1. Phylogenetic Analysis Methods

Database search and alignment

Orthologous protein and genomic sequences of TMEM18 were used to construct a multiple sequence alignment. Protein (NP_690047.2) and nucleotide (NM_152834.2) sequences for *Homo sapiens* were obtained from RefSeq (Pruitt, et al. 2013). The proteome and genome of *Branchiostoma floridae* (Assembly v2.0) were downloaded from the Joint Genome Institute's website (Putnam, et al. 2008), while all other orthologous sequences were identified by BLAST (Altschul, et al. 1990), using the human protein or gene sequences as queries against the following organisms: *Papio anubis, Mus musculus, Rattus norvegicus, Gallus gallus, Eurypyga helias, Xenopus laevis, Danio rerio, Ciona intestinalis, Oikopleura dioica, Branchiostoma floridae, Drosophila melanogaster, Bactrocera cucurbitae, Caenorhabditis elegans, Schistosoma japonicum, Trichoplax adhaerens, Amphimedon queenslandica, Saccharomyces cerevisiae, Chlorella variabilis, Albugo candida,* and *Naegleria gruberi*. Sequences were included only if e-value $10^{-4}$. The resulting protein sequences were filtered for conserved domains using the identification tool in PubMed (Marchler-Bauer, et al. 2009), then checked for transmembrane regions using TMHMM (Krogh, et al. 2001). The MAFFT (Katoh and Standley 2013) online server was used to form the alignment via the iterative refinement method (FFT-NS-i). The alignment was constructed based on three transmembrane spanning regions, and unaligned regions were removed for the phylogenetic analysis as previously described (Krishnan, et al. 2012). Coding regions of the genes were aligned according to their codons using the “view as proteins” option in Seaview.

Phylogenetic analysis

Using the protein alignment, we applied three different phylogenetic methods in parallel to construct the tree: 1) neighbor-joining, 2) maximum likelihood, and 3) Bayesian inference. The neighbor-joining approach employed MAFFT (Katoh and Standley 2013) with the JTT substitution model, 1000 bootstrap samples, and an alpha value $= 3.5$. For both the maximum likelihood and Bayesian analyses, we used ProtTest, which is based on the Akaike information criterion (AIC) (Abascal, et al. 2005; Darriba, et al. 2011), to determine the best choice for an amino acid replacement model. ProtTest suggested that the best substitution model was LG+I+G+F, where LG is the substitution model assuming that a fraction of amino acids are invariable (‘+I’), and by assigning each site to a given rate category (‘+G’) with a respective probability, and also factoring the observed amino acid frequencies (‘+F’). This model was applied used for maximum likelihood analysis using PhyML (Guindon, et al. 2005),
implemented in Seaview (Gouy, et al. 2010) with five random starting trees. We also used this model to perform the Bayesian inference analysis by applying MrBayes (Ronquist and Huelsenbeck 2003), which implemented a Markov-chain, Monte Carlo analysis with 10,000,000 generations to approximate the posterior probability and a standard deviation of split frequencies < 0.01 to indicate convergence as previously described (Ronquist and Huelsenbeck 2003). For the resulting tree, we report bootstrap values from the neighbor-joining and maximum likelihood methods, as well as list the respective posterior probabilities of each node performed by the Bayesian inference analysis.

**PAML analysis**

We used the coding DNA alignment and our final tree to investigate the ratio of non-synonymous (dN) to synonymous (dS) amino acid substitutions using the PAML program. Likelihood ratio tests (LRT) were constructed to compare the p-values of χ² square tests for selective pressure models against neutral models (Yang 1998; Yang, et al. 2005). Three levels of analysis were investigated. The first level calculates the global ω for the tree using the one-ratio model M0 (Yang 1998), where ω = dN/dS. The second level of analysis used a two-ratio branch model which allows ω value to vary among branches in the same tree (Nielsen and Yang 1998; Yang 1998). This test was used to investigate ω values for primates, Supraprimates, tunicates, and insect lineages. The third level calculates the variation across sites of the alignment. This was done by performing two tests: M1a versus M2a and M7 versus M8. The nearly-neutral model (M1a) (Nielsen and Yang 1998) includes sites under purifying selection (0<dN/dS<1) as well as sites under neutral evolution (dN/dS=1), while the positive selection model (M2a) (Nielsen and Yang 1998) includes sites that evolved under positive selection (dN/dS >1). Model M7 uses a beta distribution for ω over sites limited to the interval (0, 1), and was used as the null hypothesis. Model M8 adds another site class to M7 with the ω ratio estimated from the data (Yang and Nielsen 2002). Afterwards, Bayes Empirical Bayes (Yang et al. 2005) was used to compute the posterior probability of the resulting ω for each putative positive selected site.

2. **Trehalose, Glucose and Glycogen Analysis**

Four substrates were measured: circulating trehalose, stored trehalose, circulating glucose, and glycogen. All extracted substrates are converted to a glucose solution for final analysis via spectrophotometry. Male flies aged 5-7 days post-eclosion were collected and starved for either 0, 12, or 24 hours in 1% agarose vials and then frozen at -80°C overnight. To collect hemolymph, ten flies per replicate were weighed using a 1/10000 scale (Denver instrument company, Göttingen, Germany) and placed in phosphate buffer saline (PBS, pH 7.4) in a 1:5 ratio of PBS (mg of flies/µL of PBS) and decapitated via centrifugation (at 3000 g for 6 min at 4°C). Hemolymph was used to measure circulating glucose and trehalose. To determine stored trehalose and glycogen, the remaining bodies of the 10 flies were homogenized in a 1:10 ratio of PBS (mg of flies/µL of PBS) and homogenates were centrifuged at 12000 g for 15
min at 4° C and supernatant was collected for analysis. Trehalose from the hemolymph or supernatant was converted to glucose using porcine kidney trehalase (Sigma T8778) overnight at 37° C. Glycogen from the supernatant was converted to glucose by amylglucosidase from Aspergillus niger (Sigma A7095) overnight at 25° C. Lastly, glucose levels from all substrates were quantified using a glucose assay kit involving glucose oxidase and peroxidase (Liquick Cor-Glucose Diagnostic kit, Cormay, Poland) according to the manufacturer’s instructions. Briefly, glucose is oxidized to form gluconic acid and hydrogen peroxide by glucose oxidase. The hydrogen peroxide then reacts with 4-aminoantipyrine in the presence of peroxidase to form a colored solution where glucose concentration is proportional to absorbance of light. Absorbance to 492 nm light was measured for each replicate of each substrate on a multi-scan microplate spectrophotometer and converted to a mM concentration of glucose using a linear regression obtained by a calibration curve made from a serial dilution of a sample with a known glucose concentration.

3. Glucose Stimulus Assay

Three strains/groups were used: a control cross between Ilp2-GAL4; tGPH and w1118 flies, a cross between Ilp2-GAL4; tGPH and UAS-CG30051-RNAi flies, and negative control (no fluorescent signal) of w1118 flies crossed with UAS-CG30051-RNAi. Each group was fed blue-dyed food, so as to identify still-feeding larvae (such larvae had visible blue digestive tracts). Then 3rd-instar, still-feeding larvae were collected and starved in H2O for 30 min. Subsets of larvae were re-fed 20% sucrose for 30 min following the initial 30 minute starvation. Fat bodies from each group were dissected and prepared for immunohistochemistry analysis. Fat bodies dissected from larvae were put into a staining glass bowl containing using 4% formaldehyde in phosphate buffered saline (PBS, pH 7.4) on ice for 15 min on agitation. After fixation, tissues were washed four times for 15 min each with PBS and then with supermix (a solution of 0.25% gelatin and 0.5% triton X-100 in PBS). Tissues were incubated with primary antibody to green fluorescent protein (anti-GFP, chicken, Invitrogen) diluted in a 1:400 ratio of antibody:supermix, and kept overnight at 4° C in bowls sealed with parafilm and aluminum foil. Following incubation, tissues were washed four times for 15 min each with PBS and incubated with secondary antibody (Alexa-488 green, goat, anti-chicken, Invitrogen) diluted 1:800 in PBS- triton X (PBT) for 1 hour. Tissues were then washed once more with PBT for 15 min and incubated with DAPI diluted in supermix at a 1:1200 ratio for 5 min. Finally tissues were washed four times with PBS and mounted with mounting agent Mowiol. The GFP and DAPI signals were visualized with an Axioplan-2 Imaging upright fluorescent Microscope (Carl Zeiss). The excitation/emission wavelengths for GFP and DAPI were 488/519 and 351/461 nm respectively.

4. Improved Phylogenetic Analysis
We extended our group’s previous tree to investigate how well the TMEM18 ortholog is conserved throughout evolution in 21 species (Supplementary Figure 1A), and also investigated the degree of selection among their coding regions. Orthologs were found in basal bilateria: *Schistosoma japonicum* (diverging 150 MYA (Lawton, et al. 2011)), *Chlorella variabilis* (700 MYA (Becker 2013)), *Albugo candida* (1 BYA (Brown and Sorhannus 2010)), and *Naegleria gruberi* (1.5 BYA (Yoon, et al. 2004)). Thus, TMEM18 is likely conserved among the four main families of eukaryotes (*Archaeplastida, Unikonta, Chromalveolata*, and *Excavata*) and is ancient, as previously claimed (Almen, et al. 2010). However, no homologs were found in *Caenorhabditis elegans, Amphimedon queenslandica, or Saccharomyces cerevisiae*. Moreover, no paralogs were observed in the species we studied (Supplementary Figure 1B). An extra copy of TMEM18 was found in pigs. However, although they have different gene accession numbers (XP_003125451.1 and XP_003125449.1), they appeared in close proximity to each other on the third chromosome (LOC100515724 and LOC100515374) and their nucleotide sequences were identical, suggesting that the copies could result from sequencing errors (Silver 1995). Furthermore, PAML showed that the coding region of TMEM18 is under significant negative selection ($\omega = 0.067$, p <0.01), with the mammal lineage also significantly conserved ($\omega = 0.0008$, p <0.01). However, the Arthropoda lineage, which is known for its rapid evolution (Wyder, et al. 2007; Lin, et al. 2006; Zdobnov, et al. 2002), showed no significant selection ($w = 0.0031$, p = 0.47). In addition, only one site, a leucine at position 35 in the *Drosophila melanogaster* protein sequence (CG30051), showed positive section. And no sites in any of the sequences showed positive selection under the M1a-M2a test. From all this, TMEM18 is likely an important gene with evolutionary pressure towards conservation, and CG30051 in *Drosophila melanogaster* is likely a good ortholog of human TMEM18.

5. **Knockdown of CG30051**

We crossed UAS-CG30051-RNAi flies with the ubiquitous daughterless (da) da-GAL4 driver to express RNAi to CG30051 systemically (shown as da>CG30051-RNAi). da>CG30051-RNAi flies were viable with no obvious behavioral or morphological phenotype. We then compared expression of CG30051 transcript via qPCR in samples collected from whole flies. Compared to both control crosses, da-GAL4 x $w^{118}$ and $w^{118}$ x UAS-CG30051-RNAi, the da>CG30051-RNAi cross reduced CG30051 levels by 94% (Supplementary Figure 2A).

We further validated our results by checking for possible off-target effects. CG30051 overlaps with DUBAI. Thus, we also tested whether levels of DUBAI were affected by global knockdown of CG30051. In an experiments similar as described above, DUBAI levels in the da-GAL4 x UAS-CG30051-RNAi cross were no different from either control cross (Supplementary Figure 2B).

6. **Control Experiments for Food Intake and Diet**
We tested if the changes in substrate levels were due to excessive food intake. To this end, we used the CAFE assay, a method in *Drosophila* which measures food consumption (Ja, et al. 2007), to determine if *Ilp2>CG30051*-RNAi flies had different feeding behaviors than controls. We found no difference in the amount of food consumed in a 24 hour period between controls and the transgenic cross (Supplementary Figure 3A). We also tested *CG30051* expression when control flies were fed diets with differing ratios of macronutrients. CSORC flies were raised to adulthood on a standard diet, but shortly after eclosion were moved to a new bottle containing one of five different diets with a different ratio of sugar to yeast: 2.5:2.5, 10:10, 10:40, 40:10, and 40:40. Flies fed on the treatment diet for 5-7 days and then male flies were frozen and their heads were later processed for qPCR experiments. *CG30051* expression only changed on the 40:40 diet, which had a lower relative expression level of 0.70 ± 0.03 compared to the other diets (Supplementary Figure 3B). From all this, the changes in substrate levels caused by *CG30051* knockdown are probably due to abnormal physiological regulation rather than changes in food intake.


**Supplementary Figure 1. TMEM18 is widely conserved.** 21 organisms were chosen to investigate the evolution of TMEM18. Three methods were used to construct a multiple sequence alignment and a phylogenetic tree: 1) neighbor-joining, 2) maximum likelihood, and 3) Bayesian inference. There was consensus between all three methods for the resulting tree. The sequence for Naegleria gruberi was used as the outgroup. A) Multiple sequence alignment for TMEM18 orthologs. Protein sequences were aligned by the three putative transmembrane regions as indicated by the black lines above the sequences. Overall, sequences showed high shared identity. Black color was used for identical amino acids while similar amino acids are shown in light grey. Orthologs were absent in Amphimedon queenslandica, Caenorhabditis elegans, and Saccharomyces cerevisiae. B) Phylogenetic tree of the TMEM18 orthologs. The gene has no paralogs in any of the species tested. These results also display the gene’s ancient origin, as an ortholog is found in both amoeboflagellata (Naegleria gruberi) and water molds (Albugo candida). The tree produced using the neighbor-joining method had the highest bootstrap values (shown). Posterior probabilities from Bayesian inference analysis are shown in parentheses. Scale bar indicates evolutionary distance.

**Supplementary Figure 2. Successful knockdown of CG30051.** The Drosophila ortholog TMEM18 was successfully knocked down in a transgenic, cross. Expression of an RNAi construct for CG30051 was driven by the GAL4-UAS system using the ubiquitous daughterless da-GAL4 driver. Thus, RNAi to CG30051 was expressed in all cells. Whole, male flies, aged 5-7 days post-eclosion were collected for analysis. A) Compared to two
control crosses, the \textit{da-GAL4} x \textit{UAS-CG30051-RNAi} cross had 94% reduced expression of \textit{CG30051}. \textbf{B) To test for off-target effects, levels of \textit{DUBAI} were analyzed in the same crosses with \textit{CG30051} knocked down globally. \textit{DUBAI} was chosen as this gene overlaps with \textit{CG30051}. Levels of \textit{DUBAI} were unaffected by \textit{CG30051} knockdown (n = number of samples, 3 replicates per sample; \textit{da-GAL4} x \textit{w}^{1118}, n = 5; \textit{w}^{1118} x \textit{CG30051-RNAi}, n = 5; \textit{da-GAL4}>\textit{CG30051-RNAi}, n = 6; Tukey HSD test, *** = p < 0.001).}

\textbf{Supplementary Figure 3. \textit{CG30051} does not affect intake of food or change expression in response to diet.} \textbf{A) Flies lacking \textit{CG30051} in the insulin producing cells do not have altered feeding behavior.} The CAFE assay was used to measure the amount of food consumed in 24 hours. Flies were male, aged 5-7 days post-eclosion. There was no difference in the amount of food eaten between the \textit{Ilp2>CG30051-RNAi} cross versus either of the 2 control crosses (n = 10 for each strain). \textbf{B) Expression of \textit{CG30051} did not change in response to different diets, except for one group.} CSORC flies were fed different concentrations of a sugar:yeast ratio for 5-7 days post eclosion, and then heads were prepared for analysis via qPCR. Flies fed the 40:40 ratio had less expression of \textit{CG30051} compared to the 2.5:2.5 ratio (n = number of samples, 3 replicates per sample; n = 5 for all groups; Tukey HSD test, * = p < 0.05).