SUPPLEMENTARY MATERIAL

(Bonnefond A. *et al.* Loss-of-function mutations in *SIM1* contribute to obesity and Prader-Willi-Like-related features)

SUPPLEMENTARY METHODS

Study participants

SIM1 was sequenced in:

- 44 children presenting with at least one clinical feature of PWL syndrome (including neonatal hypotonia, short hands, intellectual disability, developmental delay, behavioural problems, facial dysmorphism, hypogenitalism or hypogonadism (1)), that was either reported by the family, clinically apparent in clinic, or revealed only after detailed assessment. None of these children had chromosomal abnormality at chromosome 15q or 6q, or microdeletions elsewhere in the genome, as assessed by cytogenetic, array comparative genomic hybridization (via Agilent CGH array) and Prader-Willi syndrome DNA methylation analyses. All children were recruited by Lille hospital (*N*=1; France), and the General Paediatric clinic/the Paediatric Endocrinology clinic/the Paediatric Genetics clinic at the University of Florida (*N*=43; USA).
- 198 children with severe early-onset obesity, recruited by the CNRS UMR8199 unit (*N*=176; France), and the General Paediatric clinic/the Paediatric Endocrinology clinic/the Paediatric Genetics clinic at the University of Florida (*N*=22; USA).
- 568 morbidly obese adults, recruited by the CNRS UMR8199 unit (*N*=221; France), the ABOS bariatric surgery study (*N*=48; France) (2), the outpatient obesity clinic at

the University of Antwerp (*N*=64; Belgium) (3) and a bariatric surgery study from Zurich (*N*=235; Switzerland) (4).

- 383 normal weight French adults from the D.E.S.I.R. prospective study, fully described elsewhere (5).

Recruitment criteria for severe early-onset obesity were a history of weight >150% of ideal BMI, or a BMI≥97th percentile, according to the recommendations of the European Childhood Obesity Group study (6). The z-score of BMI was calculated as previously described (7). In adults, the obesity status was defined as: normal weight (BMI<25 kg/m²), overweight (25≤BMI<30 kg/m²), obesity (BMI≥30 kg/m²), severe obesity (30≤BMI<40 kg/m²) and morbid obesity (BMI≥40 kg/m²).

When a rare mutation was identified, it was assessed by sequencing in carriers' relatives (when DNA was available).

Sequencing of *SIM1*

SIM1 is located on human chromosome 6q16.3-q21 and encodes a 766-amino-acid protein (NM_005068→NP_005059). Genomic DNA was amplified by PCR with primers designed to cover the 11 exons and flanking intron-exon boundaries of *SIM1*. Fragments were bidirectionally sequenced using the automated 3730xl DNA Analyzer (Applied Biosystems). Electrophoregram reads were assembled and analyzed using Sequencher software. We only analysed non-synonymous variants observed with a frequency <1%.

HRM genotyping

We genotyped seven variants (p.T46R, p.E62K, p.Q152E, p.H323Y, p.R581G, p.T714A and p.D740H) in 2,896 normal weight French individuals from the D.E.S.I.R. study, using the HRM technology (Roche), as previously described (8).

In Silico functional prediction software

Polyphen-2 (9), SNAP (10), SIFT (11), PMut (12) and Align GVGD (13) programs were used to predict the possible impact of an amino acid substitution on the structure and function of SIM1. Codes per software are (from lowest damaging risk to highest damaging risk):

- For Polyphen-2 (9), "benign" → "possibly damaging" → "probably damaging"
- For SNAP (10), "neutral" → "non neutral"
- For SIFT (11), "tolerated" → "damaging"
- For PMut (12), "neutral" → "pathological"
- For Align GVGD (13), "C0" (risk estimates between 1.10 and 1.33) → "C15" → "C25" → "C35" → "C45" → "C55" → "C65" (risk estimates > 3.00)

Homology modeling

Homology modeling was carried out with the ICM-Pro program suite (14) using the homology add-on (15; 16). Each model of the heterodimer was first created individually using the sequence for human SIM1 residues 1-225 (Uniprot number: P81133) and for human ARNT2 residues 1-330 (Uniprot number: Q9HBZ2) so as to include the bHLH and PASA domains of each protein. Using the protein data bank (PDB), we found that the CLOCK:BMAL1 structure was the closest homologous structure (PDB ID: 4F3L) (17). Therefore, a homology model of SIM1 was created using the CLOCK protein as a three-dimensional template and the ARNT2 model was created using BMAL1 as a three-dimensional model. After creation of the individual models, they were subject to regularization and model refinement using ICM-Pro (to optimize model geometry, carry out energy minimization, and alleviate clashing side-chains). The two structures were then docked using the CLOCK:BMAL1 structure as a guide. Further regularization and model refinement were then performed to ensure the integrity of the dimer interface. Finally, several loops were

subject to loop modeling to improve clashing at the dimer interface using the ICM-Pro loop modeling utility (18). Figures were created using PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC). Of note, as crystal structures showed very different PASB interactions for HIF:ARNT PASB (19), CLOCK:BMAL1 PASB (17) and the PER PASA PASB homodimer (20), we have not attempted modeling beyond the PASA domain. Furthermore, modeling is not possible for the C-terminus as there are no structures of this region for any member of the protein family.

Plasmid construction per mutation

Details of all primers used in this section are available upon request.

pDR2-hSIM1 and pML-6CWT were a kind gift from Dr. S.E. Antonarakis (Geneva University) and Dr. J. Pelletier (McGill University), respectively.

pEF-hARNT-IRES-neo has been previously described (21).

pEF-hARNT2-IRES-neo was constructed by PCR amplifying full-length human ARNT2 from 293T cDNA, digesting with MluI/XbaI and ligating into similarly digested pEF-IRES-neo.

For the generation of stable cell lines, full-length human SIM1 with two in-frame C-terminal Myc tags was generated. First, pEF-hSIM1-IRES-puro was constructed by digesting full-length hSIM1 out of pDR2-hSIM1 with AflII, blunt-ending with Klenow, and ligating into EcoRV digested pEF-IRES-puro6 (22). Then, SIM1 was PCR amplified from this vector, digested with ClaI/HpaI and ligated into ClaI/EcoRV digested pEF-mSIM2L-Myc-IRES-puro (23). This ligated the hSIM1 coding sequence in-frame with the existing 2XMyc tag to create pEF-hSIM1-Myc-IRES-puro. pcDNA5-FRT-TO-hSIM1-Myc was constructed by ligating the NheI/NotI fragment from pEF-hSIM1-Myc-IRES-puro into similarly digested pcDNA5-FRT-TO-hSIM2s-Myc. pcDNA5-FRT-TO-hSIM2s-Myc was itself constructed by ligating the

AflII/NotI fragment from pEF-hSIM2s-Myc-IRES-puro into similarly digested pcDNA5-FRT-TO (Invitrogen).

Point mutations within the hSIM1 coding sequence were then introduced into pcDNA5-FRT-TO-hSIM1-Myc as follows:

- p.T46R (ACG>AGG): overlap extension PCR.
- p.E62K (GAG>AAG): overlap extension PCR.
- p.I128T (ATT>ACT): overlap extension PCR.
- p.Q152E (CAG>GAG): overlap extension PCR.
- p.H323Y (CAC>TAC): the NheI/BamHI fragment was digested out of pcDNA3-hSIM1-p.H323Y (mutation was generated using the QuikChange site-directed mutagenesis kit (Stratagene)) and ligated into similarly digested pcDNA5-FRT-TO-hSIM1-Myc.
- p.R581G (AGA>GGA): the EcoRV/PshAI fragment was digested out of pcDNA3-hSIM1-p.R581G (mutagenesis performed as above) and ligated into similarly digested pEF-hSIM1-Myc-IRES-puro. Then the NheI/NotI fragment was digested out and ligated into similarly digested pcDNA5-FRT-TO-hSIM1-Myc.
- p.T714A (ACT>GCT): PCR amplified from pcDNA3-hSIM1-p.T714A (mutagenesis performed as above) and ligated into full-length hSIM1 in pGEM-T-Easy (Promega) using EcoRV/HpaI. Full-length hSIM1-p.T714A then subcloned into ClaI/EcoRV digested pEF-mSIM2L-Myc-IRES-puro using ClaI/HpaI, and finally subcloned into pcDNA5-FRT-TO using NheI/NotI.
- p.D740H (GAT>CAT): overlap extension PCR.

Generation of stable cell lines, cell culture, and transfection

The human embryonic kidney 293 Flp-In T-Rex cell system (Invitrogen) was used to generate a doxycycline-inducible stable cell line for each SIM1 mutant, as well as two independently derived SIM1 wild type cell lines and one control (empty vector) cell line, as per manufacturer's instructions. Transfections were performed on subconfluent cells using Fugene6 (Roche).

Luciferase reporter assays

Cells were plated at 40-50% confluency in 24-well tray format and transfected with 400ng pML-6CWT, 0.5ng phRL-CMV (Promega), and 20ng pEF-hARNT-IRES-neo or 50ng pEF-hARNT2-IRES-neo using Fugene6 (Roche). pML-6CWT is a SIM1-responsive Firefly Luciferase reporter plasmid controlled by six consecutive repeats of the CME from the toll promoter (24). Six hours after transfection, medium was replaced with fresh medium containing doxycycline (Sigma) to a final concentration of 1μg/mL. Cells were harvested after 16 hours doxycycline treatment and assayed for luciferase activity using the Dual Luciferase Assay System (Promega) and a Glomax Luminometer (Promega) as per manufacturer's instructions. Empty vector, wild type and mutant cell lines were assayed in triplicate in at least three independent experiments. Firefly luciferase activity was normalised to Renilla luciferase activity for each well, and the three normalised values were averaged. In the case of the two wild type lines, the Firefly/Renilla values for all six wells were combined into a single average value. These figures were then converted to log values for the purposes of statistical analysis. Data displayed are the mean Firefly/Renilla value +SEM for each cell type (n=3 for all mutants except p.Q152E n=5, empty vector and wild type n=9) expressed relative to wild type, which, for the purposes of clarity, has been normalised to 100%. All luciferase data are shown in **Supplementary Table 4**.

Western analysis

Cells were stimulated in complete medium supplemented with 1µg/mL doxycycline for 16 hours. Whole cell extracts were prepared as previously described (25). 30µg of whole cell extracts were subjected to 7.5% SDS-PAGE and transferred to nitrocellulose using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Proteins were detected with the anti-Myc 4A6 monoclonal antibody (Millipore). The anti-alpha tubulin monoclonal antibody MCA78G (AbD Serotec) was used as a loading control.

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SUPPLEMENTARY TABLE 1. Clinical characteristics of *SIM1* mutated probands presenting with PWL syndrome

Proband	Variant	Age (yrs)	Gender	Z-score of BMI	Intellectual disability	Developmental delay	Beha-viour*	Facial dysmorphism**	Short hands	Hypo-tonia	Hypogonadism
1	**p.I128T**	5	F	6.9	**+**	**+**	**+**	**-**	**-**	**-**	**-**
2	**p.Q152E**	4	F	7.7	**+** (IQ=97)	**-**	**+**	**+**	**-**	**-**	**+** (hypoplastic clitoris and labia minora)
3	**p.R581G**	19	F	5.2	**+** (IQ=53)	**+** (sat: 18m; spoke: 24m; walked: 36m)	**+**	**+**	**-**	**-**	**+** (No menses; no clitoris and labia minora; hypoplastic labia majora)
4	**p.T714A**	1.5	M	7.7	**+**	**+** (sat: 10m; spoke: never understandable; walked: 4yrs)	**+**	**+**	**-**	**-**	**-**

F, female; ***M***,male; ***BMI***, body mass index; ***yrs***, years; ***m***; months

*Behavioural problems: temper tantrums and/or self-injurious behaviour (e.g. skin-picking)

**Facial dysmorphism: round face with small nose and depressed nasal bridge

SUPPLEMENTARY TABLE 2. Clinical characteristics of morbidly obese adults carrying a rare variant in *SIM1*

Carrier	Variant	Age (yrs)	Gender	BMI	Other clinical features
1	**p.T46R**	40	F	57.3	-
2	**p.T46R**	32	F	56.2	Developmental delay
3	**p.T46R**	38	M	52.5	Developmental delay
4	**p.T46R**	46	F	53.5	-
5	**p.E62K**	38	F	52.2	-
6	**p.H323Y**	54	F	47.1	-
7	**p.D740H**	36	F	66.0	-

F, female; ***M***, male; ***BMI***, body mass index; ***yrs***, years

SUPPLEMENTARY TABLE 3. Clinical characteristics of the family with PWL-associated clinical features, and carrying the *SIM1* p.T714A mutation

Family member	z-score of BMI	Age at onset of obesity (yrs)	Hyperphagia	Cognitive impairment/ learning disabilities	Developmental delay	Facial dysmorphism*	Behavioural problems**	Hypogonadism
Proband	7.7	1.5	yes	yes	yes (sat: 10m; spoke: never understandable; walked: 4yrs)	mild	yes	no
Sister 1	7.4	2.5	yes	yes	mild (spoke: needs orthophonist; walked: 16m)	mild	yes	no
Brother	2.0	2	yes	yes	yes (spoke: needs orthophonist, walked: 18m)	mild	yes	no
Sister 2	3.2	4	yes	mild	no	mild	no	no (hyperandrogeny)
Mother	2.9	20	no	yes	NA	mild	no	no
Maternal aunt	1.7	-	no	no	NA	no	no	no

BMI, body mass index; ***yrs***, years; ***m***; months

*Facial dysmorphism: round face with small nose and depressed nasal bridge

**Behavioural problems: temper tantrums and/or self-injurious behaviour (skin-picking)

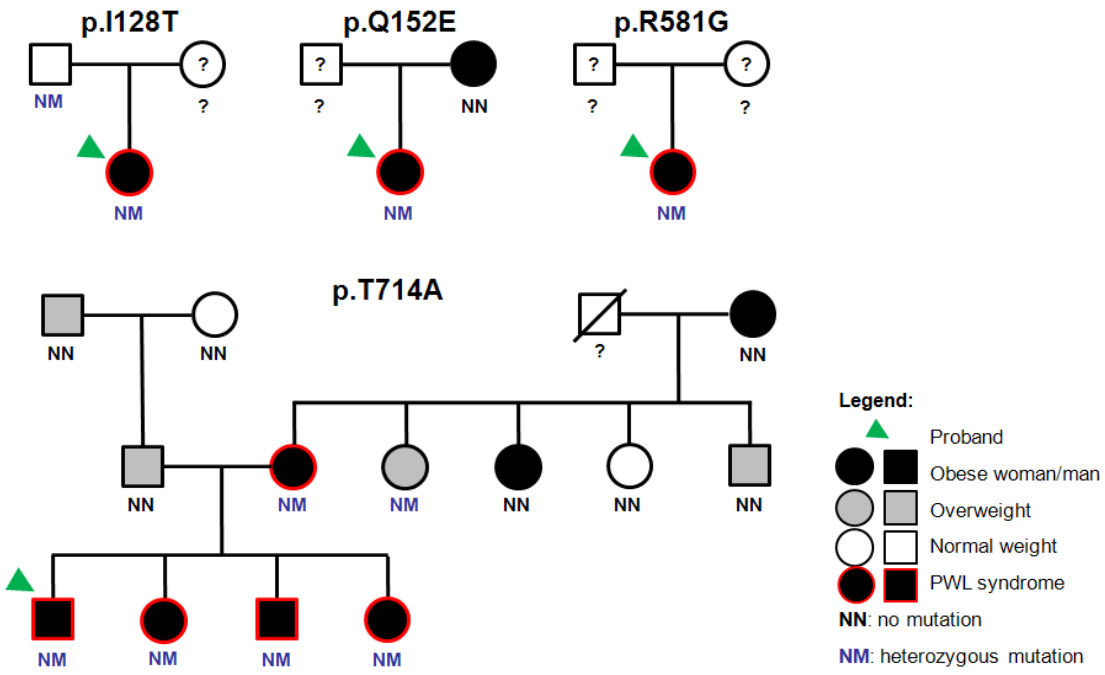
SUPPLEMENTARY TABLE 4. Luciferase data showing the effect of *SIM1* subsitutions on the transcriptional activity of SIM1

For each experiment date, the "average" value is the average of a triplicate tranfections FireflyLuc/Renilla Luc. Similarly, each "stdev" value is the stdev of a triplicate. The WT value in each experiment is the average of the 6 raw values collected for independent cell lines WT1 and WT2 in that experiment. Similarly the WT stdev is the stdev of the 6 values collected for WT1 and WT2 in that experiment.

Date			average	stdev	fold change	%WT
25/02/10	ARNT	empty	0,00043	2,00719E-05	1	0,0172
		WT	0,02502	0,000628888	58,29	1
		I128T	0,02043	0,000737236	47,60	0,8166
	ARNT2	empty	0,00044	1,02814E-05	1	0,0694
		WT	0,00633	0,000385835	14,41	1
		I128T	0,00474	5,26473E-05	10,78	0,7484
02/03/10	ARNT	empty	0,00090	1,86498E-05	1	0,0260
		WT	0,03476	0,007268399	38,48	1
		I128T	0,03820	0,001901873	42,29	1,0991
		Q152E	0,02119	0,00083006	23,46	0,6096
	ARNT2	empty	0,00100	4,96408E-05	1	0,0978
		WT	0,01025	0,001471553	10,22	1
		I128T	0,01011	0,000617858	10,09	0,9866
		Q152E	0,00662	0,000355365	6,60	0,6460
05/03/10	ARNT	empty	0,00057	6,8858E-05	1	0,0219
		WT	0,02605	0,006186361	45,70	1
		I128T	0,01805	0,002198308	31,67	0,6931
		Q152E	0,02674	0,000836352	46,91	1,0265
	ARNT2	empty	0,00063	3,42806E-05	1	0,0711
		WT	0,00880	0,002257478	14,07	1
		I128T	0,00444	0,000821157	7,10	0,5044
		Q152E	0,00722	0,000244091	11,54	0,8201
04/05/10	ARNT	empty	0,00070	2,69562E-05	1	0,0230
		WT	0,03019	0,004201944	43,41	1
		I128T	0,03113	0,002344889	44,76	1,0310
		Q152E	0,02618	0,001221121	37,64	0,8671
		H323Y	0,00785	0,003563751	11,29	0,2600
		R581G	0,02347	0,003878099	33,75	0,7775
		T714A	0,01311	0,001546903	18,85	0,4342
	ARNT2	empty	0,00055	2,5411E-05	1	0,0730
		WT	0,00750	0,002194624	13,70	1
		I128T	0,00549	0,000512928	10,03	0,7324
		Q152E	0,00632	0,000447827	11,54	0,8421
		H323Y	0,00254	0,000386828	4,63	0,3379
		R581G	0,00645	0,000860257	11,77	0,8592
		T714A	0,00467	0,000556563	8,53	0,6225
19/05/10	ARNT	empty	0,00084	0,000163926	1	0,0264
		WT	0,03193	0,00410374	37,89	1
		Q152E	0,03308	0,003296636	39,25	1,0359
		H323Y	0,00844	0,002482495	10,01	0,2643
		R581G	0,03127	0,002222249	37,10	0,9792
		T714A	0,00971	0,000425058	11,52	0,3040
	ARNT2	empty	0,00081	7,14835E-05	1	0,0782
		WT	0,01040	0,001353896	12,79	1
		Q152E	0,00918	0,000850405	11,29	0,8831
		H323Y	0,00370	0,000477633	4,55	0,3558
		R581G	0,00914	0,000845917	11,24	0,8786
		T714A	0,00378	0,000186265	4,65	0,3635

Date			average	stdev	fold change	%WT
27/05/10	ARNT	empty	0,00095	0,000163926	1	0,0197
		WT	0,04843	0,006349825	50,72	1
		I128T	0,04469	0,000844173	46,80	0,9228
		Q152E	0,03629	0,003296636	38,00	0,7493
		H323Y	0,02232	0,002482495	23,37	0,4609
		R581G	0,04970	0,002222249	52,04	1,0262
		T714A	0,02743	0,000425058	28,73	0,5664
	ARNT2	empty	0,00099	7,14835E-05	1	0,0620
		WT	0,01594	0,000624682	16,13	1
		I128T	0,01346	0,001119041	13,62	0,8447
		Q152E	0,01128	0,000850405	11,41	0,7076
		H323Y	0,00662	0,000477633	6,69	0,4151
		R581G	0,01867	0,000845917	18,90	1,1717
		T714A	0,00968	0,000186265	9,80	0,6075
24/06/11 #1	ARNT	empty	0,00054	6,95293E-05	1	0,0266
		WT	0,02025	0,004452646	37,65	1
		T46R	0,00072	4,29144E-05	1,34	0,0357
		E62K	0,03358	0,002569191	62,44	1,6585
		D740H	0,03671	0,000291338	68,26	1,8132
	ARNT2	empty	0,00059	2,27297E-05	1	0,0723
		WT	0,00823	0,000792752	13,84	1
		T46R	0,00081	3,86967E-05	1,35	0,0978
		E62K	0,00983	0,00080875	16,53	1,1943
		D740H	0,00896	0,000280238	15,07	1,0893
24/06/11 #2	ARNT	empty	0,00064	2,39818E-05	1	0,0343
		WT	0,01868	0,005465252	29,13	1
		T46R	0,00075	1,67651E-05	1,16	0,0399
		E62K	0,03056	0,002214768	47,67	1,6363
		D740H	0,03200	0,00283445	49,90	1,7131
	ARNT2	empty	0,00075	2,2055E-05	1	0,0757
		WT	0,00986	0,00103506	13,21	1
		T46R	0,00089	7,72051E-06	1,19	0,0900
		E62K	0,01086	0,000617686	14,55	1,1018
		D740H	0,00955	0,000399467	12,80	0,9689
24/06/11 #3	ARNT	empty	0,00049	1,04268E-05	1	0,0275
		WT	0,01792	0,001021704	36,42	1
		T46R	0,00060	3,23995E-05	1,21	0,0332
		E62K	0,02507	8,17332E-05	50,95	1,3990
		D740H	0,02340	0,001124947	47,55	1,3056
	ARNT2	empty	0,00049	3,66632E-06	1	0,0820
		WT	0,00592	0,000661355	12,19	1
		T46R	0,00066	1,52958E-05	1,36	0,1116
		E62K	0,00634	0,000590839	13,06	1,0714
		D740H	0,00553	7,18142E-05	11,39	0,9342

SUPPLEMENTARY FIGURE 1. Pedigrees of *SIM1* mutated probands presenting with clinical features of PWL syndrome



SUPPLEMENTARY FIGURE 2. Pedigrees of *SIM1* mutated participants presenting with morbid obesity