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Loss-of-function mutations in *SIM1* contribute to obesity and Prader-Willi–like features

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***Sim1* haploinsufficiency in mice induces hyperphagic obesity and developmental abnormalities of the brain. In humans, abnormalities in chromosome 6q16, a region that includes *SIM1*, were reported in obese children with a Prader-Willi–like syndrome; however, *SIM1* involvement in obesity has never been conclusively demonstrated. Here, *SIM1* was sequenced in 44 children with Prader-Willi–like syndrome features, 198 children with severe early-onset obesity, 568 morbidly obese adults, and 383 controls. We identified 4 rare variants (p.I128T, p.Q152E, p.R581G, and p.T714A) in 4 children with Prader-Willi–like syndrome features (including severe obesity) and 4 other rare variants (p.T46R, p.E62K, p.H323Y, and p.D740H) in 7 morbidly obese adults. By assessing the carriers' relatives, we found a significant contribution of *SIM1* rare variants to intra-family risk for obesity. We then assessed functional effects of the 8 substitutions on *SIM1* transcriptional activities in stable cell lines using luciferase gene reporter assays. Three mutations showed strong loss-of-function effects (p.T46R, p.H323Y, and p.T714A) and were associated with high intra-family risk for obesity, while the variants with mild or no effects on *SIM1* activity were not associated with obesity within families. Our genetic and functional studies demonstrate a firm link between *SIM1* loss of function and severe obesity associated with, or independent of, Prader-Willi–like features.**

Introduction

The basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) transcription factor single-minded 1 (*SIM1*) plays a key role in neuronal differentiation within the paraventricular nucleus of the hypothalamus, which is critical for food intake regulation (1). *Sim1*-haploinsufficient mice display hypodevelopment of the paraventricular nucleus of the hypothalamus and are hyperphagic, obese, and highly sensitive to diet-induced obesity, whereas *Sim1* overexpression in mice leads to a decrease in food intake (2–4). In humans, several deletions in the chromosome 6q16, the region that includes *SIM1*, have been identified in obese patients presenting with a Prader-Willi–like (PWL-like) syndrome (5), which is characterized in early infancy by global developmental delay, hypotonia, and feeding difficulties and later in life by hyperphagia, obesity, and facial dysmorphisms (6). The 4.1-Mb critical region for PWL syndrome includes *SIM1* but also 11 other genes or gene

predictions, and the specific role of *SIM1* haploinsufficiency in the development of PWL syndrome has not definitively been established (6). Nonetheless, a de novo balanced translocation disrupting *SIM1* has been described in a patient presenting with severe obesity but no other clinical features suggestive of a PWL syndrome (7). Another study reported 6 rare non-synonymous *SIM1* mutations in extremely obese patients only, but no functional analyses were performed (8).

In the present study, we aimed to assess the contribution of rare mutations in *SIM1* to monogenic and syndromic forms of obesity. Coding regions of *SIM1* were sequenced in 44 children presenting with PWL syndrome features, 198 children with severe early-onset obesity, 568 morbidly obese adults, and 383 normal-weight controls. Subsequently, we functionally characterized the effects of the mutations on *SIM1* activity and analyzed phenotypes in *SIM1*-deficient patients.

Results and Discussion

By focusing on rare coding variants only (minor allele frequency [MAF] <1%), we identified 4 substitutions (p.I128T, p.Q152E, p.R581G, and p.T714A; Figure 1) in 4 children presenting with

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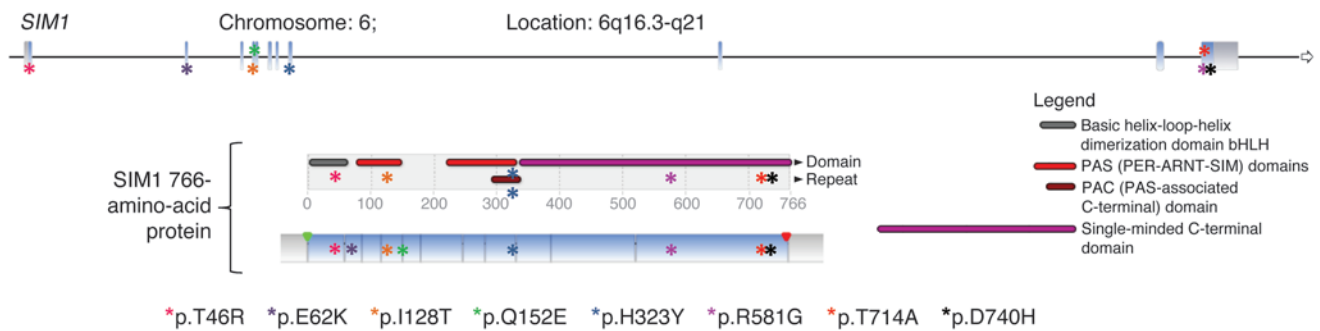


Figure 1

Location of the 8 *SIM1* rare substitutions. At the top are shown the substitution locations in exons. In the middle are shown the substitution locations in protein domains (only substitutions located in specific domains are reported). At the bottom are shown the substitution locations in the *SIM1* 766-amino-acid protein.

clinical features of PWL syndrome, including severe obesity that started between 1 and 2 years of age (Table 1 and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI68035DS1), and 4 other substitutions (p.T46R, p.E62K, p.H323Y, and p.D740H; Figure 1) in 7 morbidly obese adults (Table 1 and Supplemental Table 2).

With regard to the obese child *SIM1* mutant carriers presenting with PWL syndrome (Supplemental Table 1 and Supplemental Figure 1), the girl carrying the p.I128T variant received the substitution from her normal-weight father. The mother of the girl carrying the p.Q152E variant did not carry this substitution; the father's DNA and phenotypes were not available. The girl carrying the p.R581G variant was an adopted child. In contrast, we were

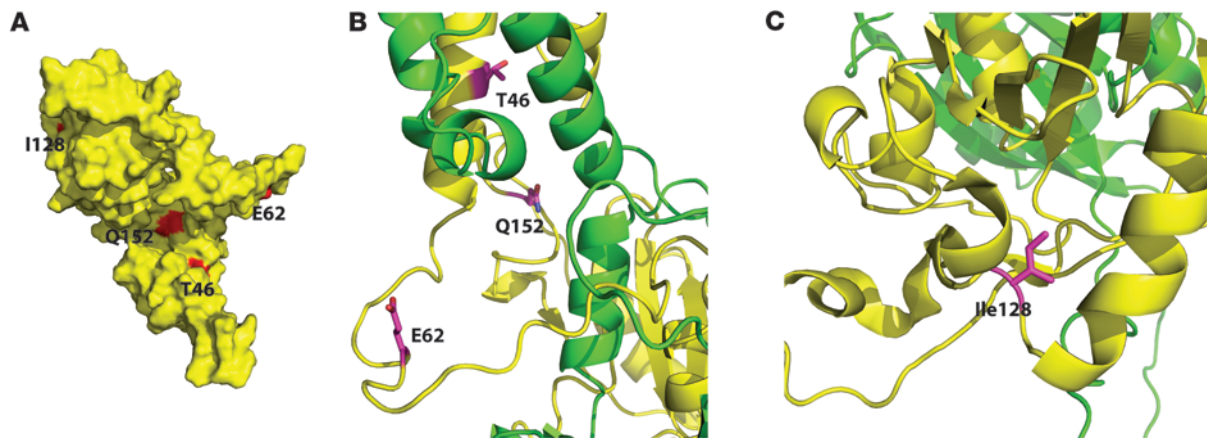
able to investigate the family of the boy carrying the p.T714A variant. His 3 siblings, his mother, and a maternal aunt also carried this substitution. The mother and her 4 children were severely obese and presented at least one other clinical feature associated with PWL syndrome, while the aunt was only overweight and without clinical abnormalities (Supplemental Table 3).

Among the morbidly obese adult *SIM1* mutant carriers (Supplemental Table 2 and Supplemental Figure 2), 4 unrelated patients carried the same p.T46R variant. In the first mutated participant, the p.T46R variant had arisen de novo. In the second mutated participant, the p.T46R variant had been inherited from his obese mother. Furthermore, this participant had an obese sister who also carried the p.T46R variant. Although parental DNA samples were

Table 1
Genetic and functional description of the 8 *SIM1* rare substitutions

Variant	p.T46R	p.E62K	p.I128T	p.Q152E	p.H323Y	p.R581G	p.T714A	p.D740H
Codon change	ACG>AGG	GAG>AAG	ATT>ACT	CAG>GAG	CAC>TAC	AGA>GGA	ACT>GCT	GAT>CAT
dbSNP135	–	rs201038781	rs138546433	rs140908824	–	–	–	–
MAF (in European Americans)	–	NA	0.06%	0.01%	–	–	–	–
No. carriers (proband and their relatives)								
PWL syndrome	0	0	1	1	0	1	5	0
Obesity	7	1	0	0	2	0	0	1
Overweight	0	0	0	0	0	0	1	0
Normal weight	0	0	1	0	0	0	0	0
In silico functional prediction								
Polyphen-2	Probably damaging	Probably damaging	Possibly damaging	Benign	Probably damaging	Possibly damaging	Benign	Probably damaging
Sift	Deleterious	Deleterious	Deleterious	Tolerated	Tolerated	Deleterious	Tolerated	Tolerated
SNAP	Non neutral	Neutral	Neutral	Neutral	Non neutral	Non neutral	Neutral	Non Neutral
Pmut	Pathological	Pathological	Pathological	Neutral	Neutral	Pathological	Neutral	Pathological
Align GVG D	C15	C0	C25	C0	C0	C0	C0	C0
Total ^A	5	3	4	0	2	4	0	3
Functional effect according to the present investigation ^B	2	1	1	1	2	0	2	1

^AThe total risk score (0 = lowest risk → 5 = highest risk) is the sum of each score calculated per software (see Supplemental Methods); 0 if Polyphen-2 = "Benign," Sift = "Tolerated," SNAP = "Neutral," Pmut = "Neutral," Align GVG D = "C0"; 1 if different than "benign," "tolerant," "neutral," or "C0." ^B0 = no effect; 1 = mild effect on *SIM1* activity with ARNT or ARNT2; 2 = strong effect on *SIM1* activity with ARNT and ARNT2. MAF is according to the NHLBI Exome Project. NA, not available.

**Figure 2**

Homology model of the SIM1:ARNT2 heterodimer. SIM1 is shown in yellow and ARNT2 in green. (A) Surface representation of SIM1, with amino acids that harbor variants shown in red. (B and C) Ribbons diagram of the SIM1:ARNT2 heterodimer, with amino acids that harbor variants depicted in pink.

not available for the third p.T46R-carrying participant, her obese son carried the substitution, while her normal-weight sister and normal-weight daughter did not. The family of the fourth p.T46R-carrying participant was not available. The patient carrying the p.E62K variant had a normal-weight mother and 2 normal-weight daughters, none of whom carried the substitution. His severely obese father has died, and no DNA was available. The carrier of the p.H323Y variant had a morbidly obese sister who was also a p.H323Y carrier, while both parents had died. No DNA was available from relatives of the p.D740H carrier.

In summary, we identified 21 (adults and children) carriers of a rare heterozygous *SIM1* variant (11 probands and 10 of their relatives) and 16 non-carrier relatives, and we found a strong contribution of *SIM1* rare variants to intra-family risk for obesity (odds ratio_[95% confidence interval]: OR = 20.9_[3.5–126.5]; $P = 9.3 \times 10^{-4}$).

Of note, 3 of the 8 rare variants have been listed in the Single Nucleotide Polymorphism database (dbSNP 137) — p.E62K/rs201038781, p.I128T/rs138546433, and p.Q152E/rs140908824 — but are very rare in the general population, according to the NHLBI Exome Project and the 1000 Genomes Project (MAF <0.1%). When we sequenced 383 normal-weight controls, we found a single carrier of the p.I128T variant (in the heterozygous state). A previous study reported that p.I128T did not cosegregate with either obesity or overweight phenotype in a family of European descent (9). Therefore, this variant is unlikely to have a significant effect on obesity or PWL-related clinical features. We then genotyped 2,896 normal-weight individuals but did not find any carriers of the substitutions p.T46R, p.E62K, p.Q152E, p.H323Y, p.R581G, p.T714A, or p.D740H.

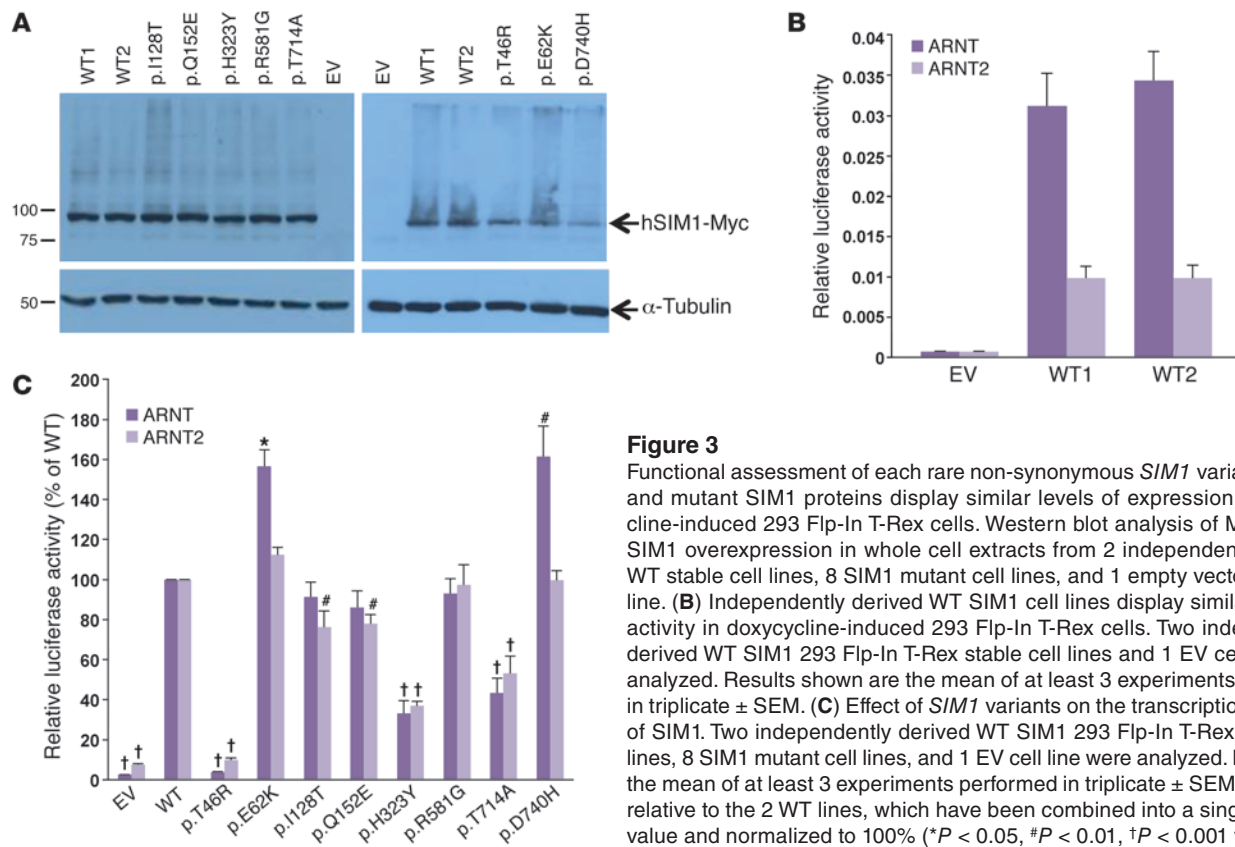
Subsequently, we aimed to functionally assess the 8 rare substitutions. Using five types of in silico prediction software, we found that the mutation with the highest risk score of damaging effect was p.T46R, while the substitutions with the lowest risk score were p.Q152E and p.T714A (Table 1). One of the aryl hydrocarbon receptor nuclear translocators, either ARNT or ARNT2, is required as a dimerization partner for SIM1 to function as a transcription factor, with mouse knockout studies suggesting that ARNT2 is the in vivo partner in the hypothalamus (10, 11). We constructed a homology model to estimate structural features of

the SIM1:ARNT2 dimer at the bHLH and PASA domains, which was based upon the crystal structure for the N-terminal half of the CLOCK:BMAL1 dimer (12). We found that the N-terminal amino acids (T46, E62, I128, and Q152) likely lie on the surface of the protein (Figure 2A). E62, I128, and Q152 amino acids were located in unstructured loops (Figure 2, B and C); thus, their possible contributions to perturbations of SIM1 structure could not be estimated. However, T46 lay in the middle of a helix that was in close proximity to a helix of ARNT2 (Figure 2B); thus, the larger arginine residue may critically perturb the dimerization interface.

We then assessed the functional effects of the 8 substitutions on the transcriptional properties of the SIM1:ARNT and SIM1:ARNT2 dimers in vitro using luciferase gene reporter assays in human 293 Flp-In T-Rex stable cell lines expressing the WT protein or one of the 8 SIM1 mutants (Figure 3, A and B). Importantly, with this system, only 1 copy of the mutated (or WT) SIM1 is integrated at a specific and predefined insertion site in the cell genome. This renders activity levels independent of the number of inserted copies and the insertion site, and allowed an accurate comparison of the mutants' activities. We found a strong loss-of-function effect of p.T46R, p.H323Y, and p.T714A mutations on SIM1 transcriptional activity with both ARNT and ARNT2 ($P < 0.001$; Figure 3C). These mutations were located in the bHLH, PASB, and C-terminal domains, respectively (Figure 1). The 3 mutations were identified in morbidly obese adults ($n = 9$ carriers of p.T46R or p.H323Y), in 1 overweight adult (carrier of p.T714A), and in severely obese subjects with PWL syndrome ($n = 4$ carriers of p.T714A). Together, these loss-of-function mutations were associated with a strong intra-family risk for obesity (OR = 28.0_[2.7–295.7]; $P = 5.6 \times 10^{-3}$). The p.I128T and p.Q152E variants, which were identified either in normal-weight participants ($n = 2$ carriers of p.I128T) or in severely obese subjects with PWL syndrome ($n = 2$ carriers of p.I128T or p.Q152E), had a milder loss-of-function effect on SIM1 activity with ARNT or ARNT2 (Figure 3C). Two substitutions (p.E62K and p.D740H) which were identified in 2 morbidly obese participants, had a gain-of-function effect on SIM1 activity with ARNT only (Figure 3C). We did not detect any effect of p.R581G on SIM1 activity (Figure 3C), which was identified in a severely obese participant with PWL syndrome. Together, the



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**Figure 3**

Functional assessment of each rare non-synonymous *SIM1* variant. **(A)** WT and mutant *SIM1* proteins display similar levels of expression in doxycycline-induced 293 Flp-In T-Rex cells. Western blot analysis of Myc-tagged *SIM1* overexpression in whole cell extracts from 2 independently derived WT stable cell lines, 8 *SIM1* mutant cell lines, and 1 empty vector (EV) cell line. **(B)** Independently derived WT *SIM1* cell lines display similar levels of activity in doxycycline-induced 293 Flp-In T-Rex cells. Two independently derived WT *SIM1* 293 Flp-In T-Rex stable cell lines and 1 EV cell line were analyzed. Results shown are the mean of at least 3 experiments performed in triplicate \pm SEM. **(C)** Effect of *SIM1* variants on the transcriptional activity of *SIM1*. Two independently derived WT *SIM1* 293 Flp-In T-Rex stable cell lines, 8 *SIM1* mutant cell lines, and 1 EV cell line were analyzed. Results are the mean of at least 3 experiments performed in triplicate \pm SEM expressed relative to the 2 WT lines, which have been combined into a single average value and normalized to 100% (* $P < 0.05$, # $P < 0.01$, † $P < 0.001$ vs. WT).

rare variants with mild or no effects on *SIM1* activity were not significantly associated with obesity within families (OR = 7.5_[0.5–122.7]; $P = 0.158$). When we compared our functional data with the in silico predictions, only 62.5% concordance was found.

Our genetic and functional studies, together with the findings of Ramachandrapa et al. (13), convincingly demonstrate a link between *SIM1* loss of function and severe to morbid obesity that may also be associated with PWL-related clinical features including developmental delay (or intellectual disability) and facial dysmorphism. The observed effects of *SIM1* loss-of-function mutations on both development and body weight regulation are in line with the key role of *SIM1* in development of the paraventricular nucleus of the hypothalamus (1). Importantly, the neccin protein (NDN), which is believed to be involved in the Prader-Willi syndrome (14), regulates the activity of the *SIM1*:ARNT2 dimer (15), reinforcing the putative role of *SIM1* in the PWL syndrome.

SIM1 non-synonymous mutations were present in numerous patients with severe obesity associated with, or independent of, PWL-related clinical features. Some mutations, such as p.T46R, dramatically reduced *SIM1* activity on a reporter gene and were only found in obese individuals, suggesting that loss-of-function *SIM1* mutants may underpin a monogenic form of obesity. However, some substitutions with marked loss-of-function effect (e.g., p.T714A) did co-segregate with overweight/obesity but were not always associated with severe obesity or PWL syndrome. Moreover, the p.I128T variant, which caused mild loss of *SIM1* function, was unlikely to contribute to obesity. Therefore, mutation of *SIM1* is not always responsible for a fully penetrant form of obesity. Similar features have been repeatedly found in *MC4R* loss-of-function

mutation carriers, where a permissive role of the environment on disease expression has been suggested (16, 17). The degree of penetrance is expected to be determined by the severity of loss of function of a particular mutant in combination with environment and genetic background. In obese patients presenting with the clinical features associated with Prader-Willi syndrome, if chromosomes 15q11 and 6q16 are found not to contain abnormalities, then *SIM1* sequencing and subsequent molecular characterization should be performed, in order to demonstrate the presence of causative loss-of-function mutations.

Methods

Further information is available in Supplemental Methods.

Statistics. For the genetic study, effects of mutation on obesity risk were calculated through univariate logistic regression models using SPSS 14.0. For the functional analysis, univariate ANOVA was performed on the log values generated for each mutant with reference to WT using SPSS 17.0. Separate analyses were performed for ARNT- and ARNT2-transfected cells. A P value less than 0.05 was considered significant.

Study approval. The study protocol was approved by the ethics committees of Lille II University, the University of Antwerp, the Canton of Zurich, and the University of Paris-Sud and the Institutional Review Board of the University of Florida, and study participants signed informed consent agreements. For children younger than 18 years, oral consent was obtained, and parents provided written informed consent.

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