The *Drosophila* ortholog of TMEM18 regulates insulin and glucagon-like signaling

Lyle Wiemerslage, Priya A Gohel, Giulia Maestri, Torfi G Hilmarsson, Michel Mickael, Robert Fredriksson, Michael J Williams and Helgi B Schiöth

Department of Neuroscience, Functional Pharmacology, Uppsala University, Uppsala, Sweden

Correspondence should be addressed to L Wiemerslage
Email
lyle.wiemerslage@neuro.uu.se

Abstract

Transmembrane protein 18 (*TMEM18*) is an ill-described, obesity-related gene, but few studies have explored its molecular function. We found single-nucleotide polymorphism data, suggesting that *TMEM18* may be involved in the regulation/physiology of metabolic syndrome based on associations with insulin, homeostatic model assessment- β (HOMA β), triglycerides, and blood sugar. We then found an ortholog in the *Drosophila* genome, knocked down *Drosophila Tmem18* specifically in insulin-producing cells, and tested for its effects on metabolic function. Our results suggest that *TMEM18* affects substrate levels through insulin and glucagon signaling, and its downregulation induces a metabolic state resembling type 2 diabetes. This work is the first to experimentally describe the metabolic consequences of *TMEM18* knockdown, and further supports its association with obesity.

Key Words

- *Drosophila*
- fly
- insulin
- obesity
- SNP
- *TMEM18*
- *CG30051*
- glucagon
- AKH

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Introduction

Obesity and its sequelae (diabetes, heart disease, cancer, etc.) arguably make it the most important health concern in modern time (Calle *et al.* 2003, Haslam & James 2005, Barness *et al.* 2007), and the molecular mechanisms of obesity, including the genes involved, must be investigated. Our laboratory has previously described orthologs of *Drosophila melanogaster* from 22 human genes related to obesity (Williams *et al.* 2012). Flies are an excellent model system for studying a variety of molecular pathways, as well as behavior. Specifically for obesity, they have organs that function similar to the human pancreas with corresponding orthologs of insulin and glucagon: insulin-like protein 2 (Ilp2) and adipokinetic hormone (AKH), respectively (Kim & Rulifson 2004,

Nassel *et al.* 2013, Cao *et al.* 2014). Moreover, the suite of genetic tools available in *Drosophila* allows for highly specific experimentation. Thus, flies are a valuable model when exploring the function of ill-described genes. We investigate the metabolic effects of transmembrane protein 18 (*Tmem18*), the *Drosophila* ortholog of human transmembrane protein 18 (*TMEM18*).

Few studies have explored the function of *TMEM18*. Genome-wide association studies (GWAS) have identified risk alleles in/near *TMEM18*, associated with body mass index (BMI) (Thorleifsson *et al.* 2009, Willer *et al.* 2009) and type 2 diabetes (Kalnina *et al.* 2013). *TMEM18* is ubiquitously expressed throughout the body and central nervous system—including the hypothalamus and other

brain areas important for metabolism (Willer *et al.* 2009, Almen *et al.* 2010, Rask-Andersen *et al.* 2012), and has decreased expression in subcutaneous adipose tissue compared with visceral adipose tissue (Rohde *et al.* 2014). In human cell lines, TMEM18 was found in the nuclear membrane and was able to bind DNA (Jurvansuu & Goldman 2011), although this DNA-binding element appears to be missing in fly (Almen *et al.* 2010). Lastly, an *in vitro* study in mouse demonstrated that TMEM18 is involved in neuronal cell migration (Jurvansuu *et al.* 2008). To date, how TMEM18 affects metabolism remains unknown. Using publically available human data and the genetically tractable *Drosophila* model system, we provide the first molecular description of how TMEM18 may be involved in obesity and type 2 diabetes.

Materials and methods

Single-nucleotide polymorphism analysis

A list of 316 human single-nucleotide polymorphisms (SNPs) for TMEM18 was downloaded from the National Center for Biotechnology Information dbSNP database on September 5, 2014. This list was then compared against the results from all available datasets (also downloaded on 5 September 2014) from the following databases: GIANT (<http://www.broadinstitute.org/collaboration/giant/>), MAGIC (www.magicinvestigators.org), DIAGRAM (<http://diagram-consortium.org/>), GLGC (<http://www.sph.umich.edu/csg/abecasis/public/lipids2010/>), and CARDIoGRAM (<http://www.cardiogramplusc4d.org/>). Each database has carried out analysis for significance and made corrections for multiple testing. We only report SNPs with a *P*-value <0.05 and individual studies are cited with their corresponding data in the results. Data from the ENCODE project (Rosenbloom *et al.* 2013) was visualized in the UCSC Genome Browser (<http://genome.ucsc.edu/>) using the Human February 2009 (GRCh37/hg19) Assembly (Rosenbloom *et al.* 2012).

Phylogenetic analysis

Orthologous protein and genomic sequences of TMEM18 were used to construct a multiple sequence alignment and evolutionary tree as well as to test selective pressure via phylogenetic analysis by maximum likelihood (PAML). A full description is available in the [Supplementary Materials and methods](#) (see section on [supplementary data](#) given at the end of this article).

Fly stocks and maintenance

Our phylogenetic analysis indicates that CG30051 is a *Drosophila* ortholog of human TMEM18; therefore, we have renamed the gene *Tmem18*. *w¹¹¹⁸*; P{v43067} flies containing *Tmem18*-RNAi were obtained from the Vienna *Drosophila* RNAi Centre (Vienna, Austria). *w*; *Ilp2-GAL4* was a gift from Dr Eric Rulifson (University of California, San Francisco, CA, USA) (Wang *et al.* 2007). *tGPH* flies were obtained from Bloomington Stock Center (Indiana University, Bloomington, IN, USA). *Ilp2-GAL4*; *tGPH* flies were bred into a homozygous strain by our laboratory. All flies, unless otherwise stated, were maintained on enriched Jazz mix standard fly food (Thermo Fisher Scientific). Flies were maintained at 25°C in an incubator at 60% humidity on a 12 h light:12 h darkness cycle. Flies crossed with *GAL4* drivers and controls were raised at 18°C until the adults emerged; once collected, adults were raised 5–7 days at 29°C. In all assays, the *GAL4* drivers and *UAS* transgenic flies were crossed to *w¹¹¹⁸* flies and their F1 progeny were used as controls.

Capillary feeding assay

This method was modified from the original (Ja *et al.* 2007) and performed as previously described (Williams *et al.* 2014b).

Trehalose, glucose, and lipid analysis

Circulating and stored carbohydrate levels were measured via a modified method from the original (Tennessen *et al.* 2011) (see [Supplementary Materials and methods](#)). Lipid measurements were performed as described previously (Williams *et al.* 2014b).

Glucose stimulus assay

To investigate InR/PI3K signaling activity, we utilized a previously described method using *tGPH* flies (Britton *et al.* 2002) ([Supplementary Materials and methods](#)).

RNA purification, cDNA synthesis, and quantitative RT-PCR

RNA purification, cDNA synthesis, and quantitative RT-PCR (qRT-PCR) was performed as described previously (Williams *et al.* 2014a). Samples were prepared from either 15 flies or 30 fly heads (depending on experiment), and cDNA synthesis was confirmed by PCR followed by agarose

gel electrophoresis. Relative expression levels of two housekeeping genes (*Rp49* and *RpL11*) and of the genes of interest were determined with qRT-PCR. The following primers were used: *Tmem18* F: 5'-GTTTGTGTTGACTC-GGCTTTC-3', R: 5'-CCATTGATCTCGTTGACCTC-3'; *Dubai* F: 5'-CAAGCAGTTCAAGTACGAT-3', R: 5'-TTACTGTGACGCTCTCAT-3'; *Rpl11* F: 5'-CCATCGGTATCTATG-GTCTGGA-3', R: 5'-CATCGTATTCTGCTGGAACCA-3'; *Rp49* F: 5'-CACACCAAATCTTACAAAATGTGTGA-3', R: 5'-AATCCGGCCTTGACATG-3'; *Ilp2* F: 5'-TCTGCAGT-GAAAAGCTCAACGA-3', R: 5'-TCGGCACC GGCGCATG-3'; *Ilp3* F: 5'-TGAACCGAACTATCACTCAACAGTCT-3', R: 5'-AGAGAACTTTGGACCCCGTGAA-3'; *Ilp5* F: 5'-GAG-GCACCTTGGGCCTATTCT-3', R: 5'-CATGTGGTGAGATTCGGAGCTA-3'; *Akh* F: 5'-CTGGTCCTGGAACCTTTT-3', R: 5'-GAGCTGTGCCTGAGATTG-3'; *AkhR* 5'-TCCGTAG-CAGTAGATGAA-3', R: 5'-AGGAGCGACTTTGATGAG-3'.

Statistical analysis

Before analysis, all distributions were tested for normality and homogeneity of variance. For experiments with multiple comparisons, differences between groups were first tested with an ANOVA followed by pairwise *t*-test comparisons with the Tukey's honestly significant difference correction. Data are reported as mean $\pm$ 1 S.E.M.

Results

TMEM18 appears to regulate metabolic syndrome via effects on insulin

To find clues for *TMEM18*'s relationship with obesity, we searched the GIANT, MAGIC, DIAGRAM, GLGC, and CARDIoGRAM databases for SNPs linked with metabolic phenotypes. Of 316 SNPs for *TMEM18*, we found 13 significant for at least one metabolic phenotype: 12 with obesity (Speliotes *et al.* 2010), 9 with fasting insulin levels (Manning *et al.* 2012), 6 with the homeostatic model assessment- β (HOMA β) (Dupuis *et al.* 2010), 5 with triglycerides (Teslovich *et al.* 2010), 4 with BMI (Speliotes *et al.* 2010), and 1 with HbA1c (Soranzo *et al.* 2010) (Table 1). Many of these SNPs were significant for multiple phenotypes (Fig. 1). Control experiments show that these results are not likely due to excessive food intake or diet (see Supplementary Materials and methods, and Supplementary Figs 2 and 3). Furthermore, these alleles with multiple phenotypes had effects in the same

direction, that is, for an unhealthy metabolic phenotype. For example, the risk allele for rs10164967 was associated with increased insulin levels, increased HOMA β , and increased triglyceride levels (Table 1). These findings suggest that *TMEM18* affects obesity through insulin signaling and/or regulation of substrate levels, and thus is likely an important gene for obesity, metabolic syndrome, and type 2 diabetes.

Next, we determined if the 13 SNPs mentioned previously appeared in important regulatory areas. The online tool RegulomeDB reported two SNPs that likely affect the binding of regulatory elements or expression of *TMEM18*: rs17042334, which is associated with increased insulin, HOMA β , and triglyceride levels, and rs17729501, which is associated with obesity (Boyle *et al.* 2012). Moreover, we found that both of these SNPs reside in the 3' UTR region of *TMEM18* and, using data from the ENCODE project, are within 1kb of an insulator (and DNase activity), which overlaps with transcription factor binding sites for CTCF, Fos, and Jun (Rosenbloom *et al.* 2012, 2013). In addition, the online tool mrSNP found 11 miRNAs whose binding may be affected by 3 of the 13 SNPs described in our analysis: rs17042334, rs17729501, and rs3187671 (notably, these include the two SNPs identified by RegulomeDB) (for an improved phylogenetic analysis see the Supplementary Materials and methods, Supplementary Figure 1, and Supplementary Tables 1 and 2 (see section on supplementary data given at the end of this article)) (Deveci *et al.* 2014). Thus, these SNPs may be important biomarkers, and *TMEM18* could be regulated by growth signaling.

The *D. melanogaster* TMEM18 ortholog has a comparable pattern of expression between humans and flies

CG30051 is the putative ortholog of *TMEM18* in the fly *D. melanogaster*. A basic local alignment search tool for protein (BLASTp) (Altschul *et al.* 1990) search using the human *TMEM18* protein sequence as a query (NP_690047.21) reported CG30051 as the only significant hit in *D. melanogaster* (*e*-value=1⁻²⁸), and previous work found high conservation in the transmembrane domains of *TMEM18* across several species, including *D. melanogaster* (Almen *et al.* 2010). We further support these findings with an improved phylogenetic analysis (see the Supplementary Materials and methods). Then we compared absolute, tissue-specific expression data of *TMEM18* from the Human eFP Browser with analogous

Table 1 *TMEM18* SNPs associated with multiple metabolic phenotypes.

Phenotype	rs ID	Major allele	Minor allele	P-value	Effect	Minor allele frequency (%)
Obesity	rs12714416	a	g	0.006	NA	17
	rs12990777	g	a	0.006		17
	rs2293083	c	g	0.005		17
	rs2293084	g	t	0.017		61
	rs2293082	g	c	0.039		8
	rs10172821	c	t	0.024		9
	rs17729501	c	t	0.013		9
	rs3187671	t	c	0.024		9
	rs10164967	c	g	0.038		8
	rs10168696	c	t	0.038		5
	rs13002433	c	t	0.038		8
	rs4241323	a	g	0.038		8
	rs10164967	c	g	0.012	0.012	8
Insulin levels (fasting)	rs10168696	t	c	0.012	−0.012	5
	rs12714416	a	g	0.017	0.009	17
	rs12990777	a	g	0.015	−0.009	19
	rs13002433	t	c	0.012	−0.012	8
	rs17042334	t	c	0.013	0.012	9
	rs2293082	c	g	0.013	−0.012	8
	rs2293083	c	g	0.015	0.009	17
	rs4241323	a	g	0.012	0.012	8
	rs10164967	c	g	0.008	0.013	8
	rs10168696	t	c	0.007	−0.013	5
HOMAb	rs13002433	t	c	0.007	−0.013	8
	rs17042334	t	c	0.010	0.013	9
	rs2293082	c	g	0.008	−0.013	8
	rs4241323	a	g	0.007	0.013	8
	rs10164967	c	g	0.034	0.011	NR
	rs10168696	t	c	0.037	−0.011	
Triglycerides	rs13002433	t	c	0.041	−0.011	
	rs17042334	t	c	0.044	0.011	
	rs4241323	a	g	0.040	0.011	
	rs12714416	a	g	0.023	NR	17
	rs12990777	g	a	0.022		17
BMI	rs2293083	c	g	0.023		17
	rs2293084	g	t	0.017		61
	rs2293084	t	g	0.015	−0.009	43
Hb1Ac	rs2293084	t	g	0.015	−0.009	43

List of significant SNPs for metabolic phenotypes. An initial list of 316 putative human SNPs for *TMEM18* was downloaded from the NCBI dbSNP database and matched with significant SNPs from the following GWAS databases: GIANT, MAGIC, DIAGRAM, GLGC, and CARDIoGRAM. Each database has tested for significance and made corrections for multiple testing. Only SNPs with a *P*-value <0.05 are reported. NA, not applicable; NR, not reported.

data of *CG30051* from FlyAtlas 2. For both genes, there was high expression in the ovaries, testes, and salivary glands. Other tissues showed even, ubiquitous expression (Winter *et al.* 2007, Robinson *et al.* 2013). Data from the Human Protein Atlas further supported this comparison, showing high expression not only for the same areas but also for pancreas and stomach (Uhlén *et al.* 2010, 2015). *CG30051* is likely an ortholog of *TMEM18*; thus, we renamed the *Drosophila* gene *Tmem18*.

Tmem18 knockdown in insulin-producing cells has effects on starvation

As *Tmem18* was associated with insulin in the human data, we wanted to limit its manipulation to the insulin-producing cells (IPCs) in *Drosophila* and test for an effect from RNAi knockdown specifically in these cells (for knockdown of *Tmem18*, see the [Supplementary Materials and methods](#)). IIPs are produced by 14 IPCs

Figure 1

Many SNPs for *TMEM18* are associated with multiple metabolic phenotypes. Venn diagram representing the overlap of SNPs for a given phenotype. SNPs associated with insulin levels were the most encompassing, whereas the SNP associated with the blood glucose test, Hb1Ac, did not overlap with any other phenotypes, as did some SNPs associated with class II obesity, and 1 with BMI and class I obesity. Obesity was broken up into three groups based on BMI: class I (BMI=30–34.99 kg/m²), class II (BMI=35–39.99 kg/m²), and class III (BMI>40 kg/m²). Interestingly, SNPs associated with the super obese (class III) overlapped completely with the HOMAb and triglyceride levels, which were also completely contained within insulin levels. Also, overlapping SNPs always had effects in the same direction, for example, the risk allele for rs10164967 was associated with increased insulin levels, HOMAb, and triglyceride levels. Thus, SNPs that overlap for multiple phenotypes may be important biomarkers. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0040>.

with physiological function similar to mammalian pancreatic beta cells (Nassel *et al.* 2013). Moreover, RNAseq experiments from laser-dissected IPCs in *Drosophila* larvae identified *Tmem18* expression in those cells (Cao *et al.* 2014). To limit *Tmem18*-RNAi specifically to IPCs, we drove expression with an *Ilp2* (*Ilp2-GAL4*) construct.

First, we performed a starvation assay using *UAS-Tmem18*-RNAi flies crossed with *Ilp2-GAL4* flies (*Ilp2>Tmem18*-RNAi). Adult flies, aged 5–7 days, were starved until death. The *Ilp2>Tmem18*-RNAi flies were 26% less resistant to starvation compared with controls (Fig. 2A). Next, we found that the level of *Tmem18* expression changes in control flies during starvation. The control flies were from a cross between *Canton-S* and *Oregon-R-C* (named *CSORC*). The expression of *Tmem18* decreased 79% by 12 h starvation (Fig. 2B). Thus, starvation affects the expression of *Tmem18*, and reduced the expression of *Tmem18* in the brain, which likely has negative consequences on metabolic functions.

Figure 2

CG30051 is involved during starvation. (A) Flies lacking *CG30051* in the IPCs were less resistant to starvation compared to two control crosses. *CG30051* was knocked down with RNAi specifically in the IPCs using the *Ilp2-GAL4* driver. Adult, male flies, aged 5–7 days post-eclosion, were starved until death in vials containing 1% agarose ($n=32$ flies for each strain). (B) *CG30051* expression decreased during starvation. The heads of adult, male flies, aged 5–7 days post-eclosion from the *CSORC* strain, were collected for qPCR analysis after 0, 12, or 24 h during starvation. *CG30051* expression decreased by 12 h and remained decreased at 24 h (n =number of samples, three replicates per sample; $n=5$ for all time points; Tukey's HSD test, *** $P<0.001$).

Knockdown of *CG30051* in IPCs changes metabolic profile

We next measured the carbohydrate and lipid levels of *Ilp2>Tmem18*-RNAi flies. Four different measurements are made for carbohydrate levels: body stores of glycogen and trehalose, and circulating levels of glucose and trehalose in hemolymph. All five substrates were measured in flies fed *ad libitum*, as well as after 12 and 24 h starvation. We found increases in all metabolic measurements when *Tmem18* was knocked down specifically in the IPCs.

For stored carbohydrates, glycogen and trehalose levels in body were increased 14 and 30%, respectively, when fed *ad libitum*, and elevations remained after 12 and 24 h starvation (Fig. 3A and B). For circulating carbohydrate stores, glucose and trehalose levels in the hemolymph increased 86 and 60%, respectively, but were not similarly maintained throughout starvation (Fig. 3C and D). Lastly, the total lipid content also increased 29% in flies fed *ad libitum*, but not significantly during starvation (Fig. 3E). Thus, *Tmem18* knockdown increases basal substrate levels, which are partially maintained

Figure 3

Knockdown of *CG30051* in IPCs changes the metabolic profile of the fly. Both circulating and body levels of carbohydrates, as well as total lipid content, were increased. *CG30051* was knocked down specifically in the IPCs and compared to two control crosses. Adult, male flies, aged 5–7 days post-eclosion, were collected for analysis at 0, 12, or 24 h starvation (n =number of samples; $n=8$ for all strains at each time point for all experiments except for lipids; Tukey's HSD test, $*P<0.05$, $***P<0.001$). (A) Glycogen stores were increased at 0, 12, and 24 h. (B) Body trehalose levels were also increased at 0, 12, and 24 h. (C) Circulating glucose in the hemolymph was increased at 0 and 12 h. (D) Circulating trehalose in the hemolymph was increased at 0 h but decreased at 24 h. (E) Lipid content per fly increased at 0 h (n =number of samples; *Ilp2-GAL4*×*w¹¹¹⁸*: 0 h, $n=7$, 12 h, $n=8$, 24 h, $n=6$; *w¹¹¹⁸*×*CG30051-RNAi*: 0 h, $n=8$, 12 h, $n=7$, 24 h, $n=5$; *Ilp2-GAL4*×*CG30051-RNAi*: 0 h, $n=7$, 12 h, $n=6$, 24 h, $n=6$; Tukey's HSD test, $**P<0.01$, $***P<0.001$).

throughout starvation. Control experiments show that these results are not likely due to excessive food intake or diet (see the [Supplementary Materials and methods](#)).

Evidence for the modulation of metabolic genes by *TMEM18*

We next determined if transcript levels of metabolic proteins were affected by *Tmem18* knockdown in the same population of cells. qRT-PCR experiments tested a subset of the insulin orthologs *Ilp2*, *Ilp3*, and *Ilp5*, as well as the *Drosophila* insulin receptor (*InR*). In addition, we tested adipokinetic hormone (*Akh*), which functions as glucagon in fly (Kim & Rulifson 2004, Buch *et al.* 2008), and its receptor *AkhR*. Expression of *Ilp2* and *InR*, and *Akh* decreased ~33, ~42, and ~59%, respectively, compared with controls, whereas increases were found in the *Ilp3* and *Ilp5* transcripts: ~43 and ~139%, respectively, compared with control levels. *AkhR* was the only transcript unchanged (Fig. 4). Thus, *Tmem18* could be affecting metabolism via regulation of metabolic proteins.

Tmem18 knockdown inhibits the response to feeding in larval fat body cells

Previous work in *Drosophila* established a method to visualize insulin signaling in the fat body cells of larvae using *Ilp2-GAL4*;tGPH transgenic flies (Britton *et al.* 2002). Briefly, a β -tubulin construct drives the expression of green fluorescent protein (GFP) tagged to the PH domain of the *Drosophila* general receptor for phosphoinositides-1 (GRP1). In starved conditions, the PH-GFP chimeric protein is kept perinuclear but is trafficked to the cell membrane after feeding, that is, during insulin signaling. To determine if *Tmem18* is involved in the cellular response to insulin signaling, we crossed *Ilp2-GAL4*;tGPH flies with either *UAS-Tmem18-RNAi* or *w¹¹¹⁸* control flies. Second instar larvae from each cross were collected and starved for 30 min and then refed for 30 min after starvation. The fat bodies were dissected from larvae in both the starved and fed states, and visualized via the GFP signal. In both crosses, the GFP signal was perinuclear during starvation, showing

Figure 4

Knockdown of *CG30051* in IPCs changes the expression profile of metabolic genes. Whole, male flies, aged 5–7 days post-eclosion, were collected and processed for qPCR. Primers for six metabolic genes (*Ilp2*, *Ilp3*, *Ilp5*, *InR*, *Akh*, and *AkhR*) were tested in the *Ilp2>CG30051-RNAi* cross versus two different control crosses. Expression in the *Ilp2>CG30051-RNAi* cross was decreased in *Ilp2*, *InR*, and *Akh*; increased in *Ilp3* and *Ilp5*; and unchanged for *AkhR* (n =number of samples, three replicates per sample; n =8 for all groups; Tukey's HSD test n , $*P<0.05$, $**P<0.01$, $***P<0.001$).

ill-defined borders (Fig. 5A and B). During the refed state, however, the GFP signal in the control cross (*w¹¹¹⁸ × Ilp2-GAL4;tGPH*) saturated the cell membrane demarcating cell borders (Fig. 5C, white arrows), whereas in the *Ilp2-GAL4;tGPH × UAS-Tmem18-RNAi* cross, the GFP signal

remained perinuclear (Fig. 5D). Thus, *Tmem18* is likely important for proper cellular responses to insulin or other signal transductions involving phosphoinositides.

Discussion

In this work, we examine the possible metabolic function of the obesity-related gene *TMEM18*. We find human SNP data, suggesting that *TMEM18* may affect obesity via insulin signaling. Based on these clues from the human data, we test the *Drosophila* ortholog of *TMEM18* (*CG30051*), hence renamed *Tmem18*, for effects on metabolic function. Using an RNAi construct with the *GAL4-UAS* system, we knock down *Tmem18* expression specifically in a cell population that functions similar to human pancreatic beta cells. We find increased levels of circulating and stored carbohydrates, as well as increased lipid levels. We also find different expression levels of metabolic proteins orthologous to insulin and glucagon. Our results suggest that *TMEM18* may affect substrate levels by disrupting insulin and glucagon signaling. This work is the first to experimentally describe the metabolic consequences of *TMEM18*, and further supports its association with obesity.

TMEM18 was initially identified by GWAS for a relationship with BMI (Thorleifsson *et al.* 2009,

Figure 5

Insulin signaling is disrupted in fat body cells lacking *CG30051*. Fat body cells from the third instar larvae were dissected from *Ilp2-GAL4;tGPH* flies crossed to either *w¹¹¹⁸* or *UAS-CG30051-RNAi* flies and stained via immunocytochemistry (nuclei shown in blue). *Ilp2-GAL4;tGPH* flies express GFP tagged to GRP1 protein, which is trafficked to the membrane from its perinuclear locale during phosphoinositide signaling cascades, for example, during insulin signaling in response to feeding. (A) Fat body cells starved for 30 min in the *Ilp2-GAL4;tGPH × w¹¹¹⁸* control cross. The GFP signal is mostly perinuclear, indicating an absence of insulin signaling. (B) Starved cells in the *Ilp2-GAL4;tGPH × UAS-CG30051-RNAi* cross. (C) Fat body cells refed for 30 min after starvation in the *Ilp2-GAL4;tGPH × w¹¹¹⁸* control cross. The GFP signal is now largely trafficked to the cell membrane, indicating a response to insulin signaling. (D) Refed cells in the *Ilp2-GAL4;tGPH × UAS-CG30051-RNAi* cross. The GFP signal remains perinuclear, indicating reduced response to insulin signaling. Images taken at 100× magnification (scale bar = 100 × 20 μm). Insets are from each respective image (white arrows in C show cell borders). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0040>.

Willer *et al.* 2009). We searched several GWAS databases for *TMEM18* SNPs associated with other metabolic phenotypes. Besides BMI, there were SNPs associated with obesity, insulin levels, HOMA β , triglycerides, and blood glucose. Many of these SNPs had overlapping significance for multiple phenotypes. Furthermore, two SNPs are predicted to affect the binding of regulatory factors and/or the expression of *TMEM18*, and three SNPs were predicted to affect the binding of 11 different miRNAs. Thus, our work reveals several SNPs (particularly rs17042334 and rs17729501) that may be interesting biomarkers.

We then extend the findings from human GWAS databases with molecular experiments using the genetically tractable *D. melanogaster*, manipulating its *TMEM18* ortholog. The transmembrane sequences of *Drosophila Tmem18* have highly shared sequence identity with other *TMEM18* orthologs (Almen *et al.* 2010). And interestingly, no paralogs of *TMEM18* were found in any species studied. This might indicate that duplication of *TMEM18* disrupts the normal functioning of a single copy. Indeed, our PAML analysis indicated that the gene is under strong negative selection. Thus, *TMEM18* is likely an important gene with strict evolutionary pressure toward conservation. Furthermore, human *TMEM18* and *Tmem18* had similar tissue-specific expression patterns. From all this, *Tmem18* is likely to be a good model to study the function of human *TMEM18*. Using an RNAi construct with expression driven by the *GAL4-UAS* system (Brand & Perrimon 1993), we knocked down *Tmem18* expression specifically in the IPCs of *Drosophila*. These cells function physiologically similar to the mammalian pancreas (Cao *et al.* 2014), releasing *Drosophila* insulin-like proteins (*Ilp*) (Nassel *et al.* 2013, Cao *et al.* 2014). Using this knockdown, we tested substrate levels and expression of metabolic genes.

Knockdown of *Tmem18* in the IPCs changed the metabolic profile of the flies, as well as the transcriptional profile of metabolic genes. All substrate levels were increased: glycogen and trehalose stored in the body, circulating glucose and trehalose in hemolymph (orthologous to mammalian blood glucose), and total lipids. The same changes in substrate levels also happened in flies with inhibited or ablated IPCs (Rulifson *et al.* 2002, Broughton *et al.* 2005, Slack *et al.* 2010) and are analogous to a diabetes-like affliction (Kannan & Fridell 2013). In this state, insulin levels are reduced and substrates are mobilized, similarly as in starvation. Indeed, *Tmem18* was also reduced during starvation in control flies, and substrate levels remained elevated throughout starvation in flies lacking *Tmem18*

Figure 6

Theoretical mechanism of *CG30051* in metabolism. *CG30051* in the IPCs may exist in the nuclear membrane, where it modulates expression of *Ilp* proteins via an unknown mechanism. Knockdown of *CG30051* resulted in decreased *Ilp2* but increased *Ilp3* and *Ilp5*. The increased *Ilp3* and *Ilp5* may have resulted in the downregulation of *Akh* in the corpora cardiaca cells. *InR* signaling was also downregulated by *CG30051* knockdown and illustrated by the failure of *tGPH* (a GFP-tagged molecule indicating signal transduction involving phosphoinositides) to be mobilized to the cell membrane following insulin signaling. Thus, the elevated levels of substrate (glucose, trehalose, and lipids), both stored and circulating, are likely the result of an insulin-resistant phenotype induced by disrupted insulin and AKH signaling. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0040>.

in IPCs. Thus, *Tmem18* knockdown appears to induce a starvation-like metabolic phenotype.

During starvation, insulin protein levels decrease, but in flies lacking *Tmem18* in the IPCs, we found both increases and decreases for the different *Ilp* transcripts. *Drosophila* has eight *Ilp* genes, but *Ilp2*, *Ilp3*, *Ilp5*, and *Ilp6* are the best studied. All are expressed in IPCs, except *Ilp6*, which is expressed in the fat body with low expression in the brain (Kannan & Fridell 2013). The different *Ilp* proteins in *Drosophila* appear to have specialized functions depending on the substrate affected and/or on the metabolic state of the fly. *Ilp2* appears to be coupled to trehalose levels (Kannan & Fridell 2013). *Ilp2*, *Ilp3*, and *Ilp5* are all secreted in response to nutrient cues (Chatterjee *et al.* 2014), mirroring mammalian insulin signaling. However, during starvation, *Ilp2* is unchanged, whereas *Ilp3* and *Ilp5* decrease expression (Ikeya *et al.* 2002). Furthermore, *Ilp3* and *Ilp5* increase with *Ilp2* knockdown (Nassel *et al.* 2013). In our results, the changes in the expression profile were the same for *Ilp2*, *Ilp3*, and *Ilp5* (decreased *Ilp2* with increased *Ilp3*

and *Ilp5*) as in previous reports where IPCs were ablated (Kannan & Fridell 2013), but not for adipokinetic hormone (*Akh*), which functions as glucagon in fly (Kim & Rulifson 2004, Lee & Park 2004, Bharucha *et al.* 2008, Buch *et al.* 2008, Braco *et al.* 2012, Matsuda *et al.* 2015) and increases when IPCs are ablated (Buch *et al.* 2008). In our experiments, *Ilp2* may be decreased in response to a cell-autonomous starvation signal, whereas *Ilp3* and *Ilp5* are increased in response to decreased *Ilp2* expression. And the increased *Ilp3* and/or *Ilp5* from the IPCs may be responsible for inhibiting *Akh* does as insulin for glucagon in humans.

Lastly, in a qualitative description of insulin signaling using *tGPH* transgenic flies, we observed that fat body cells from larva lacking *Tmem18* in the IPCs did not respond normally to feeding/insulin. Thus, loss of *Tmem18* appears to make cells insulin resistant, which makes sense given that circulating glucose and trehalose were elevated. Moreover, these same high levels of substrate in addition to the augmented *Ilp* expression are likely responsible for the decrease in *Akh* expression. Given all the above, it appears that flies lacking *Tmem18* are unable to respond to insulin or have impaired insulin secretion, leaving elevated substrate levels in circulation and storage, and disrupting the normal function of the *Ilp* proteins, *InR*, and *AKH* (Fig. 6).

This study is not without limitations. The SNPs we list as likely to be important for metabolism have yet unproven links to *TMEM18* regulation/function. Thus, we caution that these findings from the human data should be interpreted as correlative. Although these findings were enough to justify our following series of experiments, the link between these SNPs and *TMEM18* requires further validation.

In conclusion, we present a molecular description for the function of *TMEM18*. We found clues from human SNP data that *TMEM18* may be involved in metabolic signaling involving insulin. From experiments with the ortholog of *TMEM18* in *D. melanogaster*, we show that knocking down *Tmem18* in cells analogous to human pancreatic beta cells induced changes in substrate levels and insulin signaling, suggesting a metabolic state resembling type 2 diabetes or long-term starvation. From all this, we suggest that *TMEM18* is an interesting gene for further studying the etiology or treatment of obesity, metabolic syndrome, and type 2 diabetes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0040>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All authors have read and approved this version of the manuscript. Dr Wiemerslage wrote the majority of the manuscript, planned/organized a majority of the experiments, and analyzed a majority of the data. Gohel wrote parts of this manuscript and collected/analyzed results pertaining to the biochemical and molecular experiments as well as some of the behavioral experiments. Maestri performed/analyzed some of the molecular experiments. Hilmarsson collected/analyzed results pertaining to the behavioral experiments. Dr Mickael performed the phylogenetic analysis and contributed to its explanation. Dr Fredriksson was involved in the initial design of the study and provided insights into the phylogenetic analysis. Dr Williams helped with the writing of this manuscript and was involved in the planning/development of many of the experimental procedures. Dr Schiöth helped with the writing of this manuscript, oversaw the project, and was involved in the planning of many of the experimental procedures.

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