***Supplementary Information***

## Supplementary Methods

*Antibody Production and Plasmid Construction*

Rabbit polyclonal antibodies were raised against purified human ATGL in the Polyclonal Antibody Production facility at Cornell University. Full-length ATGL was produced as an MBP-fusion protein by cloning into the pMAL-2G vector (New England Biolabs). ATGL-MBP was over-expressed in *E. coli* TB1 cells and isolated from cell extracts by amylose resin affinity chromatography. Genenase I was used to cleave MBP from ATGL, and the products were separated by SDS-PAGE. ATGL was excised from Coomassie blue stained gels, electro-eluted into 50 mM acetate buffer containing 0.1% SDS, pH 7.8, and concentrated using Nanosep (PALL LifeSciences, Ann Arbor, MI). Guinea pig anti-TIP47 antibodies were from Research Diagnostics (Flanders, NJ). Mouse monoclonal antibodies against □-tubulin and □-actin were from Abcam (Cambridge, MA). Cy3- and FITC-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). For expression in mammalian cells, the ATGL coding region was amplified by PCR using pCMV-226 DNA (provided by Peter Shaw) as template. The PCR product was cloned into pEGFP-N1 from Clontech to produce pEGFP-N1-ATGL, expressing ATGL with GFP at the C-terminus. To introduce the S47A mutation into ATGL, pEGFP-N1-ATGL was used as template in the QuikChange mutagenesis kit (Stratagene, Cedar Creek, TX). The Tgl3p coding region was amplified by PCR from *S. cerevisiae* genomic DNA. The PCR product was cloned into pEGFP-N1 for expression of Tgl3p-GFP in mammalian cells. All constructs were verified by DNA sequencing.

*TIP47 displacement quantification*

For Table 1 quantifications, fixed HeLa cells transfected with the indicated constructs and grown with or without 400 μM Oleic Acid/BSA were prepared for immunofluorescence analysis using anti-TIP47 antibodies. Transfected cells were identified by observing GFP-ATGL fluorescence. In the same cell, TIP47 fluorescence on lipid droplets was compared to the corresponding TIP47 staining in neighboring untransfected cells and scored as normal, decreased or absent.

*Sequence Alignment and Phylogeny Reconstruction*

The multiple alignment shown in Fig. S1 and those used for phylogenetic analysis were constructed using T-coffee (Notredame *et al*., 2000) and then manually corrected on the basis of high-scoring sequence pairs generated by PSI-BLAST (Altschul *et al*., 1997) searches. Maximum likelihood trees were constructed using the ProtML program of the MOLPHY package by optimizing the least-square trees with local rearrangements (Adachi and Hasegawa, 1992). Bootstrap analysis was performed for each maximum likelihood tree as implemented in MOLPHY using the Resampling of Estimated Log-Likelihoods (RELL) method (Adachi and Hasegawa, 1992; Kishino *et al*., 1990). ATGL and Tgl3p families along with the patatin and phospholipase A2 families are likely to be products of ancestral duplication events specific to eukaryotes. All four families have representatives from at least two out of four distinct eukaryotic lineages (plants, animals, fungi, apicomplexa) and the corresponding branches are supported by bootstrap analysis. These families are more related to each other than to the bacterial clade, suggesting that eukaryotic duplications likely occurred after the divergence from prokaryotes.

**Legends to Supporting Figures**

Figure S1. Multiple alignment of the selected representatives of six families of patatin group lipases. The sequence of cytosolic calcium-dependent phospholipase A2 whose crystal structure has been solved (cPLA2) is shown for comparison. The sequences are denoted by Gene Identification (GI) numbers from the GenBank database and with abbreviated species names. The positions of the first and the last residue of the aligned region in the corresponding protein are indicated for each sequence. The numbers within the alignment refer to the length of inserts not shown that are poorly conserved between all four families. The alignment coloring is based on the consensus shown underneath the alignment: h indicates hydrophobic residues (A,C,F,I,L,M,V,W,Y,S,T) and t indicates turn-forming residues (A,S,T,D,N,V,G,P,E,N,R,K), s indicates small residues (A,G,S,T,C,T,N,D,P,V), charged residues are red, hydroxyl-group containing residues are blue, catalytic amino acids are shown in reverse shading. The secondary structure elements are for cPLA2 and are shown below the alignment. H indicates α-helix and E indicates extended conformation (β-strand). Six distinct phospholipase A2-like families are indicated on the right. Amino acids identical among ATGL family members are boldfaced. The catalytic serine in motif II is marked (*). Motif II is the most highly conserved sequence motif among members of the α/β hydrolase superfamily.

Figure S2. Lipase activity of ATGL and ATGL-ΔGASAG.

SF9 cell extracts, prepared from baculovirus-infected cells expressing wild type His-ATGL, His-ATGL-ΔGASAG, or vector alone, were obtained by sonication (in 50 mM Tris pH 8.0, 150 mM NaCl, 1% Igepal, 1% β-mercaptoethanol, protease inhibitors) and subsequent centrifugation. The supernatants were harvested and tested for lipase activity using a $^{3}$H-palmitate labeled mixture of neutral lipids and phospholipids in the form of mixed micelles. The substrate was prepared from 85 parts phosphatidylcholine:15 parts phosphatidylethanolamine in 0.1% Triton-X100, 100 mM HEPES, 5 mM EDTA, which was spiked with $^{3}$H-palmitate labeled lipids. For lipase assays, 200 μg of extract and 100 μg cold substrate containing 20,000 cpm labeled lipid mixture per assay were incubated for 30 minutes at 40°C. The reactions were subjected to Dole extraction and separation, and the released free fatty acid was measured in the aqueous phase. The results were normalized to the amount of each protein that was expressed as determined by Western blotting using anti-ATGL (right panel), and the fold-increases in activity of the wild type and mutant ATGL-expressing extract with respect to control extract from non-recombinant baculovirus alone treated cells is shown (left panel). Since the substrate was heterogeneous, containing phospholipids as well as triacylglycerols, the maximum activity is not shown. The results showed that extracts from cells expressing wild type ATGL had ~8-fold more activity than extracts expressing the ATGL-ΔGASAG mutant. Moreover, the lipase activity in extracts expressing the ATGL-ΔGASAG mutant protein was not significantly above that found in control extracts from vector treated cells. Note that the mutant we have tested for lipase activity is a deletion mutant that removes five amino acids including S47, as well as two amino acids upstream and downstream of this

catalytic serine. Hence it is not identical to the ATGL-S47A mutant that was tested in vivo, but is a deletion mutant that lacks this catalytic serine.

Figure S3. Phylogenetic tree for lipases of the ATGL family. Analysis and designations are the same as for Fig. 4. The phylogenetic reconstruction suggests that several animal-specific duplications occurred during the evolution of the ATGL family. The duplications gave rise to five distinct subfamilies all of which are present in the human genome: the ATGL/PNPLA2 subfamily, also referred to as iPLA2, desnutrin and transport-secretion protein 2 (TTS-2); adiponutrin/PNPLA3, also known as iPLA2, an adipose tissue specific protein that has been demonstrated to be a triglyceride lipase (Jenkins *et al* 2004); PNPLA5, an uncharacterized subfamily which is closely related to adiponutrin and is likely to be another triglyceride lipase; PNPLA1, for which the complete human sequence is not available (shown is the closely-related full-length chimpanzee (Pan troglodytes) sequence, indicated by *); and finally GS2 (also called iPLA2 or PNPLA4), which has been demonstrated to have triglyceride lipase activity and to be widely expressed in all tissues as is ATGL. PNPLA stands for "patatin-like phospholipase domain containing".

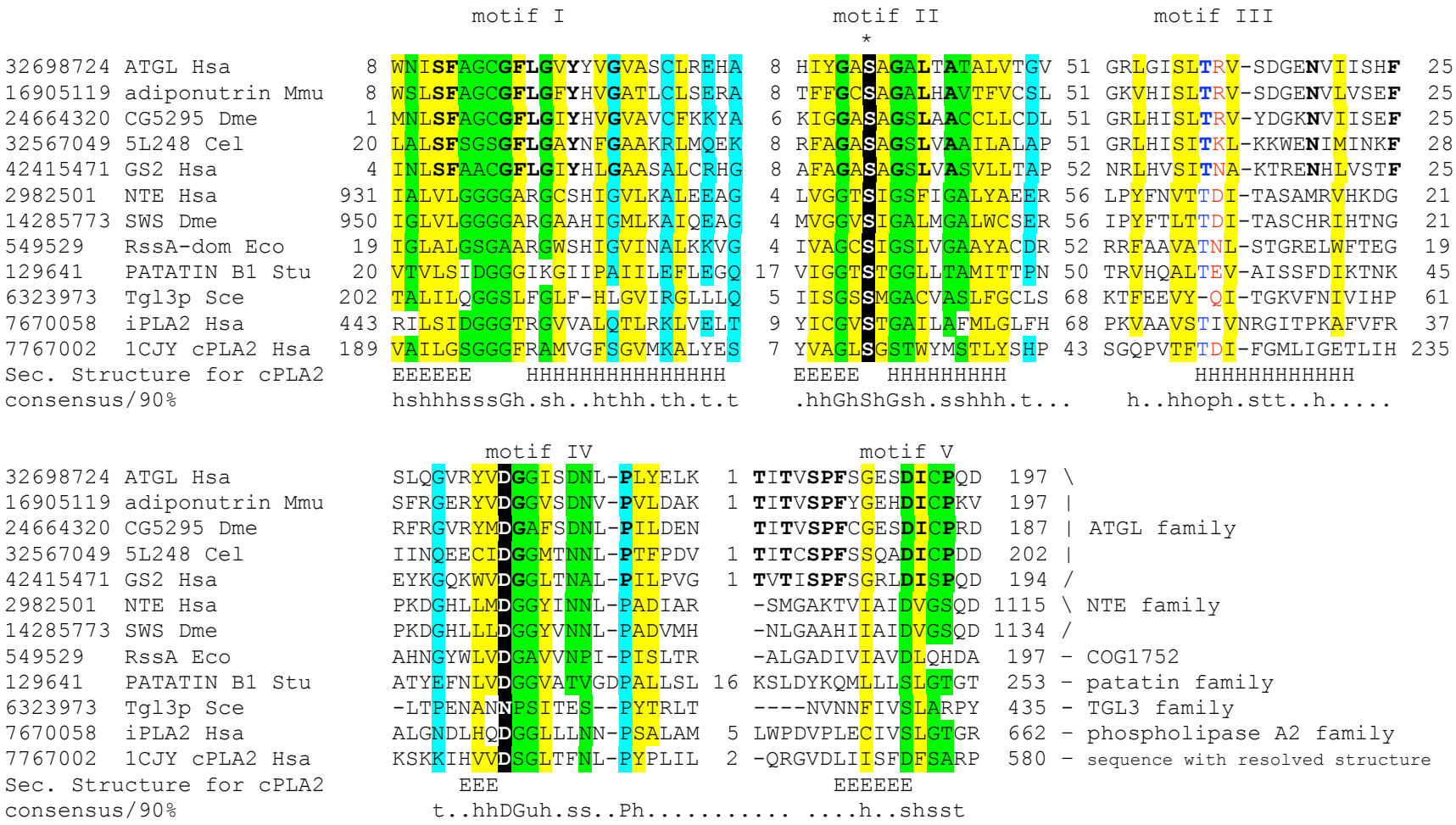
**Supplementary References**


Adachi, J. and Hasegawa, M. (1992) MOLPHY: Programs for molecular phylogenetics. In *Computer Science Monographs*. Institute of Statistical Mathematics, Tokyo, Vol. 27.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389-3402.

Kishino, H., Hasegawa, M. and Miyata, T. (1990) Maximum likelihood inference of protein phylogeny and the origin of chloroplasts. *J Mol Evol* **31**: 151-160.

Notredame, C., Higgins, D.G. and Heringa, J. (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* **302:** 205-217.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motif II
motif III
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motif V
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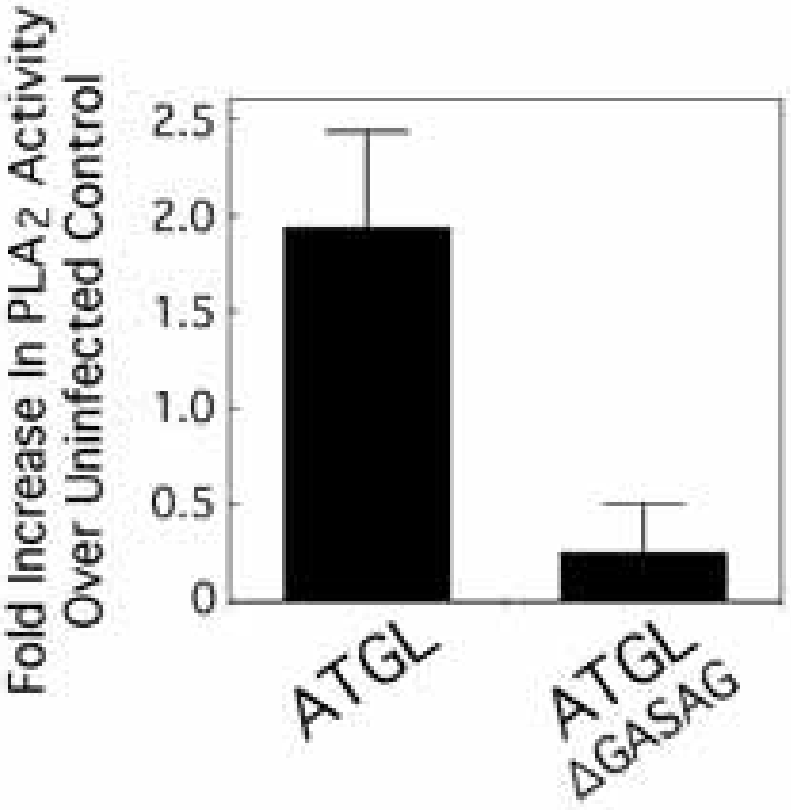
Figure S1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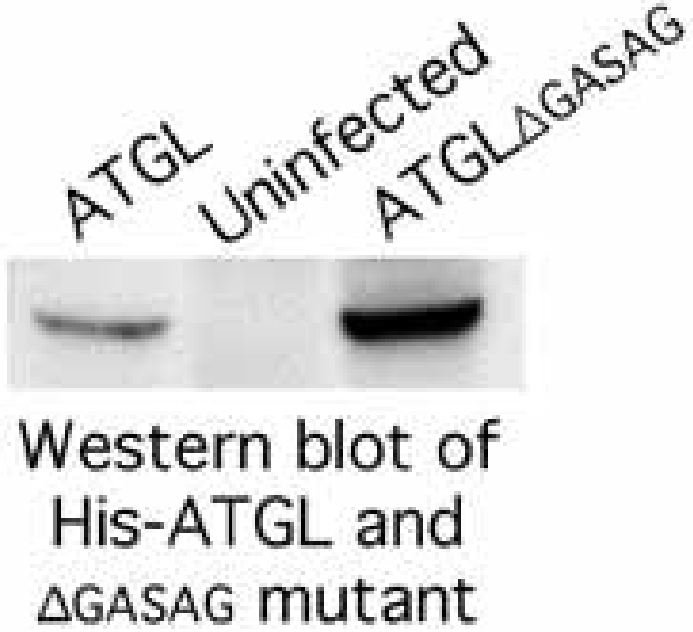
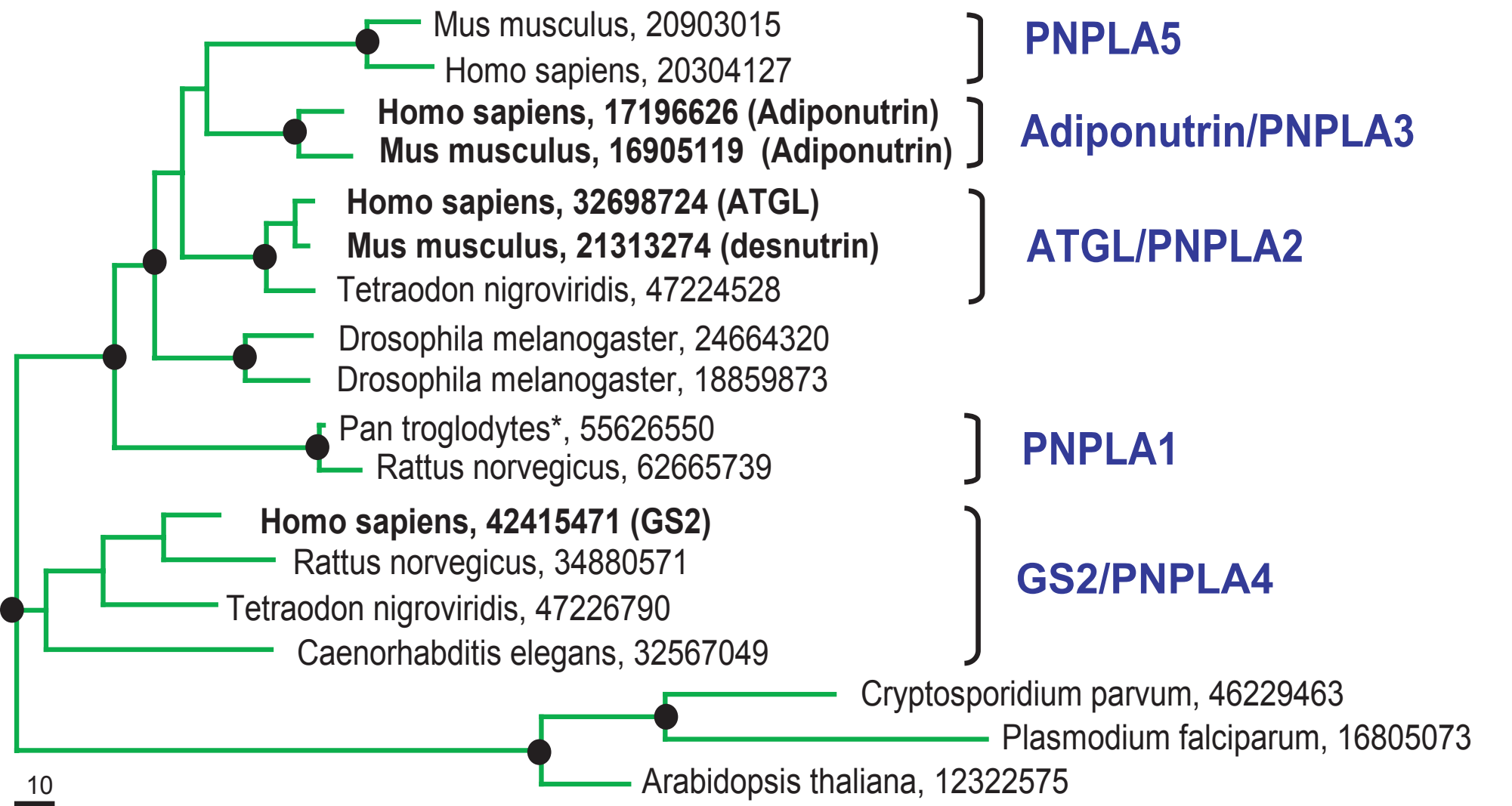
ATGL
ΔGASAG


ATGL
Uninfected
ATGLΔGASAG
Western blot of
His-ATGL and
ΔGASAG mutant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