nature genetics

Rare *MTNR1B* variants impairing melatonin receptor 1B function contribute to type 2 diabetes

Amélie Bonnefond[1,2,32], Nathalie Clément[3–5,32], Katherine Fawcett[6], Loïc Yengo[1,2], Emmanuel Vaillant[1,2], Jean-Luc Guillaume[3–5], Aurélie Dechaume[1,2], Felicity Payne[6], Ronan Roussel[7,8], Sébastien Czernichow[9,10], Serge Hercberg[11,12], Samy Hadjadj[13,14], Beverley Balkau[15,16], Michel Marre[7,8], Olivier Lantieri[17], Claudia Langenberg[18], Nabila Bouatia-Naji[1,2], The Meta-Analysis of Glucose and Insulin-Related Traits Consortium (MAGIC)[19], Guillaume Charpentier[20], Martine Vaxillaire[1,2], Ghislain Rocheleau[21,22], Nicholas J Wareham[18], Robert Sladek[23,24], Mark I McCarthy[25,26], Christian Dina[27–29], Inês Barroso[6,30], Ralf Jockers[3–5,33] & Philippe Froguel[1,2,31,33]

© 2012 Nature America, Inc. All rights reserved.

Genome-wide association studies have revealed that common noncoding variants in *MTNR1B* (encoding melatonin receptor 1B, also known as MT_2) increase type 2 diabetes (T2D) risk[1,2]. Although the strongest association signal was highly significant ($P < 1 \times 10^{-20}$), its contribution to T2D risk was modest (odds ratio (OR) of ~1.10–1.15)[1–3]. We performed large-scale exon resequencing in 7,632 Europeans, including 2,186 individuals with T2D, and identified 40 nonsynonymous variants, including 36 very rare variants (minor allele frequency (MAF) <0.1%), associated with T2D (OR = 3.31, 95% confidence interval (CI) = 1.78–6.18; $P = 1.64 \times 10^{-4}$). A four-tiered functional investigation of all 40 mutants revealed that 14 were non-functional and rare (MAF < 1%), and 4 were very rare with complete loss of melatonin binding and signaling capabilities. Among the very rare variants, the partial- or total-loss-of-function variants but not the neutral ones contributed to T2D (OR = 5.67, CI = 2.17–14.82; $P = 4.09 \times 10^{-4}$). Genotyping the four complete loss-of-function variants in 11,854 additional individuals revealed their association with T2D risk (8,153 individuals with T2D and 10,100 controls; OR = 3.88, CI = 1.49–10.07; $P = 5.37 \times 10^{-3}$). This study establishes a firm functional link between *MTNR1B* and T2D risk.

Disruption of central and peripheral circadian rhythms, including the pancreatic clock, may lead to metabolic disorders and type 2 diabetes (T2D)[4,5]. The neurohormone melatonin (MLT) is mainly secreted from the pineal gland in a circadian pattern, with higher levels being observed during the night. MLT targets two high-affinity G protein–coupled receptors (GPCRs), melatonin receptor 1A (also known as MT_1, encoded by *MTNR1A*) and MT_2 (encoded by *MTNR1B*), which modulate both G_i protein–adenylyl cyclase and ERK1 and ERK2 (ERK1/2) pathways[6,7]. Genome-wide association studies (GWAS) recently revealed an association between SNPs in *MTNR1B* and T2D risk in humans[1,2]. However, the contribution of these SNPs to T2D risk was typical for common alleles[1–3]. So far, the effects seen for all common T2D-associated SNPs identified by GWAS explain less than 10% of T2D heritability[3,8]. The missing heritability of T2D might be partially explained by numerous rare mutations that have a stronger functional effect[9]. However, rare variant effects cannot easily be identified via GWAS because of problems of statistical power. Such rare variant effects have been discovered in GWAS-identified susceptibility genes for type 1 diabetes[10,11] and hypertriglyceridemia[12] by large-scale resequencing. Recently, two T2D-associated genes identified by GWAS (*HHEX* and *KCNJ11*) were sequenced in 13,715 individuals, and an excess of rare recent variants were found, consistent with explosive population growth[13]. The authors of this study suggested that increased disease (T2D) risk in contemporary

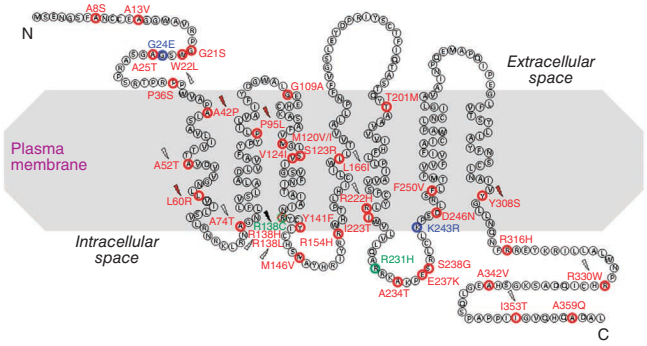

Figure 1 Distribution of the 40 nonsynonymous MT_2 variants identified by exon resequencing. MAF calculations were based on the whole-sequencing dataset (7,632 individuals, including 2,186 with T2D). Nonsynonymous variants are colored: blue, MAF ≥1%; green, MAF between 0.1% and 1%; and red, MAF < 0.1%. Red flash, mutants that completely lack melatonin binding (and associated downstream signaling); white flash, mutants with only impaired G_i protein–dependent signaling; black flash, mutants with impaired G_i protein–dependent signaling and ERK1/2 activation. Nomenclature for variants refers to functional protein sequences.

A full list of author affiliations appears at the end of the paper.

Received 15 September 2011; accepted 2 December 2011; published online 29 January 2012; doi:10.1038/ng.1053

Table 1 Contribution of nonsynonymous *MTNR1B* variants to T2D risk

Variants	Position on chr. 11	Amino acid change	Allele change	MAF_1 (%)	MAF_2 (%) T2D (cases)	IFG	NG (controls)	OR (95% CI)	*P*
G24E	92,342,610	G>E	G>A	≥1	6.43	5.45	6.96	0.87 (0.75–1.00)	0.06
K243R	92,354,765	K>R	A>G	≥1	2.42	2.26	2.76	0.86 (0.67–1.11)	0.25
R138C	92,354,449	R>C	C>T	0.1≤*x*<1	0.16	0.23	0.14	1.00 (0.40–3.20)	0.81
R231H	92,354,729	R>H	G>A	0.1≤*x*<1	0.66	0.93	0.76		
A8S	92,342,561	A>S	G>T	<0.1	0	0	0.01	3.31 (1.78–6.18)	1.64×10^{-4}
A13V	92,342,577	A>V	C>T	<0.1	0	0	0.01		
G21S	92,342,600	G>S	G>A	<0.1	0	0	0.01		
W22L	92,342,604	W>L	G>T	<0.1	0.02	0	0		
A25T	92,342,612	A>T	G>A	<0.1	0	0	0.01		
P36S	92,342,645	*P*>S	C>T	<0.1	0.02	0	0		
A42P	92,342,663	A>P	G>C	<0.1	0.02	0	0		
A52T	92,342,693	A>T	G>A	<0.1	0.02	0	0		
L60R	92,342,718	L>R	*T*>G	<0.1	0.11	0.08	0.06		
A74T	92,342,759	A>T	G>A	<0.1	0.02	0	0.01		
P95L	92,354,321	*P*>L	C>T	<0.1	0.02	0	0		
G109A	92,354,363	G>A	G>C	<0.1	0	0	0.01		
M120V	92,354,395	M>V	A>G	<0.1	0	0	0.02		
M120I	92,354,397	M>I	G>A	<0.1	0	0.08	0.01		
S123R	92,354,406	S>R	C>G	<0.1	0	0.08	0.01		
V124I	92,354,407	V>I	G>A	<0.1	0.11	0.16	0.08		
R138L	92,354,450	R>L	G>T	<0.1	0	0	0.02		
R138H	92,354,450	R>H	G>A	<0.1	0	0	0.01		
Y141F	92,354,459	Y>F	A>T	<0.1	0.02	0	0		
M146V	92,354,473	M>V	A>G	<0.1	0	0	0.01		
R154H	92,354,498	R>H	G>A	<0.1	0.02	0	0.01		
L166I	92,354,533	L>I	C>A	<0.1	0	0	0.01		
T201M	92,354,639	*T*>M	C>T	<0.1	0.02	0	0		
R222H	92,354,702	R>H	G>A	<0.1	0.02	0	0		
I223T	92,354,705	I>T	*T*>C	<0.1	0.02	0	0		
A234T	92,354,737	A>T	G>A	<0.1	0	0	0.03		
E237K	92,354,746	E>K	G>A	<0.1	0	0	0.01		
S238G	92,354,750	S>G	G>A	<0.1	0.02	0	0		
D246N	92,354,774	D>N	G>A	<0.1	0.02	0	0		
F250V	92,354,786	*F*>V	*T*>G	<0.1	0.02	0	0		
Y308S	92,354,963	Y>S	A>C	<0.1	0.02	0	0		
R316H	92,354,984	R>H	G>A	<0.1	0	0	0.01		
R330W	92,355,025	R>W	C>T	<0.1	0.02	0	0		
A342V	92,355,062	A>V	C>T	<0.1	0.07	0	0		
I353T	92,355,095	I>T	*T*>C	<0.1	0.05	0	0.02		
A359Q	92,355,113	A>Q	C>A	<0.1	0	0	0.01		

Nonsynonymous *MTNR1B* variants were identified by exon resequencing in 7,632 European individuals. Mutation position is based on human genome build NCBI36/hg18, and amino acid position is based on the protein reference sequence NP_005950. There were 2,186 individuals with T2D, 642 individuals with impaired fasting glucose and 4,804 individuals with normal fasting glucose in the study. Chr., chromosome; IFG, impaired fasting glucose; NG, normal fasting glucose. MAF_1 calculation was based on the whole sequencing dataset (7,632 individuals). MAF_2 calculations were based on T2D, IFG or NG participants.

populations might be heavily influenced by the distribution of rare variants. However, they did not test for T2D association or function for any of the variants[13].

Based on the strong evidence for the association between the *MTNR1B* gene and T2D risk, together with the fact that MT_2 belongs to the superfamily of GPCRs that are privileged drug targets, we wanted to identify rare nonsynonymous variants with putative effect on the function of MT_2 that associate with T2D risk. We sequenced the two exons of *MTNR1B* in 7,632 unrelated European individuals with known glycemic status, including 2,186 subjects with T2D (**Supplementary Table 1**). We identified 40 nonsynonymous variants, including two common SNPs (MAF >1%; rs8192552 encoding a G24E variant and rs61747139 encoding a K243R variant), two less common variants (MAF between 0.1 and 1%; rs61746674 encoding an R138C variant and rs8192553 encoding an R231H variant) and 36 very rare variants (MAF <0.1%), which were not previously listed in public SNP databases (**Fig. 1** and **Table 1**). No association was found between T2D risk and either of the common SNPs (rs8192552 or rs61747139) (**Table 1**). The rarer variants were analyzed by pooling them according to their MAFs: we analyzed one pool of the two variants with MAFs between 0.1 and 1% and a second pool of the 36 variants with MAFs of <0.1%. By using the kernel-based adaptive cluster (KBAC) method[14] embedded in a logistic regression model, we found that the variants with intermediate MAFs did not associate with T2D risk ($N_{controls}$ = 4,804; N_{cases} = 2,186, where cases are individuals with T2D; odds ratio (OR) = 1.00, 95% confidence interval (CI) = 0.40–3.20; $P = 0.81$; **Table 1**), whereas the rarest variants (with MAF <0.1%) contributed strongly to increased T2D risk (OR = 3.31, 95% CI = 1.78–6.18; $P = 1.64 \times 10^{-4}$; **Table 1**).

© 2012 Nature America, Inc. All rights reserved.

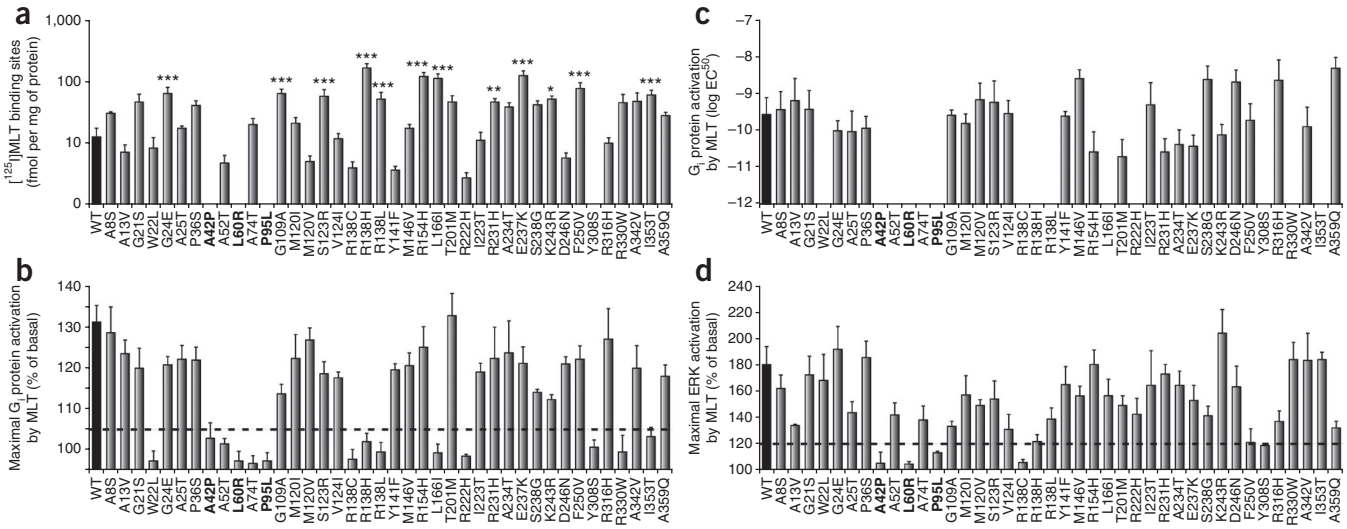

Figure 2 Functional characterization of wild-type and mutant MT_2 receptors. (**a**) Expression levels in Flp-In HEK293 cells determined by [^{125}I]MLT saturation binding experiments. (**b**) Maximal G_i protein–dependent signaling. (**c**) Half-maximal effective concentration (EC_{50}) values of G_i protein–dependent signaling determined in MLT dose-response curves. (**d**) ERK activation in the presence of 100 nM MLT. The threshold lines in **b** and **d** were defined according to the known variability in each assay. MT_2 mutants deficient in ligand binding are highlighted in bold. Data are presented as mean ± s.e.m. for at least three independent experiments, each performed in duplicate. Significant differences relative to wild-type MT_2 are indicated; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. WT, wild type; [^{125}I]MLT, 2-[^{125}I]-iodomelatonin.

Subsequently, we wanted to determine which nonsynonymous *MTNR1B* variants altered MT_2 function. Thus, we assessed the effects of all 40 nonsynonymous *MTNR1B* variants on MT_2 cell surface expression and 2-[^{125}I]-iodomelatonin ([^{125}I]MLT) binding, as well as on MLT-dependent G_i protein and ERK1/2 activation in human HEK293 cells. We found that cell surface expression of all 40 MT_2 mutants was indistinguishable from that of the wild-type MT_2 receptor when measured using immunohistochemical techniques (**Supplementary Fig. 1a**). [^{125}I]MLT saturation binding experiments revealed a marginal (fourfold) variation in the dissociation constant (K_d) for most mutants (**Supplementary Fig. 1b**) and a tenfold variation in the number of [^{125}I]MLT binding sites (**Fig. 2a**). Notably, four MT_2 mutants (the A42P, L60R, P95L and Y308S variants) showed no [^{125}I]MLT binding ability (**Fig. 2a** and **Supplementary Fig. 1b**). These same mutants did not activate downstream G_i protein–dependent (**Fig. 2b**,**c**) or ERK1/2 (**Fig. 2d**) signaling pathways. Ten additional mutants with intact MLT binding (the W22L, A52T, A74T, R138C, R138H, R138L, L166I, R222H, R330W and I353T variants) showed impaired G_i protein–dependent signaling, whereas the other mutants had similar activity to the wild-type MT_2 receptor (**Fig. 2b**,**c**). Of the ten mutants with impaired G_i protein signaling, only the R138C variant was unable to activate the ERK1/2 pathway, indicating the pathway-specific effect of the other nine mutants (**Fig. 2d**). It is noteworthy that all of the variants that ablated MLT binding and all but one of the variants that inhibited signaling downstream of MT_2 were very rare and had MAFs <0.1%.

The four MT_2 mutants deficient in MLT binding contain amino acid substitutions in the predicted transmembrane domains I (p.Ala42Pro and p.Leu60Arg), II (p.Pro95Leu) and VII (p.Tyr308Ser) (**Fig. 1**). The ten MT_2 mutants with impaired G_i protein signaling have amino acid changes localized to the predicted I (p.Ala52Thr), II (p.Ala74Thr), III (p.Arg138Cys, p.Arg138His and p.Arg138Leu), IV (p.Leu166Ile) and V (p.Arg222His) transmembrane domains, N terminus (p.Trp22Leu), and the C terminus (p.Arg330Trp and p.Ile353Thr) (**Fig. 1**). Four of these alterations affect conserved motifs in MT_2: p.Tyr308Ser alters a conserved NP(A)XXY motif (where X represents any amino acid), and p.Arg138Cys, p.Arg138Leu and p.Arg138His alter the D(N)RY motif, both of which are involved in GPCR activation[15].

Table 2 Contribution of nonsynonymous *MTNR1B* variants to increased T2D risk according to their consequences on MT_2 function

Consequences on MT_2 function	Variants	N_{T2D} (% of variant carriers)	N_{NG} (% of variant carriers)	OR (95% CI)	P
No melatonin binding (and downstream signaling)	A42P, L60R, P95L, Y308S	8,153 (0.22)	10,100 (0.13)	3.88 (1.49–10.07)	5.37×10^{-3}
No G_i protein–dependent signaling	W22L, A52T, A74T, R138C, R138H, R138L, L166I, R222H, R330W, I353T	2,186 (0.64)	4,804 (0.42)	2.66 (1.01–7.00)	0.047
No ERK activation	R138C	2,186 (0.32)	4,804 (0.27)	1.24 (0.44–3.51)	0.680
Loss-of-function variants with MAF <0.1%	W22L, A42P, A52T, L60R, A74T, P95L, R138H, R138L, L166I, R222H, Y308S, R330W, I353T	2,186 (0.69)	4,804 (0.27)	5.67 (2.17–14.82)	4.09×10^{-4}
Neutral variants with MAF <0.1%	A8S, A13V, G21S, A25T, P36S, G109A, M120V, M120I, S123R, V124I, Y141F, M146V, R154H, T201M, I223T, A234T, E237K, S238G, D246N, F250V, R316H, A342V, A359Q	2,186 (0.73)	4,804 (0.52)	2.15 (0.93–4.95)	0.072

Loss-of-function variants are variants that impair MT_2 function according to our four-tiered functional investigation. N_{T2D}, number of individuals with T2D; N_{NG}, number of individuals with normal fasting glucose.

© 2012 Nature America, Inc. All rights reserved.

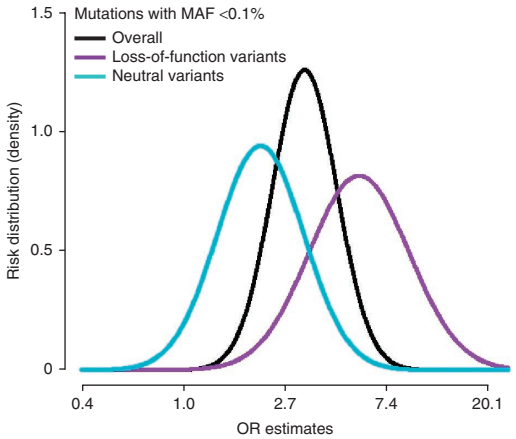

Figure 3 Odds ratio estimates of partial or total loss-of-function variants compared to neutral very rare (MAF <0.1%) variants for T2D risk determined on the basis of *MTNR1B* sequencing data. For each pool of variants (purple, loss-of-function; cyan, neutral; black, combined), we generated an estimate of the effect size and its associated s.e.m. The figure represents the assumed normal distributions of the ORs (log scale) centered on the effect sizes, with s.d. equal to the respective estimated s.e.m. The relative impact of functional variants to that of all variants with MAFs <0.1% is shown. Loss-of-function variants are variants determined to have impaired MT_2 function in our four-tiered functional investigation.

To further assess the observed deficits in MT_2 function, we compared our functional data with sequence- and structure-based predictions made by Polymorphism Phenotyping (PolyPhen-2) v2 software (**Supplementary Table 2**)[16]. By defining variants that were predicted to be 'possibly' or 'probably' damaging by PolyPhen-2 as loss-of-function mutants, we found 60% concordance between our functional data and the PolyPhen-2 predictions (**Supplementary Table 3**). Furthermore, the software predicted 30% false positive and 7.5% false negative loss-of-function variants compared to our own data. It is noteworthy that the most recent version of PolyPhen-2 (v2.0.23; released 9 December 2010) is more sensitive than the previous version (v2.0.22), but it identified more potentially damaging variants that were not biologically confirmed (**Supplementary Table 3**).

We then assessed whether the four total loss-of-function MT_2 variants had a significant effect on increased T2D risk, determining whether we could confirm what was previously observed for all rare variants with MAF <0.1%. We genotyped the portion *MTNR1B* encoding the A42P, L60R, P95L and Y308S variants in 11,854 additional French subjects, including 5,967 individuals with T2D. By analyzing these variants as a pool using the KBAC method embedded in a logistic regression model, we found that the A42P, L60R, P95L and Y308S variants had a strong and significant effect on increased T2D risk ($N_{controls}$ = 10,100; N_{cases} = 8,153; OR = 3.88, 95% CI = 1.49–10.07; $P = 5.37 \times 10^{-3}$; **Table 2**), confirming the association between the total loss of function of MT_2 variants and T2D risk. A study has described impaired G protein–dependent MT_2 function caused by an *MTNR1B* mutation encoding a p.Leu60Arg alteration[17]. No association with T2D was found, probably as a result of statistical power issues[17], emphasizing the need for extensive composite sequencing-genotyping-biological studies in very large populations.

The pool of ten partial loss-of-function MT_2 variants (which had impaired G_i protein signaling without MLT binding deficiency) showed a trend toward association with T2D risk ($N_{controls}$ = 4,804; N_{cases} = 2,186; OR = 2.66, 95% CI = 1.01–7.00; P = 0.047; **Table 2**). This suggests that G_i protein–dependent signaling might contribute to T2D risk. Of note, the original group of 36 nonsynonymous variants with MAF <0.1% that associated with T2D (**Table 1**) can be split into two categories after our current biological assessment: a group of 13 loss-of-function variants that are strongly associated with T2D risk ($N_{controls}$ = 4,804; N_{cases} = 2,186; OR = 5.67, 95% CI = 2.17–14.82; $P = 4.09 \times 10^{-4}$; **Fig. 3** and **Table 2**) and a second group of 23 seemingly neutral variants that did not associate with T2D risk (**Fig. 3** and **Table 2**). Therefore, the association between rare nonsynonymous *MTNR1B* variants and T2D risk is further supported by the association of mutants that showed evidence of melatonin signaling impairment.

Based on RNA expression data, it has been reported that the GWAS-identified at-risk allele for T2D at rs10830963 (located in the unique *MTNR1B* intron) was associated with increased *MTNR1B* transcript levels in human islets[18]. As pharmacological doses of MLT inhibit insulin secretion from murine β-cell lines[18,19], it was suggested that inhibiting MLT receptors could be a therapeutic avenue for T2D treatment[18]. Our functional analyses at the protein level, showing an association between defective MT_2 receptor function and T2D risk, suggest that the previously observed increased *MTNR1B* expression is not causal but could instead be a result of the absence of negative feedback regulatory events under conditions of impaired MT_2 receptor signaling. Plausible mechanisms are loss of the acute inhibitory effect of melatonin on insulin secretion or loss of the effect of melatonin on circadian rhythm entrainment. Hence, MLT receptor agonists that are currently prescribed for sleep and circadian rhythm disorders as well as for depression[20] may be beneficial for T2D therapy.

Our data confirm that resequencing GWAS-identified susceptibility genes for a complex disease (like T2D), followed by the careful biological evaluation of newly identified rare but potentially damaging variants, can contribute to a more complete picture of the genetic architecture of disease risk.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We are sincerely indebted to all participants in the genetic study. We thank M. Deweirder and F. Allegaert for their technical assistance and their invaluable management of DNA samples and B. Gardiola-Lemaître for her invaluable advice. This study was supported by the French Agence Nationale de la Recherche (ANR-08-GENOPAT to P.F. and ANR-11-blanc 'MLT2D' and ANR-11-META 'MELA-BETES' to R.J. and P.F.), the Contrat de Projets Etat-Région Nord-Pas-De-Calais (CPER 2007-2013 'Axe Cardio-Diabète' to P.F.), the Société Francophone du Diabète (to A.B.), the Fondation Recherche Médicale ('Equipe FRM' to R.J.), Inserm and CNRS. I.B. acknowledges funding from the Wellcome Trust (077016/Z/05/Z) and from the UK National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre and the MRC Centre for Obesity and Related Metabolic Diseases.

AUTHOR CONTRIBUTIONS

A.B., R.J. and P.F. wrote the manuscript, and N.C., J.-L.G., M.V., C.D., R.S., M.I.M. and I.B. reviewed and/or edited the manuscript and contributed to discussion. R.J. and P.F. managed the project. L.Y. performed the statistical analyses, and A.B., G.R. and C.D. contributed to statistical analyses. A.B. and K.F. performed the sequencing. E.V. performed the genotyping. A.B., N.C., E.V., J.-L.G. and A.D. performed the functional analyses. F.P., R.R., S.C., S. Hercberg, S. Hadjadj, B.B., M.M., O.L., C.L., N.B.-N., MAGIC, G.C., N.J.W., M.I.M. and I.B. contributed to cohort study samples and researched data.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

© 2012 Nature America, Inc. All rights reserved.

Published online at http://www.nature.com/naturegenetics/.
Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Bouatia-Naji, N. *et al.* A variant near *MTNR1B* is associated with increased fasting plasma glucose levels and type 2 diabetes risk. *Nat. Genet.* **41**, 89–94 (2009).
2. Prokopenko, I. *et al.* Variants in *MTNR1B* influence fasting glucose levels. *Nat. Genet.* **41**, 77–81 (2009).
3. Voight, B.F. *et al.* Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat. Genet.* **42**, 579–589 (2010).
4. Bass, J. & Takahashi, J.S. Circadian integration of metabolism and energetics. *Science* **330**, 1349–1354 (2010).
5. Marcheva, B. *et al.* Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature* **466**, 627–631 (2010).
6. Dubocovich, M.L. *et al.* International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein–coupled melatonin receptors. *Pharmacol. Rev.* **62**, 343–380 (2010).
7. Jockers, R., Maurice, P., Boutin, J.A. & Delagrange, P. Melatonin receptors, heterodimerization, signal transduction and binding sites: what's new? *Br. J. Pharmacol.* **154**, 1182–1195 (2008).
8. Manolio, T.A. *et al.* Finding the missing heritability of complex diseases. *Nature* **461**, 747–753 (2009).
9. Schork, N.J., Murray, S.S., Frazer, K.A. & Topol, E.J. Common vs. rare allele hypotheses for complex diseases. *Curr. Opin. Genet. Dev.* **19**, 212–219 (2009).
10. Nejentsev, S., Walker, N., Riches, D., Egholm, M. & Todd, J.A. Rare variants of *IFIH1*, a gene implicated in antiviral responses, protect against type 1 diabetes. *Science* **324**, 387–389 (2009).
11. Downes, K. *et al.* Reduced expression of *IFIH1* is protective for type 1 diabetes. *PLoS ONE* **5**, e12646 (2010).
12. Johansen, C.T. *et al.* Excess of rare variants in genes identified by genome-wide association study of hypertriglyceridemia. *Nat. Genet.* **42**, 684–687 (2010).
13. Coventry, A. *et al.* Deep resequencing reveals excess rare recent variants consistent with explosive population growth. *Nat. Commun.* **1**, 131 (2010).
14. Liu, D.J. & Leal, S.M. A novel adaptive method for the analysis of next-generation sequencing data to detect complex trait associations with rare variants due to gene main effects and interactions. *PLoS Genet.* **6**, e1001156 (2010).
15. Nygaard, R., Frimurer, T.M., Holst, B., Rosenkilde, M.M. & Schwartz, T.W. Ligand binding and micro-switches in 7TM receptor structures. *Trends Pharmacol. Sci.* **30**, 249–259 (2009).
16. Adzhubei, I.A. *et al.* A method and server for predicting damaging missense mutations. *Nat. Methods* **7**, 248–249 (2010).
17. Andersson, E.A. *et al.* *MTNR1B* G24E variant associates with BMI and fasting plasma glucose in the general population in studies of 22,142 Europeans. *Diabetes* **59**, 1539–1548 (2010).
18. Lyssenko, V. *et al.* Common variant in *MTNR1B* associated with increased risk of type 2 diabetes and impaired early insulin secretion. *Nat. Genet.* **41**, 82–88 (2009).
19. Peschke, E. & Muhlbauer, E. New evidence for a role of melatonin in glucose regulation. *Best Pract. Res. Clin. Endocrinol. Metab.* **24**, 829–841 (2010).
20. Dubovsky, S.L. & Warren, C. Agomelatine, a melatonin agonist with antidepressant properties. *Expert Opin. Investig. Drugs* **18**, 1533–1540 (2009).

[1]Centre National de la Recherche Scientifique (CNRS) Unité Mixte de Recherche (UMR) 8199, Lille Pasteur Institute, Lille, France. [2]Ecole Doctorale de Biologie–Santé, Lille Nord de France University, Lille, France. [3]Institut National de la Santé et de la Recherche Médicale (Inserm) U1016, Institut Cochin, Paris, France. [4]CNRS UMR 8104, Paris, France. [5]Department of Medicine, Paris Descartes University, Paris, France. [6]Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK. [7]Department of Endocrinology, Diabetology and Nutrition, Bichat-Claude Bernard University Hospital, Assistance Publique des Hôpitaux de Paris (AP-HP), Paris, France. [8]Inserm U695, Paris 7 University, Paris, France. [9]Department of Nutrition, Ambroise Paré Hospital, AP-HP, Boulogne-Billancourt, France. [10]Unité de Formation et de Recherche (UFR) des Sciences de la Santé, University of Versailles Saint-Quentin, Boulogne-Billancourt, France. [11]Inserm U557, Institut National de la Recherche Agronomique Unit 1125, Conservatoire National des Arts et Métiers, Centre de Recherches en Nutrition Humaine, Paris 13 University, Bobigny, France. [12]Department of Public Health, Avicenne Hospital, AP-HP, Bobigny, France. [13]Department of Endocrinology and Diabetology, Centre Hospitalier Universitaire Poitiers, Poitiers, France. [14]Inserm U927, Clinical Investigation Centre of Poitiers (Biotheque CIC0802), Poitiers, France. [15]Inserm U780, Centre for Research in Epidemiology and Population Health, Villejuif, France. [16]Santé Publique, Paris-Sud 11 University, Orsay, France. [17]Institut Inter-Régional pour la Santé (IRSA), La Riche, France. [18]Medical Research Council (MRC) Epidemiology Unit, Institute of Metabolic Science, University of Cambridge, Cambridge, UK. [19]A full list of consortium members is provided in the **Supplementary Note**. [20]Department of Endocrinology and Diabetology, Corbeil-Essonnes Hospital, Essonnes, France. [21]Centre de Recherche, Centre Hospitalier de l'Université de Montreal, Montreal, Quebec, Canada. [22]Prognomix, Montreal, Quebec, Canada. [23]Department of Human Genetics, Faculty of Medicine, McGill University, Montreal, Quebec, Canada. [24]Genome Quebec Innovation Centre, Montreal, Quebec, Canada. [25]Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Oxford, UK. [26]Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK. [27]Inserm U915, Institut du Thorax, Nantes, France. [28]CNRS Equipe de Recherche Labellisée 3147, Nantes, France. [29]Department of Biology, Medicine and Health, Nantes University, Nantes, France. [30]Metabolic Research Labs, Institute of Metabolic Science, Addenbrooke's Hospital, University of Cambridge, Cambridge, UK. [31]Department of Genomics of Common Disease, School of Public Health, Imperial College London, Hammersmith Hospital, London, UK. [32]These authors contributed equally to this work. [33]These authors jointly directed this work. Correspondence should be addressed to P.F. (p.froguel@imperial.ac.uk) or R.J. (ralf.jockers@inserm.fr).

© 2012 Nature America, Inc. All rights reserved.

ONLINE METHODS

The study design is summarized in **Supplementary Figure 2**.

Study participants. Clinical characteristics and phenotypic data collected from the study populations are reported (**Supplementary Table 1**). Fasting plasma glucose levels and T2D status were determined for all participants. All subjects in this study were unrelated and of European ancestry.

Sequencing of the two *MTNR1B* exons and genotyping of the four total loss-of-function *MTNR1B* mutations (encoding p.Ala46Pro, p.Leu60Arg, p.Pro95Leu and p.Tyr308Ser alterations) were performed in several general European cohort studies and T2D case-control studies (**Supplementary Table 1**). The non-diabetic subjects were obtained from five European studies: (i) the Data from the Epidemiological Study on the Insulin Resistance Syndrome (D.E.S.I.R.) cohort, which is a longitudinal study in the French general population, fully described elsewhere[21], (ii) the Ely study, which was established in 1990 as a prospective population-based cohort study of the etiology and pathogenesis of T2D (ref. 22), (iii) the Hertfordshire Cohort Study, which consists of men and women born between 1931 and 1939 and still residing in the English county of Hertfordshire, of whom almost 3,000 have been extensively characterized[23], (iv) obese probands from French pedigrees of obesity who were recruited at CNRS UMR 8199 through an ongoing national media campaign[24] and (v) the French SU.VI.MAX study, which is a randomized double-blind, placebo-controlled primary prevention trial designed to assess the usefulness of daily supplementation with antioxidant vitamins and minerals in reducing the frequency of major health problems in industrialized countries[25]. In total, 685 individuals with T2D were ascertained from the five previously described studies. The majority of subjects with T2D were recruited from (i) the Endocrinology-Diabetology Department of the Corbeil-Essonnes Hospital[26], (ii) the French Diabhycar/Diab2-Néphrogène/Surdiagène study[27] and (iii) the CNRS UMR 8199 (ref. 26).

For each individual, glycemic status was defined according to the 1997 American Diabetes Association criteria[28]: normal glucose was defined as fasting plasma glucose levels of <6.1 mmol/l without treatment with antidiabetic agents, impaired fasting glucose was defined as fasting plasma glucose levels of ≥6.1 mmol/l and <7.0 mmol/l without treatment with antidiabetic agents, and T2D was defined as fasting plasma glucose levels of ≥7.0 mmol/l or by treatment with antidiabetic agents.

All samples used were collected with appropriate informed consent consistent with their use in the present study. All cohort studies followed ethical principles defined in the Helsinki declaration (revised in 1996), and they were approved by local ethical committees from Lille (France), Paris (France), Poitiers (France) and Cambridge (UK).

Sequencing. Sanger sequencing of the *MTNR1B* exons was performed in 7,632 unrelated European subjects, including 2,186 subjects with T2D. *MTNR1B* is located on human chromosome 11q21-q22 and encodes a 362-amino-acid protein (NM_005959.3 and NP_005950.1). The two *MTNR1B* exons were analyzed in five fragments using previously described primer sequences and PCR conditions[1]. Fragments were bidirectionally sequenced using the automated 3730xl DNA Analyzer (Applied Biosystems). Electrophoregram reads were assembled and analyzed using Variant Reporter software (Applied Biosystems). Each newly identified nonsynonymous mutation was confirmed by a second bidirectional Sanger sequencing reaction. The location of each variant was determined relative to the translation initiation codon using Human Genome Variation Society (HGVS) nomenclature for the description of sequence variations. The position of mutations was indicated according to the human genome build NCBI36/hg18.

Genotyping. Genotyping of the four total loss-of-function *MTNR1B* mutations (encoding p.Ala46Pro, p.Leu60Arg, p.Pro95Leu and p.Tyr308Ser amino acid substitutions) was performed in 14,909 additional unrelated European individuals. The DNA samples were pooled and screened by High-Resolution Melting (HRM); samples that potentially contained sequence polymorphisms were studied individually using Sanger sequencing. We used the LightCycler 480 HRM Master kit (Roche Diagnostics) according to the manufacturer's protocol. Briefly, for genotyping of each mutation, 10 ng of DNA per sample was used in 5 µl of Master Mix (2×) with 1 µl of Primer Mix (4 µM) and 1.2 µl of $MgCl_2$ (25 mM). The sequences for primers are provided (**Supplementary Table 4**). PCR and HRM were performed in 384-well plates using the LightCycler 480 Real-Time PCR System (Roche Diagnostics) with the following protocol: (i) pre-incubation: one cycle; 95 °C for 5 min; (ii) touchdown: 10 cycles; 95 °C for 10 s, 70 °C to 63 °C for 15 s (−0.7 °C per cycle), 72 °C for 25 s; (iii) amplification: 45 cycles; 95 °C for 10 s, 63 °C for 15 s, 72 °C for 25 s; (iv) HRM: one cycle; 95 °C for 1 min, 40 °C for 1 min, temperature gradient from 63 °C to 95 °C for 1 s at each increase of 1 °C; and (v) cooling: 40 °C. We determined that pools of four DNA samples per well provided accurate genotypes (**Supplementary Fig. 3**), but acting conservatively, we chose to pool three DNA samples in each reaction. Each 384-well plate contained two positive controls: one well with only a mutated DNA sample and one well with a mutated sample and two wild-type DNA samples (**Supplementary Fig. 3**). When an HRM profile matched the profile of a positive control, we resequenced the three DNA samples following a standard protocol. A total of 3,055 samples were both directly sequenced for the two *MTNR1B* exons and subsequently genotyped for the four mutations. We found 100% concordance (3,055/3,055) between the two protocols.

Statistical analyses. We independently assessed the effect of frequent variants (with MAF ≥1%) on T2D risk using logistic regression adjusted for age, gender and body mass index (BMI) under an additive model. Rarer variants were analyzed by pooling them on the basis of their MAFs (between 0.1 and 1% or <0.1%) or their functional consequences. We assessed the effect of these pooled variants on T2D risk via the KBAC method[14] embedded in a logistic regression model adjusted for age, gender and BMI. The KBAC method was developed to overcome the problems of detecting rare variant associations in the presence of misclassification. This method was extensively compared to three widely accepted methods (the weighted-sum statistic (WSS), the combined multivariate and collapsing method (CMC) and the comparison of rare variants found exclusively in cases to those found only in controls (RVE)) and was determined to be the most powerful under the assumption of phenotypic effects inversely correlated with MAF[14]. It is noteworthy that the age adjustment used in the assessment of the effect of total loss-of-function *MTNR1B* variants on T2D risk showed a positive impact on OR estimates. To further examine this phenomenon, we performed a Mantel-Haenszel stratified analysis using three or four strata based on the quantiles of the age distribution without any adjustments for other confounders. Both the ORs and the corresponding *P* values for T2D risk measured for the three- and four-strata analyses had the same magnitude as those reported by the logistic regression model adjusted for age, gender and BMI, which shows the robustness of our analysis. To address a possible effect of population stratification on our results, we used SNP array data (Illumina Metabochip or Illumina CNV370-Duo DNA arrays), which were available for carriers of partial or total loss-of-function *MTNR1B* variants. By using the population-specific genotype database released by the HapMap project, we found that 39,000 SNPs present on both chips were identified as good markers of ancestry, as a primary principal-component analysis (PCA) of those variants allowed us to clearly distinguish individuals of European descent (HapMap Utah residents of Northern and Western European ancestry (CEU)), Africans (HapMap Yoruba from Ibadan (YRI)) and Asians (HapMap Japanese from Tokyo (JPT) and Han Chinese from Beijing (CHB)) on the first factorial plan. We then achieved a secondary PCA, including the mutation carriers so as to detect potential admixture. This analysis revealed that it was highly unlikely that the mutation carriers did not have European ancestry (**Supplementary Fig. 4**). All statistical analyses were performed with R (version 2.12) and SPSS software (version 14.0 for Windows).

Functional characterization of the MT_2 mutants. Human *MTNR1B* cDNA was fused at its 5′ end with a sequence encoding a mouse Myc epitope and subcloned into the Flp-In pcDNA5/FRT plasmid (Invitrogen). Each of the 40 nonsynonymous mutations was generated in this plasmid using the QuikChange site-directed mutagenesis kit (Stratagene) and was confirmed by Sanger sequencing of the full-length clone. We established 41 stable Flp-In HEK293 cell lines stably expressing the wild-type receptor or one of the 40 mutant MT_2 receptors according to the manufacturer's recommendations (Invitrogen). Briefly, Flp-In HEK293 cells were co-transfected with each MT_2-encoding Flp-In pcDNA5/FRT plasmid and the pOG44 vector encoding

© 2012 Nature America, Inc. All rights reserved.

doi:10.1038/ng.1053

the Flp recombinase at a ratio of 1:9 using FuGENE HD transfection reagent (Roche Diagnostics). At 48 h after transfection, medium containing 100 μg/ml hygromycin B (Invitrogen) was added to cells, and cells were grown for 2–3 weeks. Hygromycin B–resistant cells were collected, and expression of MT_2 mutants was assessed by [^{125}I]MLT (PerkinElmer) binding and protein blotting (data not shown). Use of Flp-In cells was an attractive approach, as only one copy of the mutated *MTNR1B* gene is integrated at a specific, predefined insertion site in the cell genome. This renders expression levels independent of the number of inserted copies and the insertion site and allowed comparison of the expression levels of the 40 mutants solely on the basis of the receptors' intrinsic properties.

Surface expression of MT_2 variants was evaluated by In-Cell Western experiments (LI-COR) using human HEK293T cells transfected with each plasmid and seeded onto sterile poly-L-lysine–coated 24-well plates 1 d after transfection. After 24 h, cells were fixed with a 4% paraformaldehyde (PFA) solution (in PBS) for 15 min. After a 10-min permeabilization step in 0.1% Triton X-100 (in PBS), cells were blocked for 1 h with 3% BSA (in PBS). Cells were immunolabeled for 1 h with primary antibody (monoclonal antibody to Myc at 0.2 μg/ml (sc-40, Santa Cruz Biotechnology)). Immunoreactivity was detected using IRDye secondary antibody (LI-COR) and quantified using the LI-COR Odyssey infrared imaging system (Sciencetec).

Radioligand saturation binding experiments were performed in stable MT_2 Flp-In HEK293 cell lines. As previously described[29], saturation binding assays were carried out on crude membrane preparations with increasing concentrations of [^{125}I]MLT to determine dissociation constants (K_d) and expression levels (B_{max}) of wild-type and mutant MT_2 receptors using PRISM software (GraphPad).

G_i protein activation was determined in HEK293T cells expressing each MT_2 mutant and the chimeric $G\alpha_{q/i9}$ protein, which links G_i-coupled receptors to the activation of the phospholipase C pathway and inositol phosphate production. Cells were incubated for 30 min with different MLT concentrations (10^{-13}–10^{-6} mol/l) and inositol phosphate levels determined with the IP-one HTRF kit (Cisbio Bioassays) according to the manufacturer's instructions. EC_{50} values were calculated using PRISM software.

ERK1/2 activation was monitored in Flp-In HEK293 cell lines stably expressing MT_2 mutants. Cells were stimulated for 2, 5, 7.5 and 10 min with 100 nM MLT, and the level of ERK1/2 phosphorylation was determined. Reactions were stopped in Laemmli sample buffer, and total cell lysates separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and ERK1/2 phosphorylation determined by immunoblotting using antibody to phosphorylated ERK1/2 (pERK1/2) (sc-7383, Santa Cruz). Immunoreactivity was detected using IRDye secondary antibody and quantified using the LI-COR Odyssey infrared imaging system. The amount of loaded proteins was verified by performing protein blotting with an antibody to total ERK2 (sc-154, Santa-Cruz). ERK1/2 phosphorylation was transient, with peak levels occurring at 2–5 min after the addition of MLT. No difference in kinetics was observed between wild-type and mutant MT_2 receptors.

21. Balkau, B. An epidemiologic survey from a network of French Health Examination Centres (D.E.S.I.R.): epidemiologic data on the insulin resistance syndrome. *Rev. Epidemiol. Sante Publique* **44**, 373–375 (1996).
22. Williams, D.R. *et al.* Undiagnosed glucose intolerance in the community: the Isle of Ely Diabetes Project. *Diabet. Med.* **12**, 30–35 (1995).
23. Syddall, H.E. *et al.* Cohort profile: the Hertfordshire cohort study. *Int. J. Epidemiol.* **34**, 1234–1242 (2005).
24. Meyre, D. *et al.* A genome-wide scan for childhood obesity-associated traits in French families shows significant linkage on chromosome 6q22.31-q23.2. *Diabetes* **53**, 803–811 (2004).
25. Hercberg, S. *et al.* A primary prevention trial using nutritional doses of antioxidant vitamins and minerals in cardiovascular diseases and cancers in a general population: the SU.VI.MAX study—design, methods, and participant characteristics. Supplementation en VItamines et Mineraux Antioxydants. *Control. Clin. Trials* **19**, 336–351 (1998).
26. Sladek, R. *et al.* A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* **445**, 881–885 (2007).
27. Hadjadj, S. *et al.* Prognostic value of the insertion/deletion polymorphism of the ACE gene in type 2 diabetic subjects: results from the non-insulin-dependent Diabetes, Hypertension, Microalbuminuria or Proteinuria, Cardiovascular Events, and Ramipril (DIABHYCAR), Diabete de type 2, Nephropathie et Genetique (DIAB2NEPHROGENE), and Survie, Diabete de type 2 et Genetique (SURDIAGENE) studies. *Diabetes Care* **31**, 1847–1852 (2008).
28. American Diabetes Association. Standards of medical care in diabetes. *Diabetes Care* **31**, (suppl.), S12–S54 (2008).
29. Ayoub, M.A. *et al.* Monitoring of ligand-independent dimerization and ligand-induced conformational changes of melatonin receptors in living cells by bioluminescence resonance energy transfer. *J. Biol. Chem.* **277**, 21522–21528 (2002).

© 2012 Nature America, Inc. All rights reserved.