

Identification of Novel Type 2 Diabetes Candidate Genes Involved in the Crosstalk between the Mitochondrial and the Insulin Signaling Systems

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Abstract

Type 2 Diabetes (T2D) is a highly prevalent chronic metabolic disease with strong co-morbidity with obesity and cardiovascular diseases. There is growing evidence supporting the notion that a crosstalk between mitochondria and the insulin signaling cascade could be involved in the etiology of T2D and insulin resistance. In this study we investigated the molecular basis of this crosstalk by using systems biology approaches. We combined, filtered, and interrogated different types of functional interaction data, such as direct protein–protein interactions, co-expression analyses, and metabolic and signaling dependencies. As a result, we constructed the mitochondria-insulin (MITIN) network, which highlights 286 genes as candidate functional linkers between these two systems. The results of internal gene expression analysis of three independent experimental models of mitochondria and insulin signaling perturbations further support the connecting roles of these genes. In addition, we further assessed whether these genes are involved in the etiology of T2D using the genome-wide association study meta-analysis from the DIAGRAM consortium, involving 8,130 T2D cases and 38,987 controls. We found modest enrichment of genes associated with T2D amongst our linker genes ($p = 0.0549$), including three already validated T2D SNPs and 15 additional SNPs, which, when combined, were collectively associated to increased fasting glucose levels according to MAGIC genome wide meta-analysis ($p = 8.12 \times 10^{-5}$). This study highlights the potential of combining systems biology, experimental, and genome-wide association data mining for identifying novel genes and related variants that increase vulnerability to complex diseases.

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Introduction

Insulin resistance is a common trait present in complex disorders such as type 2 diabetes (T2D), obesity or metabolic syndrome (MetS). Around 340 million people suffer from diabetes worldwide, 90% of whom have T2D (<http://www.who.int/>

[diabetes/facts/en](http://www.who.int/diabetes/facts/en)). Unlike type 1 diabetes, overt T2D is usually diagnosed several years after its onset due to its milder presenting symptoms, which in part explains why several devastating complications such as cardiovascular related diseases tend to develop soon after or have already arisen at the moment of the initial diagnosis.

Author Summary

It has been shown that the crosstalk between insulin signaling and the mitochondria may be involved in the etiology of type 2 diabetes. In order to characterize the molecular basis of this crosstalk, we mined and filtered several interaction databases of different natures, including protein–protein interactions, gene co-expression, signaling, and metabolic pathway interactions, to identify reliable direct and indirect interactions between insulin signaling cascade and mitochondria genes. This allowed us to identify 286 genes that are associated simultaneously with insulin signaling and mitochondrial genes and therefore could act as a molecular bridge between both systems. We performed *in vitro* and *in vivo* experiments where the insulin signaling or the mitochondrial function were disrupted, and we found deregulation of these connecting genes. Finally, we found that common variants in genomic regions where these genes lie are enriched for genetic associations with type 2 diabetes and glycemic traits according to large genome-wide association meta-analyses. In summary, we reconstructed the network implicated in the crosstalk between the mitochondria and the insulin signaling and provide a list of genes connecting both systems. We also propose new potential type 2 diabetes candidate genes.

There has been growing interest in identifying genes and processes that could trigger insulin resistance beyond defects on the insulin signaling cascade itself. As a result, defective mitochondrial activity has been indirectly related to insulin resistance in insulin-targeted tissues, such as skeletal muscle [1,2,3] and liver [4]. In particular, patients with T2D and, more importantly, non-diabetic subjects with type 2 diabetic relatives showed mitochondrial dysfunction and lower expression of PPAR gamma co-activator 1 alpha and 1 beta (PGC-1 α and PGC1-1 β), which are key regulators of mitochondrial biogenesis and function. In addition, subjects with early-onset type 2 diabetes typically show defective activation of PGC-1alpha in response to physical activity [5], and similarly, morbid obese type 2 diabetic patients show a defective activation of mitochondrial gene expression in response to weight-loss surgery [5]. Whether there is a heritable component involved in the alterations in expression of mitochondrial genes/proteins in these common forms of T2D remains to be determined.

Despite all of these efforts and lines of evidence, the mechanisms and the molecular contributors to the connection between mitochondria and the insulin signaling and resistance are still unknown. The availability of a wide range of functional interaction data, including metabolomics, genomics, transcriptomics and proteomics and the integration of all these data using systems biology approaches make it now possible to investigate in detail the molecular basis of the interaction between the insulin signaling cascade and mitochondrial biology in healthy and pathological scenarios, particularly in the context of T2D.

In addition, and despite substantial progress achieved in the identification of candidate genes involved in specific complex processes or diseases through genome-wide association studies (GWAS), for most diseases, including T2D, less than 10% of the heritability (percentage of variance attributable to genetic variation) can be explained by the identified genetic associations [6]. Some hypotheses suggest that a portion of the *missing heritability* stays behind multiple small effect size variants that have not yet reached genome-wide significance in GWAS meta-analyses when tested individually, due to insufficient sample sizes. If many of the

modest effect variants are assumed to implicate genes that function in a limited number of biological processes, collective analysis of variants based on prior biological knowledge could substantially enhance association detection power. In that sense, the application of systems biology approaches to analyze GWAS data may have the potential to increase the chances of unraveling susceptibility genes or biological processes for complex diseases.

In this study, we applied systems biology approaches to screen and identify novel candidate T2D genes. The search has been guided by the hypothesis that the functional components of the crosstalk between the insulin signaling pathway and the biology of the mitochondria may play a role in the etiology or the evolution of the disease. We have also generated and analyzed gene expression data on insulin resistance and mitochondria perturbed scenarios to support these candidate genes. We finally tested whether particular genetic variants in loci that contain the identified genes could be collectively associated with T2D.

Results

Generation of the MITIN network

In order to identify genes specifically involved in the crosstalk between the insulin signaling pathway and the mitochondria, we looked for all possible direct and indirect functional interactions between mitochondria and insulin signaling genes (Figure 1). We started by building reliable models and parts lists for these two systems. We first explored and manually filtered several public versions of the insulin signaling pathway to end up with a confident collection of 197 proteins/genes (see Methods). At the same time, we extracted data from a database of nuclear and mitochondrial-encoded mitochondrial proteins (MitoP2) to generate the corresponding list of 682 mitochondria genes [7].

Once both parts lists were constructed, we screened several large functional interaction databases to identify direct and indirect connections involving any of the protein/genes of each of the systems. We applied several filters and cutoffs to be able to isolate, from all available interactions, a reliable collection that will be used further in our study. For example, from protein-protein interaction (PPI) data, we only considered those protein pairs whose interactions were reported by two or more independent laboratories (PPI_{high}) and whose pair of genes were reported to be expressed both in any of the insulin-sensitive tissues (adipose tissue, muscle, liver and heart, [8]); or any other PPI interaction reported only by a single laboratory, simultaneously expressed in any of the insulin-sensitive tissues and that also showed co-expression (gene-expression correlation) in a dataset of 427 healthy human liver samples [9] (these interactions are here termed PPI_{corr}). As a third layer of functional interaction, we also linked those proteins observed to belong to the same protein complex as described in the CORUM protein complex database [10]. The fourth source of interaction consisted of pairs of genes coding for enzymes that participate in linked metabolic reactions, i.e. those reactions that are adjacent in a metabolic reaction map according to the Biochemical Genetic and Genomic (BiGG_{met}) and the Kyoto Encyclopedia of Genes and Genomes database (<http://www.genome.jp/kegg/kegg2.html>; KEGG_{met}) [11,12,13]. Finally, we also included those interactions between genes coding for complexes or genes linked in a signaling pathway, as defined by KEGG (KEGG_{path}) [12]. This final functional interactome comprised 57,751 high confidence functional interactions involving 6963 genes, which represent a whole functional network of insulin-targeted tissues or cells.

From the pool of selected high quality interactions (affecting 6963 genes), we finally selected those interactions that, either

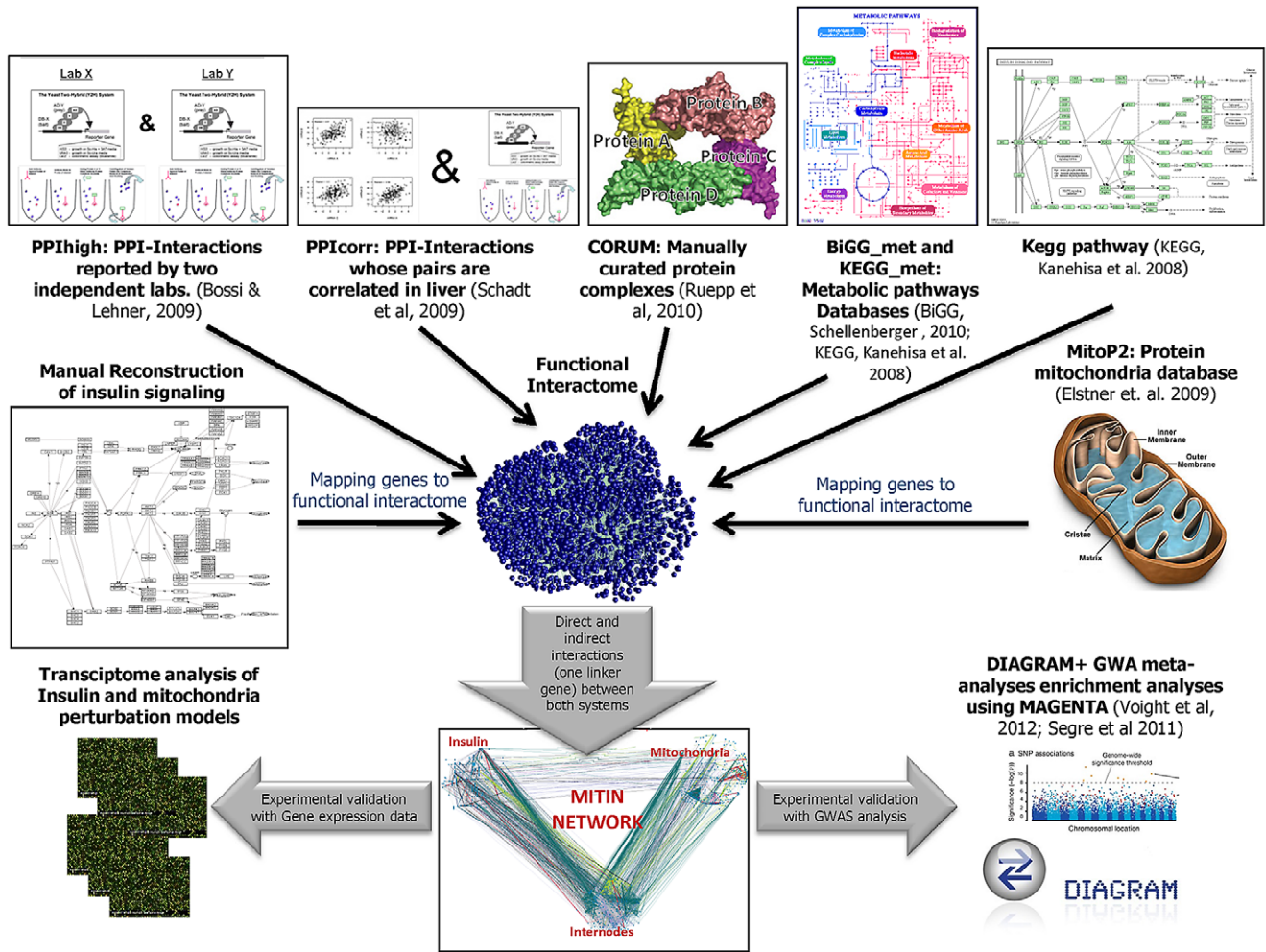


Figure 1. Schematic flow chart of the generation and evaluation of the MITIN network. The different sources of functional interaction are combined to generate a functional interactome. The resulting network is used to identify the direct and indirect interactions between the insulin signaling and mitochondria systems. The relevance of the MITIN network is tested analyzing gene expression data of models perturbing either insulin signaling or mitochondria function, and testing the variability within or near the MITIN network genes using GWA meta-analyses from DIAGRAM consortium. *In all PPIhigh and PPIcorr, both pair of interacting proteins have to be simultaneously expressed in any of the insulin-targeted tissues (adipose tissue, muscle, liver and heart). doi:10.1371/journal.pgen.1003046.g001

directly or indirectly, provide a link between the mitochondrial and the insulin signaling cascade genes. We defined indirect interactions as those mediated by genes, termed linker or internode genes, that do not belong to either the insulin or the mitochondria parts list, but that are simultaneously connected to both systems. By applying these filters, we finally generated the mitochondria-insulin (MITIN) network consisting of 886 genes and a total of 1259 interactions, 70 direct (Table S1) and 1194 indirect. The 70 direct interactions involved 44 insulin genes and 37 mitochondria genes, most of them showing only one evidence of interaction. Both the insulin and mitochondria genes that were directly connected were linked to a median of two genes from the other system. Direct connections showed heterogeneous sources of interaction: PPIhigh, PPIcorr, Corum Complexes, BiGG_met, KEGG_met, Kegg_pathway, contributed 41, 9, 13, 2, 3, 12 links, respectively. Indirect interactions involved 286 linker internode genes (Figure S1, Dataset S1, Table S2 and S3). These internodes genes were connected to a mean number of 2.1 Insulin and 1.7 mitochondria genes and showed a mean of 2.6 and 2.0 lines of evidence of interaction with insulin and mitochondria, respective-

ly. Regarding the 1194 indirect connections, PPI, PPIcorr, Corum Complexes, BiGG_met, KEGG_met, Kegg_pathway, contributed 570, 472, 1263, 42, 160, 169 interactions, respectively.

While the majority of the internode genes seem to be novel, as their bridging role connecting both systems has not yet been described, some of them have already been shown to interact with both systems, which constitutes an internal positive control of our underlying search methodology. For example, TRAF2 shows interactions within our MITIN network with four insulin and two mitochondrial genes (Table 1). Interestingly, other independent studies and approaches also identified five of these interactions. In particular with MAP3K1 (MEKK1), CAV1 (caveolin-1) and MTOR (mTOR), from the insulin signaling [14,15,16] and MAP3K5 (ASK1) and CASP8 (caspase-8) from the mitochondria [17,18] (Figure 2). Another example is NFKB1, for which we found interactions with four insulin signaling and three mitochondrial genes. As above, NFKB1 has been also reported to interact with the *IKKBK* [19,20], *AKT2* [21], *MAP3K1* [22] and *SOC3* insulin genes, as well as to *BCL2L1* [22] [23] and *BCL2* [24] (Figure 2).

Table 1. Strong candidates linking both insulin and mitochondria genes.

Internode gene	Total links	Total evidences	Insulin associated genes	Evidences linking to		Evidences linking to mitochondria	Insulin functional associations	Mitochondria functional associations
				insulin signaling	Mitochondria associated genes			
ABL1	12	21	10	18	2	3	YWHAH CBL SRC CRK BCR PTK2 CRKL GRB2 CSK NCK1	WASF1 TP53
ALDOA	6	8	2	4	4	4	FBP1 FBP2	ALDH1B1 ALDH1A3 ALDH2 GPD2
ALDOB	5	7	2	4	3	3	FBP1 FBP2	ALDH1A3 ALDH1B1 ALDH2
ALDOC	5	7	2	4	3	3	FBP2 FBP1	ALDH1B1 ALDH1A3 ALDH2
DHX9	5	6	2	3	3	3	NOLC1 RPS6	BRCA1 SLC25A5 TUFM
HNRNP1	6	6	3	3	3	3	NOLC1 YWHAQ RPS6	C1QBP SLC25A5 TUFM
HSP90AA1	13	16	9	11	4	5	CALM1 YWHAG YWHAH IKKBK SRC YWHAB PDPK1 YWHAQ AKT1	TP53 TOMM34 TOMM70A HSPD1
ILF3	6	6	3	3	3	3	NOLC1 RPS6 YWHAQ	SLC25A5 C1QBP TUFM
MYBBP1A	6	8	3	4	3	4	PKLR RPS6 NOLC1	POLG SLC25A5 TUFM
NCL	7	10	4	6	3	4	RPS6 PRKCZ PPARGC1A NOLC1	TUFM TP53 SLC25A5
NFKB1#	7	9	4	6	3	3	IKKBK SOCS3 MAP3K1 AKT2	BCL2L1 BCL2 MTIF2
NFKBIA	5	8	3	5	2	3	SRC CAPN1 IKKBK	TP53 SLC25A5
NOS1*	8	9	3	3	5	6	HRAS PRKACA CALML3	SLC25A15 SLC25A2 OTC ASS1 PRKCA
NOS3	6	8	2	3	4	5	CAV1 AKT1	SLC25A2 ASS1 SLC25A15 OTC
NPM1	6	7	3	3	3	4	RPS6 GRB2 NOLC1	TUFM SLC25A5 TP53
PKM2	4	6	1	3	3	3	PKLR	POLG AK3L1 AK2
PRNP	6	7	3	4	3	3	CAV1 MAP2K1 GRB2	HSPD1 BAX SOD1
RB1	8	9	3	3	5	6	INS MAPK1 PIK3R3	C1QBP BRCA1 TGM2 PHB TRAP1
RELA	7	10	4	7	3	3	IKKBK CALM1 IKKBK PRKCZ	ETHE1 MTIF2 ESRT
RPL10	5	6	2	3	3	3	RPS6 NOLC1	PRKCA SLC25A5 TUFM
RPL11	4	6	2	3	2	3	RPS6 NOLC1	SLC25A5 TUFM
RPL30	5	6	2	3	3	3	RPS6 NOLC1	TUFM SLC25A5 MRPS15
RPL35A	5	6	2	3	3	3	NOLC1 RPS6	SLC25A5 TUFM MPG
RPS14	4	6	2	3	2	3	RPS6 NOLC1	TUFM SLC25A5
RPS9	5	6	2	3	3	3	NOLC1 RPS6	TUFM SLC25A5 TOMM40
SNRNP1	7	7	4	4	3	3	EIF4E PPP1CA YWHAB YWHAQ	C1QBP HSPD1 TRAP1
TOP1	5	6	2	3	3	3	RPS6 NOLC1	TUFM SLC25A5 TP53
TRAF2	6	9	4	6	2	3	MAP3K1 CAV1 MAPK10 MTOR	CASP8 MAP3K5
TRAF6	9	12	5	7	4	5	PRKCZ SRC MAP3K1 MAPK10 MAP2K1	NDUFA1 MT-CO2 HADHA NDUFAF1
U2AF1	6	7	2	3	4	4	NOLC1 RPS6	SLC25A5 TRAP1 TUFM HSPD1

Table 1. Cont.

Internode gene	Total links	Total evidences	Evidences linking to		Evidences linking to mitochondria	Insulin functional associations		Mitochondria functional associations	
			Insulin associated genes	linking to insulin signaling		Mitochondria associated genes	Insulin functional associations	Mitochondria functional associations	
YBX1	6	8	3	3	5	RPS6 NOLC1 AKT1	SLC25A5 TUFM TP53		

The internode genes listed in the table have at least three lines of evidence that link them to the mitochondria and three to insulin signaling.

Above 95% percentile of T2D association gene scores based on DIAGRAM meta-analysis (see Table 2).

*Associated to HOMA-IR(9.74E-6) [42].

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The same MITIN network also allowed us to define which mitochondrial genes are more connected to insulin signaling, and *vice-versa*, either directly or indirectly. The top five insulin signaling genes most connected to mitochondria are *NOLC1*, *RPS6*, *IKKBK*, *PKLR*, *SRC*, with a total of 99, 40, 31, 28 and 22 indirect connections with mitochondria, respectively. Similarly, the five most connected mitochondrial genes with the insulin cascade were *TUFM*, *TP53*, *SLC25A5*, *POLG*, *ESR1*, with a total of 93, 36, 29, 25, and 19 indirect connections, respectively (Table S4).

We next explored whether our collection of internode genes were enriched in particular functions or processes by querying the Molecular Signatures Database [25]. We found up to 148 functional signatures for which internode genes were significantly enriched ($5.7 \times 10^{-107} < p \text{ value} < 4.41 \times 10^{-6}$, $1.94 < \text{Odds ratio} < 20.1$; Table S5). Besides several enriched categories related to translation, *Reactome Regulation of Expression in Beta Cells* ($p = 3.5 \times 10^{-87}$, Odds Ratio = 15.8), *Reactome Insulin Synthesis and Secretion* ($p = 4.46 \times 10^{-79}$, Odds ratio = 14.0), and *Reactome Diabetes pathways* ($p = 1.39 \times 10^{-35}$, Odds ratio = 5.5) were also highly enriched among our set of internode genes. No significant categories were found after correcting for multiple testing in a set of internode genes identified from a simulated network made of randomly generated interactions.

In order to facilitate the selection of any of these genes for further studies, we have ranked them according to their number of connections to each of the systems. Hence, we provide a confident subset of 31 genes with at least three lines of evidence linking insulin signaling and mitochondria genes simultaneously (Table 1).

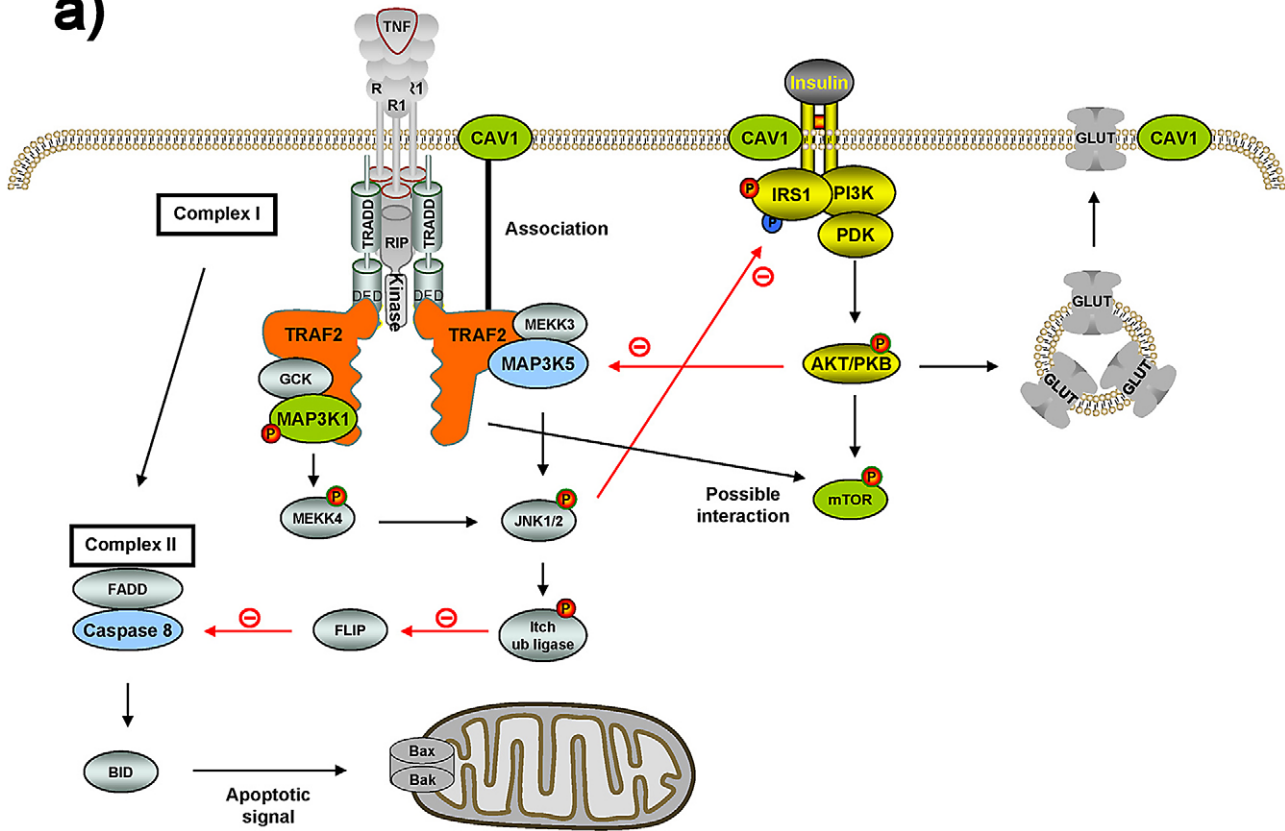
Internode gene expression is altered in insulin resistance and mitochondrial dysfunction experimental models

As further support of the functional relationship between internode genes and both, the mitochondria and the insulin signaling pathway, we explored whether the expression of these identified internode genes is modified after perturbing each of the mitochondria or insulin signaling systems independently.

To test the effect of the insulin signaling perturbation, we performed gene expression profiling of C2C12 differentiated myotubes that were either left untreated or treated with 100 nM insulin for 2 days in order to induce an insulin resistance state. This treatment resulted in the downregulation of the insulin receptor and subsequently significantly reduced insulin signaling cascades [26]. We used the gene set enrichment analysis method (GSEA, [25]) to look for enrichment of differential expression using our set of internode, mitochondria, and insulin genes as molecular signatures. Using the collection of all 6963 genes with identified interactions as a background, we found significant enrichment of upregulation within the internode genes (Normalized Enrichment Score (NES) = 1.7; False Discovery Rate (FDR) = 0.0013), while observed downregulation enrichment within the insulin signaling genes (NES = -1.4; FDR = 0.028) (Figure 3a). We also explored a second model of insulin signaling cascade perturbation through the analysis of transcriptome data from myotubes treated with RNAi against DOR (also named *Tp53imp2*). This gene is dysregulated in muscle of Zucker diabetic rats, participates in the myogenic differentiation and mediates a feed-forward loop between ecdysone receptor and the insulin signaling in flies [27,28]. In this model, we also found that there was an enrichment of upregulated internode genes (NES = 1.4; FDR = 0.004) and enrichment of downregulated insulin (NES = -1.35; FDR = 0.007) and mitochondrial (NES = -1.36; FDR = 0.001) genes (Figure 3c).

In a parallel experiment we tested how perturbations of mitochondria affect the expression of the MITIN network genes.

a)



b)

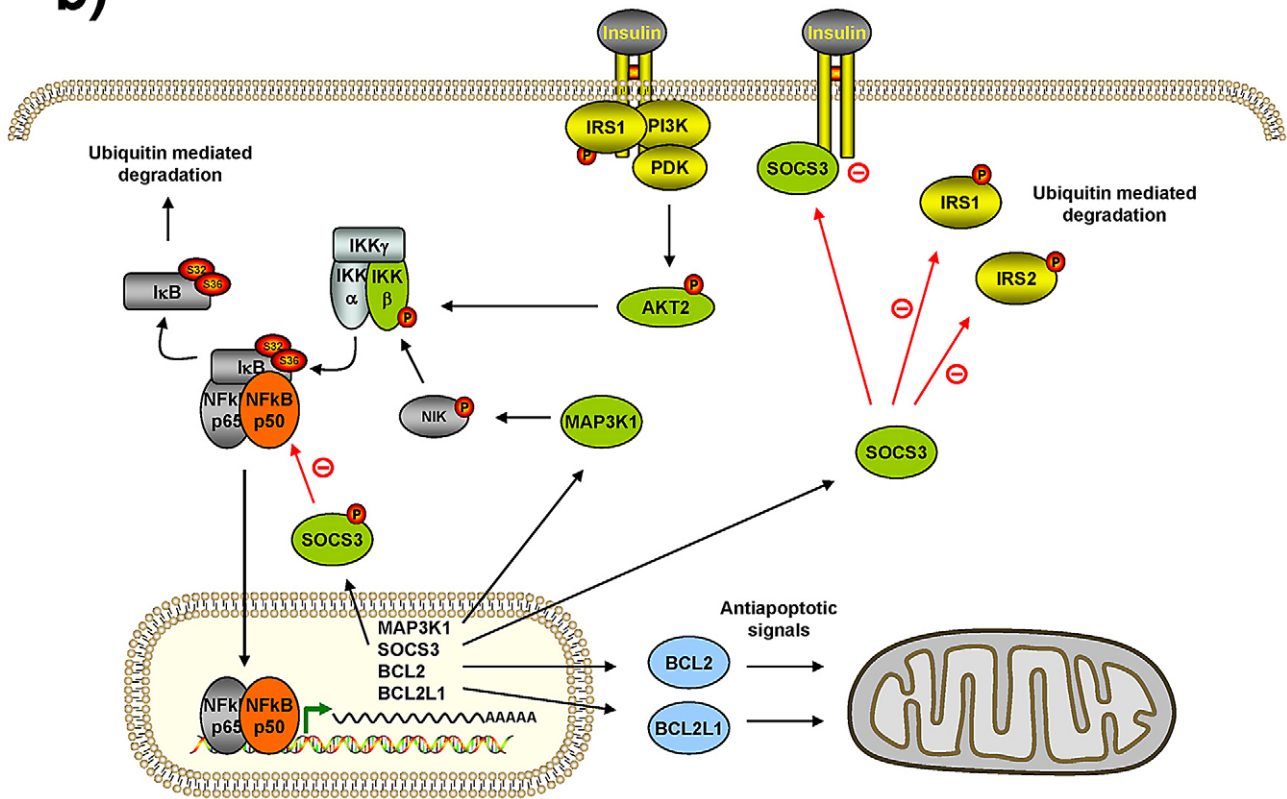


Figure 2. Connections of two internode genes, TRAF2 and NFKB1, with insulin genes and mitochondria genes. Two strong candidates linking both insulin and mitochondria genes from Table 1 were chosen and their connections to insulin genes and mitochondria genes verified using literature published in the PubMed. See main text for detailed description. A) TRAF2 has been reported to be connected to MAP3K1 (MEKK1) and CAV1 (caveolin 1) insulin genes, and to MAP3K5 (ASK1) and CASP8 (caspase-8) mitochondrial genes. A possible connection to MTOR (mTOR) has also to be considered. MAP3K5 = ASK1; MAP3K1 = MEKK1. B) NFKB1 (NF- κ B1) is connected to IKBKB (IKK β), AKT2, MAP3K1 and SOCS3 insulin genes, and BCL2 and BCL2L1 mitochondrial genes. NFKB1 = NFKBp50; IKBKB = IKK β = IKK2; MAP3K1 = MEKK1; P65 = RelA. Green boxes represent insulin genes reported to interact with TRAF2 or NFKB1 according to our network; and light-blue represents mitochondrial genes reported to interact with TRAF2 or NFKB1 according to our network. Yellow boxes represent insulin signaling genes. doi:10.1371/journal.pgen.1003046.g002

For this, we analyzed gene expression from the heart of Peroxisome-proliferator-activated-receptor γ coactivator 1 beta (PGC-1 β) knock-out mice. PGC-1 β is a co-activator that regulates

mitochondrial biogenesis and function [29,30,31,32]. The analysis of heart gene expression of these mice showed an overrepresentation of upregulated genes within the internodes (NES = 1.3;

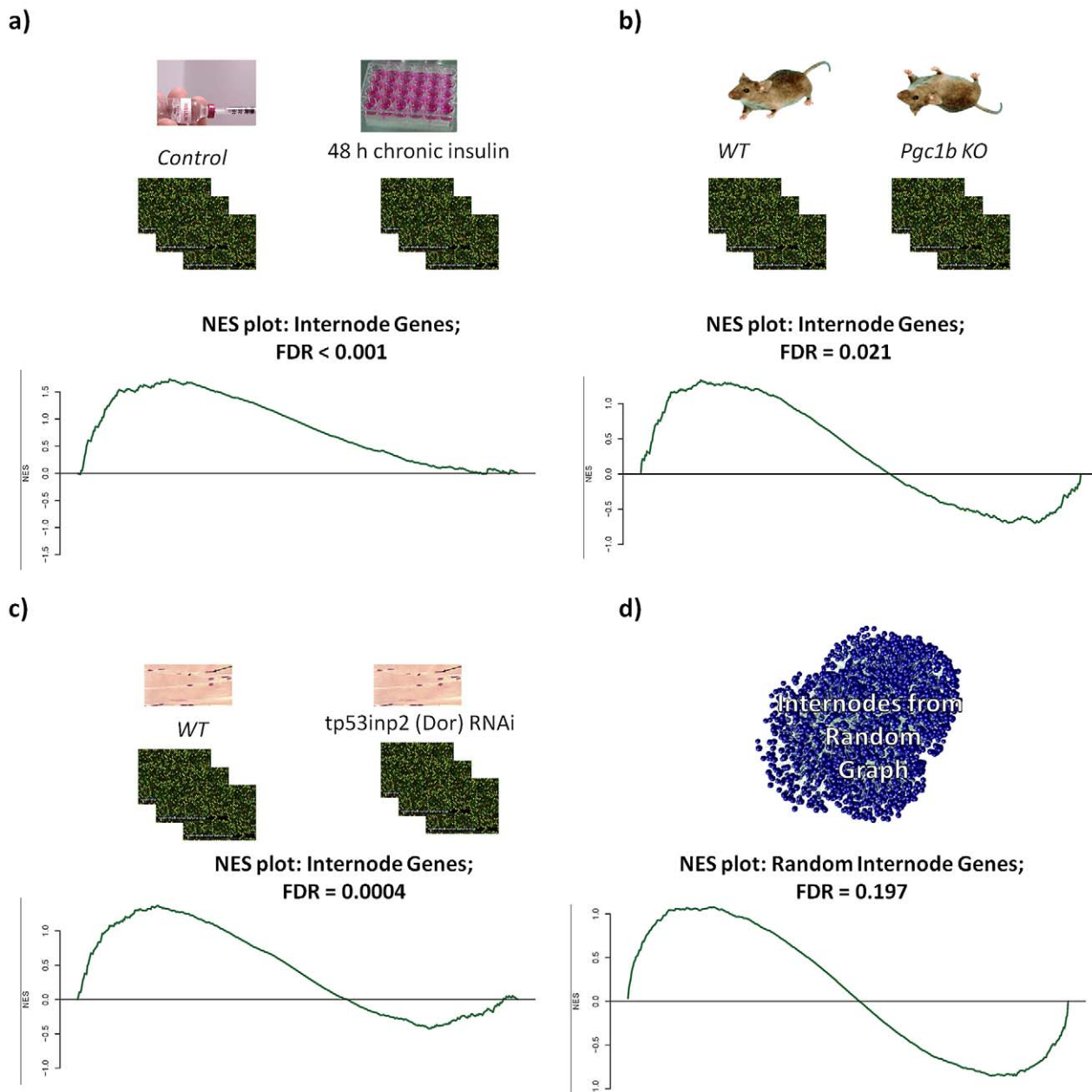


Figure 3. Gene set enrichment analysis. Gene set enrichment analysis of models with impaired Insulin (a, c) or mitochondrial (b) function. In all cases there was enrichment of upregulated genes within the internodes, except for the case when internodes were generated from a random network (d). doi:10.1371/journal.pgen.1003046.g003

FDR = 0.02), enrichment of upregulated genes within the insulin genes (NES = 1.6; FDR = 0.0012), and enrichment of downregulated mitochondria genes (NES = -2.63; FDR = <0.0001) (Figure 3b). Again, as a control from our experiment, randomly generated internode genes did not show any enrichment in any of these experiments (Figure 3d).

Clinical implications of the Global MITIN Network

We next investigated whether any of these genes has been associated to phenotypes related to insulin resistance or energy metabolism. For this, we searched through the OMIM database (<http://www.ncbi.nlm.nih.gov/omim>) those internode genes that are involved in mendelian and complex disorders [33].

We found that, among all 286 internode genes, 191 (66%) were in genomic loci associated to complex diseases or traits (SNPs within 250 kb from internode gene were considered) and 17 (6%) were involved in mendelian diseases. Interestingly 53 of the genes (18%) contained or were near polymorphisms associated to T2D or related traits such as obesity, adiposity, response to glucose challenge, hypertension or coronary artery disease (Table S6). 10,000 random simulations showed that finding 53 genes associated to T2D related traits was modestly more than what expected by chance ($p = 0.0535$). In contrast, the 10,000 random simulations also showed that we did not find more associations with any complex trait (not restricting to T2D related traits), than would be expected by chance, suggesting that the enrichment for associations of the identified internode genes is specific for T2D and related metabolic traits.

Scanning T2D genome-wide association meta-analyses for variants in the internode genomic regions shows enrichment of T2D associations

In order to further investigate the potential involvement of the internode genes in the etiology of T2D, we screened the DIAGRAM consortium GWAS dataset, which consisted on the largest T2D meta-analysis available at the time of the study (DIAGRAM meta-analysis): 8,130 cases and 38,987 controls [34]. To analyze enrichment of associated genes within the internodes, we used MAGENTA [35], a software specifically designed for large genome-wide association study meta-analyses, where individual genotypes are typically not available. We found that our internode gene list showed nominal enrichment for modest to strongly associated genes within the top 5% of T2D scores, with 18 genes observed, including three already confirmed T2D associated SNPs [34,36,37], compared to the 12 expected by chance ($p = 0.0549$, Table 2). These results were robust to the enrichment cutoff used ($p = 0.0368$ when testing for enrichment above the 97.5th percentile of all gene scores; 6 genes expected above cutoff, 11 observed). Unlike the collection of internode genes, no significant enrichment for T2D associations was found for gene-sets belonging only to the insulin signaling ($p = 0.71$) or to the mitochondrial ($p = 0.52$) systems. The insulin and mitochondria genes directly interacting with each other were also not enriched for T2D associations ($p = 0.53$).

To further support the involvement of at least some of these 18 internode SNPs in glucose metabolism regulation, we also computed how the best associated SNPs in the 18 regions increased the risk of altered glycemic traits, available from MAGIC consortium datasets [38,39,40,41,42], using an approximation approach developed by Toby Johnson [43]. Among the seven traits tested, we found a significant association risk score for fasting glucose ($p = 8.12 \times 10^{-5}$ including the 18 top ranked SNPs and $p = 0.004$ including 15 out of the 18 SNPs not previously

associated with T2D). In order to evaluate the probability of finding such a highly statistical p-value, when using the top T2D associated genes (and best local SNPs) we ran MAGENTA on 10,000 simulated random gene-sets, and extracted for each simulation the p-values of the most significant SNP per gene for all genes that ranked above the 95th percentile. The empirical p-value was then calculated as the frequency of random gene-sets whose p-values were smaller than the one obtained with the real data and whose effect size was higher than 0. We found that 8.12×10^{-5} is significantly lower than what one can expect by chance ($p = 0.0144$), confirming the association of our set of internode genes, not only with T2D, but also to fasting glucose levels.

Genetic variants in internode genomic regions associated with T2D are also associated with metabolic related quantitative traits

To further explore the involvement of the internode genes associated with T2D (see above) in related metabolic traits we explored several available GWA meta-analyses pertaining to obesity-related traits from the GIANT consortium [44,45], seven glycemic traits from MAGIC datasets [38,39,40,41,42], and cardiovascular disease traits from the ICBP consortium [46]. We found that in 10 of the 18 internode genomic loci with modest to strong associations, there was at least one SNP showing association ($p < 10^{-5}$) to one of these metabolic traits. For example, rs6453220, located in the *IQGAP2* intron, was associated to circulating glycosylated hemoglobin ($p = 4.19 \times 10^{-6}$) and rs13107325, located upstream of *NFKB1*, was strongly associated with diastolic blood pressure ($p = 7.53 \times 10^{-7}$), body mass index ($p = 1.37 \times 10^{-7}$), high density lipoprotein levels ($p = 7.2 \times 10^{-11}$), and systolic blood pressure ($p = 2.57 \times 10^{-7}$).

Discussion

Understanding the molecular basis of insulin resistance is essential for the early diagnosis, treatment and prevention of T2D and related co-morbidities, such as hyperlipidemia or cardiovascular disease. In this study we explored the molecular basis of insulin resistance beyond the known role of insulin signaling genes, and, implicitly screened for novel candidate T2D genes. Based on published evidence that connects the function of the mitochondria with insulin resistance and T2D [5,47,48,49], we hypothesized that there are genes responsible for the crosstalk between the mitochondria and the insulin signaling system, which makes them good candidates for T2D. By screening and filtering a variety of available functional interaction data, we have first generated a conservative network (MITIN) containing all genes involved in or connected to the insulin signaling or mitochondrial systems, not only through PPI but also based on interactions of other nature, including co-expression, protein complexes, and signaling and metabolic interactions. From there, we then selected a fraction of 286 internode genes that show connections to genes of both systems and are, therefore, likely to be involved in the functional crosstalk between the insulin signaling cascade and the mitochondria.

We have examined these genes at different levels to validate their bridging role and their potential implication in T2D or co-morbidities. In order to provide a more stringent list amenable to low throughput molecular biology experiments in future studies on insulin resistance and diabetes, we ranked these genes on the basis of their level of connectivity to insulin and mitochondrial genes and generated a high confidence subset of 31 genes showing three or more functional connections to each of the systems.

Table 2. Internode genes that fall in the 95% percentile of T2D association gene scores based on DIAGRAM meta-analysis using MAGENTA, and putative associations with T2D-related traits.

HGCN Name	Gene p-value	Chr	Best local SNP	Best SNP Chr Pos (bp)**	Best SNP pval	Best SNP OR	Distance from gene (Kb)	Previously reported T2D associations	Other quantitative associated Traits (Trait: rsid; p value; GeneLoc; distance in Kb)
NFKB1#	1.26E-05	4	rs7674212	104,208	1.70E-07	1.114	450.8		DBP: rs13107325; 7.53e-07; UP; 233.8 BMI: rs13107325; 1.37e-07; UP; 233.8 HDL: rs13107325; 7.2e-11; UP; 233.8 SBP: rs13107325; 2.57e-07; UP; 233.8
IQGAP2	1.66E-05	5	rs4457053	76,461	4.15E-08	0.8607	421.0	[34]	HbA1c: rs6453220; 4.19e-06; inGene; 0
RPL32	1.31E-03	3	rs6802898	12,366	3.18E-06	1.1537	485.2		LDL: rs11709504; 2.15e-07; UP; 201.8 TC: rs2290159; 4.21e-09; UP; 247.1 TG: rs17819328; 5.09e-07; UP; 386.7
IQGAP1*	3.03E-03	15	rs8042680	89,322	8.19E-06	1.1018	475.9	[34]	DBP: rs2521501; 6.06e-07; DW; 391.9 SBP: rs2521501; 1.2e-07; DW; 391.9
RPS13	6.03E-03	11	rs5215	17,365	1.60E-05	1.0934	309.4	[36,37]	DBP: rs381815; 1.22e-07; UP; 193.7 SBP: rs381815; 2.45e-09; UP; 193.7
LRPRC	9.98E-03	2	rs11899863	43,472	1.04E-05	1.1688	494.6		LDL: rs4299376; 1.73e-47; UP; 40.8 FastGluc: rs11899863; 2.58e-06; UP; 494.5 HOMA-B: rs10203174; 9.8e-06; UP; 423.3 SBP: rs10206724; 5.57e-06; inGene; 0 TC: rs4299376; 4.03e-45; UP; 40.8
LSM8	1.04E-02	7	rs10244364	117,317	2.97E-05	1.0916	294.4		BMI: rs2287019; 3.18e-07; DW; 288.1 HDL: rs4420638; 4.4e-21; UP; 486.6 LDL: rs4420638; 8.72e-147; UP; 486.6 2 hrGluc: rs10423928; 3.33e-06; DW; 268.3 TC: rs4420638; 5.2e-111; UP; 486.6 TG: rs439401; 1.14e-30; UP; 495.0
CD3EAP	1.06E-02	19	rs4420638	50,115	5.41E-05	1.1468	486.5		
KPNA2	1.08E-02	17	rs11655081	63,894	5.55E-05	1.2423	420.4		
PPP3CA	1.10E-02	4	rs1426558	102,669	1.99E-05	0.914	181.6		
POLH	1.58E-02	6	rs6933958	43,932	5.13E-05	1.1031	235.4		WHR: rs1358980; 1.38e-10; DW; 177.8 SBP: rs10948071; 5.89e-06; UP; 263.2 TG: rs998584; 4.06e-07; DW; 171.2

Table 2. Cont.

HGCN Name	Gene p-value	Chr	Best local SNP	Best SNP Chr Pos (bp)**	Best SNP pval	Best SNP OR	Distance from gene (Kb)	Previously reported T2D associations	Other quantitative associated Traits (Trait: rsid; p value; GeneLoc; distance in Kb)
BCAR1	2.48E-02	16	rs9927309	73,804	6.61E-05	1.1388	16.5		
RAB4A	2.73E-02	1	rs3767331	227,831	6.53E-05	0.8521	323.9		
SRSF1	3.15E-02	17	rs886929	53,156	6.76E-05	1.0941	277.6		
GRM5	3.24E-02	11	rs16913724	87,621	2.70E-05	1.1309	259.7		
RPL22	3.67E-02	1	rs7368256	6,558	1.31E-04	1.1085	375.5		
H1FX	3.79E-02	3	rs9847824	130.8	2.52E-04	1.1309	282.5		WHR: rs2301573; 3.68e-06; DW; 270.8
RPL10A	4.67E-02	6	rs10484578	35,354	1.62E-04	1.0865	189.9		HDL: rs7742443; 6.27e-06; UP; 427.5 LDL: rs3800406; 2.89e-07; UP; 303.1 FastIns: rs13210323; 6.32e-06; UP; 431.1 TC: rs3800406; 7.31e-09; UP; 303.1

Associations were looked-up in GWAS meta-analyses from the MAGIC, GIANT and ICBP consortiums for SNPs within 500 kb from the internode gene boundaries.

*Upregulated in our chronic insulin treatment (fold change = 1.4; FDR = 0.004) and in the Dor RNAi experiment (fold change = 1.33; FDR = 0.1).

**Chromosome position based on genome build 36 (hg18).

#Within the high confidence internode set.

BMI: Body Mass Index; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; 2 hrGluc: 2 hours glucose challenge; TC: total Cholesterol; WHR: Waist to Hip Ratio; TG: Triglycerides; DBP: Diastolic Blood Pressure; SBP: Systolic Blood Pressure; HbA1C: Glycaeted Hemoglobin; FastGluc: Fasting Glucose levels; HGNC: HUGO Gene Nomenclature committee.
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While there are no reported confirmatory data for the majority of the 286 internode genes, some have been already found to be linked to both systems, and even to T2D and related metabolic processes. For example *TRAF2* [14,15,17,18], *NFKB1* [19,20,21,22,23,24] (Figure 2) and *SMAD3* [50], which show multiple connections to insulin signaling and to mitochondrial genes in our MITIN network, have also been described elsewhere to interact with genes of both systems. In addition, variants near the *NFKB1* gene have been associated to T2D based on the DIAGRAM dataset (best nearby SNP p-value = 1.6×10^{-5}), while *SMAD3* has been recently found to protect against diet-induced obesity as well as coronary artery disease [50,51]. Other genes that also emerge as connecting internode genes in our MITIN network, such as the chaperone *HSP90AA1* gene, have not been previously described as linked to the insulin or the mitochondrial systems, but have been linked to insulin resistance conditions and hence to T2D [52,53].

On top of the previous knowledge on some of the internode genes, we provide here further evidence that supports the robustness of our search strategy and of this collection of genes as potential molecular connectors of these systems, as well as insulin resistance or T2D candidate genes. First, the 286 internode genes showed significant enrichment of functional categories, like “regulation of beta cell development” ($p = 2.1 \times 10^{-79}$), “insulin synthesis and secretion” ($p = 3.4 \times 10^{-79}$) and “diabetes pathways” ($p = 1.9 \times 10^{-35}$). Second, experimental models of mitochondria and insulin signaling perturbation caused a significant upregulation of the internode genes. This could be the result of direct regulation or a mechanism that compensates these perturbed metabolic scenarios. In all cases, the expression analyses helped us to confirm that these genes are indeed functionally connected to both systems. Furthermore, the deregulation of these internode genes under experimental conditions of insulin resistance suggests their involvement in T2D.

Encouraged by our positive functional and expression results supporting the connecting role of the internode genes and their impact on T2D, we went one step further and used the MITIN network as a basis for the identification of genetic signatures associated with T2D, contributing to unraveling its *missing heritability*. We tested for enrichment of T2D associations within the newly identified internode genes, by analyzing the results from the DIAGRAM GWA meta-analyses [34] using MAGENTA to define gene association scores and enrichment of gene associations [35]. We found enrichment of T2D variants within this group of genes, involving 18 associated genes compared to the 12 that were expected by chance ($p = 0.0549$). Our study also confirms the absence of significant signal when we tested insulin signaling and mitochondria gene-sets for enrichment of T2D associations. This is in agreement with previous studies, where no enrichment was found for mitochondrial or insulin signaling genes [34,35], and suggests that the genes involved on the crosstalk between the insulin and mitochondria networks are more susceptible to harbor T2D risk variants than those that belong to either the insulin cascade or the mitochondria alone. The best local SNP in each of the 18 top ranked regions showed a combined risk score of increased fasting glucose levels according to MAGIC consortium data-sets ($p = 8.12 \times 10^{-5}$). Also supporting these results, several variants in the internode genomic regions identified by MAGENTA were also associated with many metabolic related quantitative traits, as reported by the MAGIC [38,39,40,41,42], GIANT [44,45] and ICBP [46] consortia (Table 2).

Interestingly, the best-associated SNP in four of the 18 genes were among the 43 already validated loci of susceptibility for T2D, which in the former reports were assigned to *ZBED3*, *BCL11A*,

PRC1, and *KCNJ11* genes, based only in proximity [34,36,37]. Taking into account the intrinsic challenge in linking an associated variant to its causal gene, we cannot exclude that these SNPs may be proxies for causal variants affecting our group of identified internode genes. Accordingly, recent findings suggest that a fraction of regulatory variants can be more than 500 Kb away from their regulated gene and that a single locus can expand more than 1 Mb, and even contain more than one independent causal variant [54,55,56]. Among the top 18 top ranked internode genes identified by MAGENTA analyses of T2D GWAS meta-analysis, there are independent lines of evidence suggesting the involvement on the development of T2D or insulin resistance. For example, two members of the IQ-motif-containing GTPase-activating protein (IQGAP) family, scaffold proteins involved in a wide range of cellular and signaling processes, including cytoskeletal organization, cell adhesion, and tumorigenic processes [57,58], appear in the top 95th percentile for association with T2D according to MAGENTA analysis. IQ motif containing GTPase activating protein 2 (*IQGAP2*), the second ranked gene according to MAGENTA analysis, contained an intronic low frequency SNP (rs6453220; MAF = 0.05), which was strongly associated with glycated haemoglobin according to MAGIC WGA-meta-analyses (Hb1Ac; $p = 4.19 \times 10^{-6}$), providing more evidence that variants in *IQGAP2* may contribute to insulin resistance. In addition, another gene of the same family, *IQGAP1* (top four according to MAGENTA), was recently reported to bind the target of rapamycin complex 1 (mTORC1) having a potential negative feedback loop role upstream mTORC1/S6K1 AKT1 activation [59]. Furthermore, *IQGAP1* associates with PKA and AKAP79 in pancreatic Beta cells, suggesting a role in the Beta-cell development and physiology [60]. It is also worth mentioning that *IQGAP1* was also found upregulated in our chronic insulin treatment experiment (fold change 1.4; FDR < 0.01) and the *Tp53imp2* RNAi treatment in myotubes experiment (fold change = 1.33; FDR = 0.1). These results, together with the general role of scaffolding proteins as hubs of signaling pathways further supports the implication of the IQGAP protein family in the insulin signaling and the mitochondrial systems crosstalk and its association to T2D. *RAB4A* (Best SNP p value = 3.5×10^{-5}) is a GTPase that regulates glucose transporter GLUT4 [61], and is suggested to participate in metabolic remodeling in the diabetic heart [62]. Finally, breast cancer anti-estrogen resistance 1 (*BCAR1*), (Best SNP p value = 6.61×10^{-5} , distance from gene = 16.5 Kb) is another gene that deserves attention, as is connected to 10 insulin genes, according to our network: *CRK*, *SRC*, *PTPN1*, *PTK2*, *CRKL*, *PIK3R1*, *GRB2*, *PTPRF*, *RHOA* and *PTPRA*. Interestingly, a SNP in an intronic region 16 Kb upstream this gene was reported to be strongly associated with type 1 diabetes [63].

In summary, this study contributes to untangling the molecular basis linking the mitochondria and the insulin signaling systems and provides a subset of novel T2D candidate genes for further genetic, molecular and clinical studies. This study also constitutes a proof of concept of the utility of combining several integrative systems biology approaches with the analysis of gene expression and large GWA meta-analyses to uncover novel associations with complex diseases of otherwise hidden candidate genes.

Materials and Methods

Mitochondria and insulin parts lists

We constructed a consensus insulin pathway from several public resources, including (Biocarta; www.biocarta.com, Kegg [12]; www.genome.jp/kegg/, and PID; [64]; <http://pid.nci.nih.gov/>)

and a commercial resource (Biobase; www.biobase.de). This pathway was manually curated and refined by the participation of molecular biologists in the field.

In order to select the parts lists that compose mitochondrial proteins or genes, we have selected a total set of 900 proteins from the mitoP2 database (www.mitop2.de/; [7]). As it was done for the insulin pathway, the set has been manually curated by the participation of the expert groups in the consortium.

To allow for transferability of the results to other species, we have identified each mouse orthologous gene/protein for all involved proteins.

Generation of the MITIN network

To identify protein-protein interactions we used a non-redundant set of 23 protein interaction datasets and only included those interactions reported independently by two different laboratories (PPIhigh) [8].

For the gene co-expression analysis, we used the dataset of Schadt et al. [9], which consists of expression data of 427 healthy human liver samples and constituted the largest insulin-sensitive human transcriptome dataset. We evaluated the overlap between gene co-expression in liver and low confident PPIs (those reported only by a single lab) to provide a new source of high confident interactions.

Third, we added those interactions that pertained to the CORUM complex database [10], considering that two genes are functionally linked if they both pertain to a common complex.

The fourth source of interaction consisted of pairs of genes coding for those enzymes that participate in linked (or consecutive) metabolic reactions as described in KEGG or BiGG databases [11,12,13].

Finally, we also considered those interactions between genes coding for complexes or genes linked any signaling pathway, as defined by KEGG [12].

Identification of enriched signatures

We used the Molecular Signatures Database from the Broad Institute ([25]; <http://www.broadinstitute.org/gsea/msigdb>) and for a total of 6770 gene sets, we computed an enrichment score based on a Chi-Square test. The corrected significant p-value after applying Bonferroni's correction for all the tests was 4.41×10^{-6} . We only considered gene sets that had at least 10 genes within the group of internode genes.

Microarray data analysis and GSEA

All statistical analyses were performed using Bioconductor (Gentleman et al., 2004). Microarray data was normalized via quantile normalization and summarized to probeset expression estimates via robust multi-array average (RMA) (Irizarry et al., 2003) using the function `rma` from the `oligo` package. All the newly generated data was deposited in the Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) database (GSE3932).

We used gene set enrichment analysis (GSEA) (Subramanian et al., 2005) as implemented in the Bioconductor library `phenoTest` [65] to assess the degree of association between gene expression and the following signatures: insulin, mitochondria and internodes. As indicated in Subramanian et al. [25], P-values were computed restricting attention to simulated ES with the same sign as ES_{obs} .

Chronic insulin treatment

All chemicals and reagents were purchased from Sigma-Aldrich, (Poole, UK). Briefly, C2C12 cells were cultured in Dulbecco's modified Eagle media (DMEM) supplemented with 10% Fetal

bovine serum, and penicillin/streptomycin. To induce differentiation media was replenished by DMEM containing 2% (v/v) of horse serum with penicillin/streptomycin. Myotubes between days 4 and 7 following the induction of differentiation were used for experiments. For chronic insulin treatment cells were either left untreated or incubated with 100 nM insulin in DMEM for 48 h in fusion medium to induce an insulin resistance state. Medium was changed every 24 h.

Pgc1b knock-out model

Hearts were quickly collected and snap frozen in liquid nitrogen from wild-type and PGC-1 β KO on a mix background (sv129 and C57BL/6) generated as previously reported [31]. Animal procedures were performed in accordance with the UK Home Office regulations and the UK Animal Scientific Procedures Act [A(sp)A 1986]. Animals were housed in a temperature-controlled room with a 12-h light/dark cycle. Food and water were available ad libitum.

Dor silencing

Lentiviruses encoding scrambled or DOR siRNA were used as reported [27]. Fifteen million C2C12 myoblasts grown on 12-well plates were transduced at moi 100 and cells were amplified during 5–7 days. Transduced cells (GFP-positive) were then sorted with a MoFlo flow cytometer (DakoCytometry, Summit v 3.1 software), obtaining between 93%–99% GFP-positive cells. Confluent C2C12 myoblasts previously infected with lentiviruses encoding scrambled RNA or DOR siRNA were allowed to differentiate in 5% horse serum-containing medium for 4 days. Total RNA was purified and microarrays were performed by using an Affimetrix platform.

Enrichment of T2D associations in internode genes

We used the latest DIAGRAM T2D GWA meta-analysis comprising 8,130 cases and 38,987 controls [34] and the MAGENTA software was used to test for enrichment of associations in the 286 internode genes [35]. Briefly, we assigned to each gene a set of SNPs that lie within 500 Kb upstream and downstream of the gene's most extreme transcript boundaries. This boundaries were based on the fact that a fraction of regulatory variants can be up to 500 Kb distal to their regulated gene and that a single locus may harbor more than one causal variants, and extend to more than 1 Mb from the locus top hit [54,55,56]. For each gene, a score was assigned based on the most significant SNP, followed by correction for confounders, including gene size, number of independent SNPs, and linkage disequilibrium-based properties. Once all the association scores were computed, MAGENTA tested for over-representation of genes in a given gene set above a predetermined gene score rank cutoff, which in this case was the 95th percentile of all gene scores. The enrichment is evaluated against a null distribution of gene sets of identical set size that were randomly sampled from the 6963 genes that constitute our complete interactome based on all identified functional interactions.

Risk score analyses using multi-SNP predictors in glycemic traits from MAGIC consortium dataset

We computed how the best associated SNPs in the 18 regions could collectively increase the risk of altered glycemic traits available from MAGIC consortium datasets [38,39,40,41,42]. We used the method described in [43]. An unweighted genetic risk score was defined for each individual as the sum of the number of risk increasing alleles at each of the 18 SNPs of interest. If one had access to individual-level data, association between SNP score and glycemic traits could be tested using the usual approach. However,

when the risk score involves SNPs in linkage equilibrium, it was shown [43] that association between risk score and trait can be assessed using meta-analysis results only, without going back to individual-level data. The effect of the risk score on the phenotype is estimated by

$$\frac{\sum_{j=1}^k a_j^2 \hat{\beta}_j}{\sum_{j=1}^k a_j^2}, \text{ with standard error } \sqrt{\frac{1}{\sum_{j=1}^k a_j^2}},$$

where $\hat{\beta}_j$ is the meta analysis effect size for SNP j , and a_j is the inverse of the standard error estimate of $\hat{\beta}_j$.

The assumption of no Linkage Disequilibrium (LD) is required for the contribution of each SNP to be independent and for the standard error estimate to be valid. P-value for the risk score association can be assessed using the ratio of the SNP score effect estimate divided by its standard error, and assessing the significance of the ratio by comparing it to the standard normal distribution.

This large sample procedure will result in valid p-values under the null hypothesis of no relationship between the trait and variants included in the risk score.

Supporting Information

Appendix S1 List of authors and affiliations of the MITIN and the DIAGRAM+ consortia.

(DOC)

Dataset S1 Cytoscape file of the MITIN network.

(ZIP)

Figure S1 Graphical representation of the MITIN network.

(TIF)

Table S1 Direct interactions between mitochondria and insulin cascade genes.

(XLS)

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Table S2 Indirect interactions between mitochondria biology and insulin signaling cascade genes.

(XLS)

Table S3 Full list and description of internode genes.

(XLS)

Table S4 Number of direct and indirect connections of each mitochondria and in insulin gene to the insulin cascade and mitochondria systems, respectively.

(XLS)

Table S5 Functional enrichment of internodes in gene sets extracted from the Molecular Signatures Database.

(XLS)

Table S6 SNPs with distance less than 250 Kb to the internode genes associated with complex diseases or traits related to type 2 diabetes.

(DOC)

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Author Contributions

Conceived and designed the experiments: JMM DT JCF AZ AV-P SM. Performed the experiments: JMM MP AVS SR-C VR CY SM. Analyzed the data: JMM MP AVS EP JD SB-G SD ES DS. Contributed reagents/materials/analysis tools: AVS JD. Wrote the paper: JMM ES DS SM AV-P AZ DT.

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