular Probes, Invitrogen) diluted at 1:400 in blocking solution for 2 h at room temperature. Sections were then rinsed in PBS, coverslipped with Mowiol, and imaged using an inverted laser scanning Axio observer confocal microscope (LSM 710, Zeiss; BICeL Imaging Core Facility of the University of Lille 2, France).

Cell-Based Functional Assays

SEMA3F and PLXNA3 protein expression

A cDNA containing the entire coding region of the human SEMA3A transcript variant 1 (GenBank: NM_004186.5) was inserted into a modified pcDNA3.1+ expression vector containing a his-tag at the 5'end (GeneCust). Similarly, plasmid encoding *SEMA3F* mutants were obtained using modified pcDNA3.1+ expression vector containing a myc-tag at the 5'end of the coding region (GeneCust). A cDNA containing the entire coding region of the human *PLXNA3* (GenBank: NM_017514.5) was inserted into a modified pcDNA3.1+ expression vector containing a his-tag at the 5'end (GeneCust).

Similarly, plasmid encoding PLNXA3 mutants were obtained using modified pcDNA3.1+ expression vector containing a myc-tag at the 5'end of the coding region (GeneCust).

To assess the impact of the variants on *SEMA3F* expression and secretion, and on PLNXA3 signaling, we performed western-blot assays as previously described [4; 5].

Cell cultures

Kidney cells (HEK 293T) from human embryo were cultured under standard conditions in a DMEM-based medium containing 5% fetal bovine serum and appropriate selection antibiotics; they were re-plated before reaching 80% confluency and were passaged < 20 times. HEK 293T were authenticated based on morphology, and DNA staining revealed no mycoplasma contamination.

Transfection was performed in 6- or 12-well plates using Fugene6 (Promega) according to the manufacturer's protocol, in a transfection rate of 3:1 (Fugene6:DNA) and for 48 h at 37°C. Conditioned media were collected for secretion assays, and the plates were quickly rinsed with PBS and frozen on dry ice for subsequent expression assays. Proteins from conditioned media and cells were subjected to western blot analysis as described below.

Western blots

Homemade Tris-Triton lysis buffer was used for cell lysis and protein extraction, and the protein concentration was determined for each sample using the Pierce BCA protein assay kit (Thermofisher). For SEMA3F and PLXNA3 assays, 10 µg or 20 µg of proteins were loaded in each well of 6% or 8% hand-casted acrylamide gels, respectively. Migration was carried out in Tris/Glycine/SDS buffer and proteins were subsequently transferred to a 0.45 µm nitrocellulose membrane in Tris/Glycine/Methanol. The membranes were cut into strips and blocked in TBS-T (TBS, 0.05% Tween 20) + 5% milk for 1h at RT, followed by incubation with primary antibodies in TBS-T + 5%

Bovine Serum Albumin overnight at 4°C. The next day, membranes were incubated with HRP-conjugated secondary antibodies in TBS-T + 5% milk for 1h at RT. Finally, the strips were incubated in SuperSignal West Pico PLUS (ThermoFisher) and revelation was performed in a dark room using X-ray films. Quantification was performed in FIJI, and GAPDH was used for normalization. Primary antibodies: Sheep anti-Semaphorin 3F (R&D AB3237, 1:1000), Goat anti-Plexin A3 (R&D AB4075, 1:500), Rabbit anti-GAPDH (Sigma G9545, 1:5000). Secondary antibodies: Anti-rabbit HRP-conjugated (Cell Signaling 7074S, 1:10000), Anti-Sheep HRP-conjugated (R&D HAF016, 1:5000), Anti-Goat HRP-conjugated (Vector Labs PI-9500 , 1:5000).

Immunocytochemistry on transfected HEK293 cells

HEK293T cells were seeded on poly-L-lysin coated coverslips in a 24 wells-plate, and were transfected for 48h with *PLXNA3* variants using Fugene 6 (Promega) and following manufacturer's recommendations. Cells were fixed with paraformaldehyde 4% in PBS for 10 minutes, and rinsed three times before immunocytochemistry to assess the cellular distribution of PLEXIN A3 (PLXNA3). Immunocytochemistry was performed as follows: cells were first blocked and permeabilized for 1h in blocking buffer (PBS, 0.1% Triton, 5% Normal Donkey Serum) then incubated overnight in blocking buffer with primary antibodies (goat anti-PlexinA3 1:500, AF4275 R&D Systems, rabbit anti-Calnexin 1:1000, ab92573 Abcam), rinsed in PBS, and incubated for 2 hours in blocking buffer with secondary antibodies (donkey anti Goat/Rabbit, Alexa Fluor 488/568 conjugated, 1:500, Invitrogen). Cells were rinsed again in PBS, and nuclei were stained with DAPI 1:5000 in PBS for 2 minutes before mounting with Mowiol. Slides were imaged on an inverted laser scanning Axio observer confocal microscope (LSM 710, Zeiss; BICeL Imaging Core Facility of the University of Lille, France) using a 63x/1.40 oil-immersion objective, and 15 to 20 single-plane pictures were taken for each condition.

PLEXIN A3 cellular localization analysis

Signal analysis was performed using Fiji: PLEXIN A3 and endoplasmic reticulum stainings from single-plane acquisitions were thresholded with constant parameters. Plexin A3 signal was used as a mask to segment the ER staining and subsequently quantify the proportion of Plexin A3 in the ER. Statistical analysis was performed in Graphpad Prism 7: data was first subjected to D'Agostino & Pearson normality test, and analyzed using a one-way ANOVA with Dunnett's multiple comparisons test.

Statistical analysis

Statistical analyses were performed using Prism 5 (GraphPad Software). Data sets were assessed for normality (Shapiro-Wilk test) and variance. Where appropriate a one-way or two-way ANOVA followed by post hoc testing (specified in the figure legends) was performed and for non-Gaussian distributions, a Kruskal-Wallis test followed by Dunn's multiple comparison test was used – indicated in figure legends.

Supplementary References

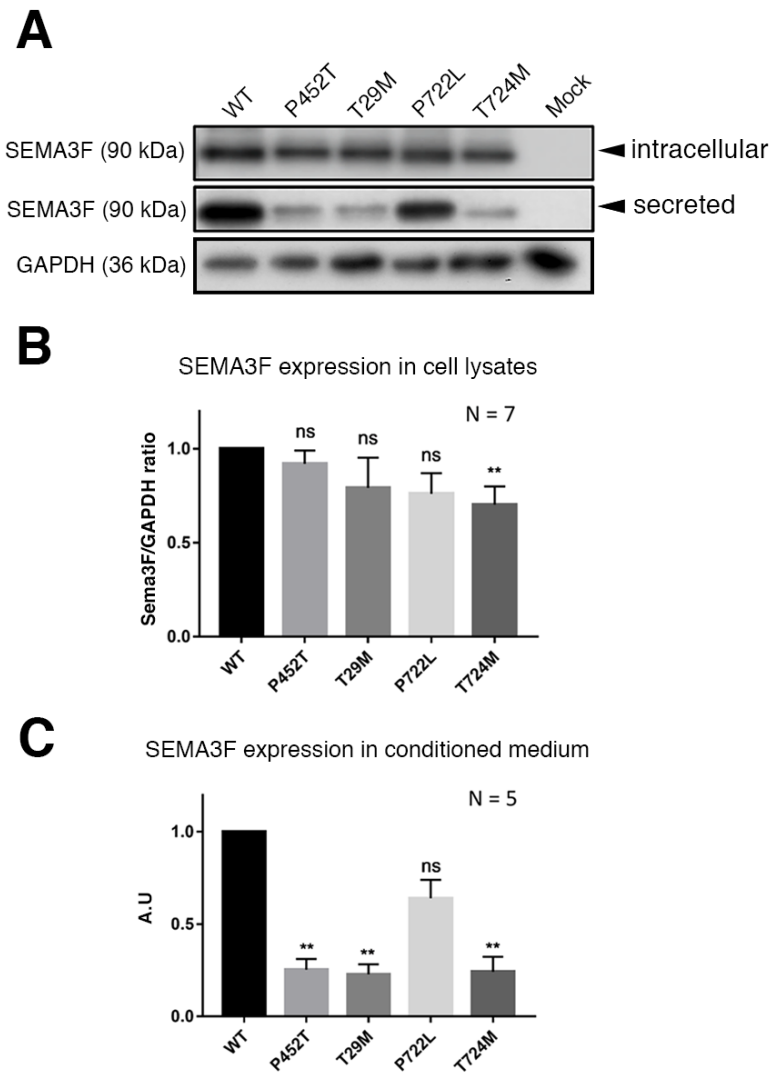
1. Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W.W., Hegde, M., Lyon, E., Spector, E., et al. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 17, 405-424.
2. Guo, M.H., Plummer, L., Chan, Y.M., Hirschhorn, J.N., and Lippincott, M.F. (2018). Burden Testing of Rare Variants Identified through Exome Sequencing via Publicly Available Control Data. Am J Hum Genet 103, 522-534.
3. Casoni, F., Malone, S.A., Belle, M., Luzzati, F., Collier, F., Allet, C., Hrabovszky, E., Rasika, S., Prevot, V., Chedotal, A., et al. (2016). Development of the neurons controlling fertility in humans: new insights from 3D imaging and transparent fetal brains. Development 143, 3969-3981.
4. Malone, S.A., Papadakis, G.E., Messina, A., Mimouni, N.E.H., Trova, S., Imbernon, M., Allet, C., Cimino, I., Acierno, J., Cassatella, D., et al. (2019). Defective AMH signaling disrupts GnRH neuron development and function and contributes to hypogonadotropic hypogonadism. Elife 8.

5. Hanchate, N.K., Giacobini, P., Lhuillier, P., Parkash, J., Espy, C., Fouveaut, C., Leroy, C., Baron, S., Campagne, C., Vanacker, C., et al. (2012). SEMA3A, a gene involved in axonal pathfinding, is mutated in patients with Kallmann syndrome. PLoS Genet 8, e1002896.

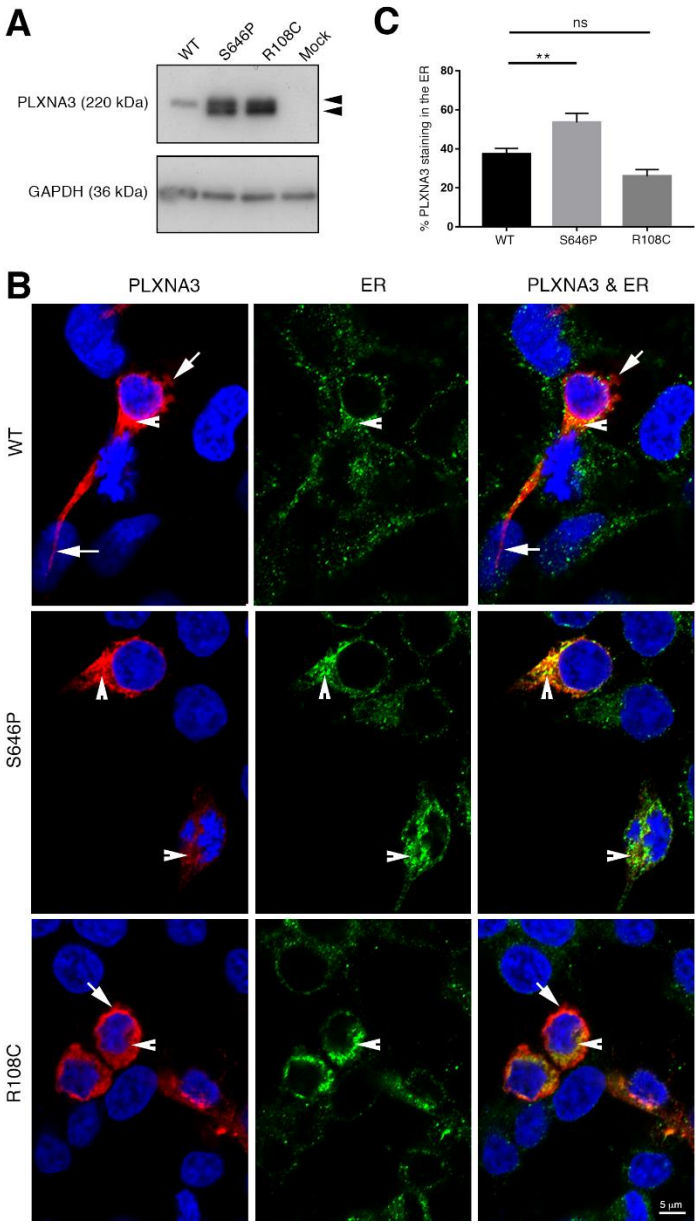
Supplementary Table 1. The molecular genetic characteristics of the *SEMA3F* and *PLXNA3* variants. Hom, homozygous; Het, heterozygous; gnomAD, The Genome Aggregation Consortium; TOPMed, The NHLBI Trans-Omics for Precision Medicine Whole Genome Sequencing Program; InterVar, interpretation of genetic variants by the ACMG/AMP 2015; VUS, variant uncertain significance; PM, pathogenic moderate; PP, pathogenic supporting; BP, benign supporting. Variants are described according to the RefSeq numbers following the gene names: SEMA3F, NM_004186; PLXNA3, NM_017514; TACR3, NM_001059; IGSF10, NM_178822; CHD7, NM_017780; FGFR1, NM_023110; and DCHS, NM_003737. In silico pathogenicity prediction tests are Mutation Taster; SIFT, Sorting Intolerant From Tolerant; LRT, Likelihood Ratio Test; PolyPhen-2, Polymorphism Phenotyping v2. The column depicts number of harmful predictions out of 4 tests.

Family/individual no	Gene	Variant at cDNA level	Variant at protein level	CADD score	TOPMed	gnomAD	InterVar	*In silico* * pathogenicity	Other IHH gene mutation/zygosity
A I-2, II-1, II-2	*SEMA3F*	c.1354C>A	p.Pro452Thr	26.9	absent	0.00001	VUS: PM1, PM2, PP3	4/4	None
B II-1	*SEMA3F*	c.1354C>A	p.Pro452Thr	26.9	absent	0.00001	VUS: PM1, PM2, PP3	4/4	None
C II-1	*SEMA3F*	c.2095C>T	p.Arg699Trp	26.2	<0.00001	0.00003	VUS: PM1	2/4	*TACR3* p.Gly93Asp Hom
D II-2	*SEMA3F*	c.86C>T	p.Thr29Met	3.2	0.00001	0.00003	VUS: PM1, BP4	0/4	*IGSF10* p.Pro1997Leu Het, *CHD7* p.Met340Val Het
E II-1	*SEMA3F*	c.2165C>T	p.Pro722Leu	16.5	absent	0.00001	VUS: PM1, PM2	1/4	None
F I-1	*SEMA3F*	c.1954G>T	p.Ala652Ser	15.8	absent	absent	VUS: PM1, PM2	0/4	*FGFR1* p.Arg209Cys Het
G II-3, II-6	*SEMA3F* *PLXNA3*	c.2171C>T c.1936T>C	p.Thr724Met p.Ser646Pro	14.4 24.6	0.00001 absent	0.00002 absent		1/4 4/4	*DCHS1* p.Asp1765Glu Het
H II-1, II-2	*PLXNA3*	c.322C>T	p.Arg108Cys	31.0	absent	0.00001	VUS: PM1, PM2	4/4	*TACR3* p.Pro353Ser Het
I II-1	*PLXNA3*	c.322C>T	p.Arg108Cys	31.0	absent	0.00001	VUS: PM1, PM2	4/4	None

J II-1	*PLXNA3*	c.3256C>G	p.Leu1086Val	14.7	absent	absent	VUS: PM1, PM2	0/4	None
K II-2	*PLXNA3*	c.4075C>T	p.Arg1359Cys	29.3	absent	0.00005	VUS: PM1	4/4	*CHD7* p.Asn2335Ser



Supplementary Figure 1. Variants of *SEMA3F* are loss of function. The expression and secretion profiles of several *SEMA3F* variants, and the signaling of *PLXNA3* variants were assessed using HEK293T cells transfected for 48h. (**A**) Representative western blot for intracellular and secreted SEMA3F. (**B, C**) While only the T724M *SEMA3F* variant was associated with a significant decrease in expression, the variants strongly affected SEMA3F secretion with an approximate threefold decrease in three out of four variants (P452T, T29M, T724M) relative to the WT. Each experiment was performed three times independently. ** $P < 0.01$ (Kruskal-Wallis test followed by Dunn's multiple comparisons test).



Supplementary Figure 2. Functional validation of *PLXNA3* variants. (A) Representative western blot for PLXNA3 in HEK293T transfected or not with *PLXNA3* WT and variants (S646P and R108C). Detection of two isoforms for the S646P and R108C variants (arrowheads), but not the WT variant of *PLXNA3*, suggests post-translational modifications affecting only or more strongly the mutant variants. Each experiment was performed three times independently. ** $P < 0.01$ (Kruskal-Wallis test followed by Dunn's multiple comparisons test). **(B)** Representative fluorescent

immunocytochemistry for PLXNA3 and Calnexin, a marker of the endoplasmic reticulum (ER), in transfected HEK293 cells. *PLXNA3* WT and R108C variant are localized mainly at the plasma membrane (arrows), even though some signal is also detectable in the ER (arrowheads). S646P variant is only visible in the ER (arrowheads) but not in the membrane. (**C**) Quantitative analysis showing the relative proportion of PLXNA3 staining in the ER in HEK293T cells transfected with *PLXNA3* WT and *PLXNA3* variants. ** $P < 0.01$ (One-way ANOVA with Dunnett's multiple comparisons test, data is shown as mean ± SEM). ER, endoplasmic reticulum. Scale bar: 5 μm.