



# Hkat, a novel nutritionally regulated transmembrane protein in adipose tissues

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White adipose tissue is an active endocrine organ regulating many aspects of whole body physiology and pathology. Adipogenesis, a process in which premature cells differentiate into adipocytes, is a complex process that includes orchestrated changes in gene expression and cell morphology in response to various nutritional and hormonal stimuli. To profile transcriptome changes in response to nutritional stimulation, we performed RNA-seq on fat in mice treated with either a high-fat diet or fasting. We identified a novel nutritionally regulated gene, Gm12824, named Hkat (heat, kidney, adipose-enriched transmembrane protein). We show that both fasting and obesity dramatically reduce *Hkat* in white adipose tissue, and that fasting reduces while obesity increases its expression in brown fat. Hkat is localized to the plasma membrane and induced during adipogenesis. Therefore, Hkat is a novel nutritionally regulated gene that is potentially involved in metabolism.

White adipose tissue, the major energy reservoir in higher eukaryotes, plays a central role in maintaining energy homeostasis<sup>1–6</sup>. During periods of nutritional abundance, white adipose tissue increases uptake of glucose and fatty acids and stores them in the form of triglycerides, which are released in the form of free fatty acids and glycerol during nutritional deprivation.

Adipose tissue secretes numerous cytokines, called adipokines, which are involved in regulating insulin sensitivity, energy expenditure, food intake, blood pressure and inflammatory responses. Some examples of these adipokines include Leptin<sup>7</sup>, Adiponectin<sup>8–10</sup>, Resistin<sup>11</sup> and TNF $\alpha$ <sup>12</sup>. Expansion of white adipose tissue, a hallmark of obesity, is a major risk factor for various pathological conditions, such as type 2 diabetes, hypertension and cardiovascular diseases<sup>13–15</sup>. Conversely, absence of adipose tissue, lipodystrophy, also exhibits diabetes phenotypes<sup>16,17</sup>. Therefore, although traditionally adipose tissue was regarded as an inactive energy reservoir only; the past two decades have seen a dramatic increase in our knowledge about adipocyte biology, indicating that adipose tissue is an active endocrine organ regulating many aspects of whole body physiology and pathology<sup>18–22</sup>.

Adipogenesis, a process in which premature cells differentiate into adipocytes, is a complex process that includes orchestrated changes in gene expression and cell morphology in response to various nutritional and hormonal stimuli. Adipogenesis relies on the accurate regulation of a complicated cascade of transcription factors, such as peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), members of the CCAAT/enhancer-binding proteins (C/EBPs), and sterol regulatory element-binding protein 1 (SREBP1)<sup>23–31</sup>. Identification of novel genes and pathways in influencing adipocyte differentiation is helpful to understand the pathology of metabolic syndromes, and this is highlighted by the current obesity epidemic<sup>32,33</sup>.

To profile transcriptome changes in response to nutritional stimulation, we performed RNA-seq experiments on fat in mice treated with either a high-fat diet or fasting. We identified a novel nutritionally regulated gene, Gm12824, named Hkat (heat, kidney, adipose-enriched transmembrane protein). We show that both fasting and obesity dramatically reduce *Hkat* in white adipose tissue. Fasting reduces and obesity increases its expression in brown fat. Hkat, a transmembrane protein, is induced during adipogenesis, and it is therefore a novel nutritionally regulated gene that is potentially involved in adipogenesis.

## Results

**Hkat is evolutionarily conserved.** To comprehensively identify nutritionally regulated genes, we performed RNA-seq experiments on white adipose tissue, in mice treated with 3-month HFD or 24-hour fasting. Here, we focus on the analysis of the novel gene Hkat, which was one of the identified genes that were sensitive to nutritional stimulation. The symbol for mouse Hkat is Gm12824, and its human homolog is LOC388630 (Table 1).



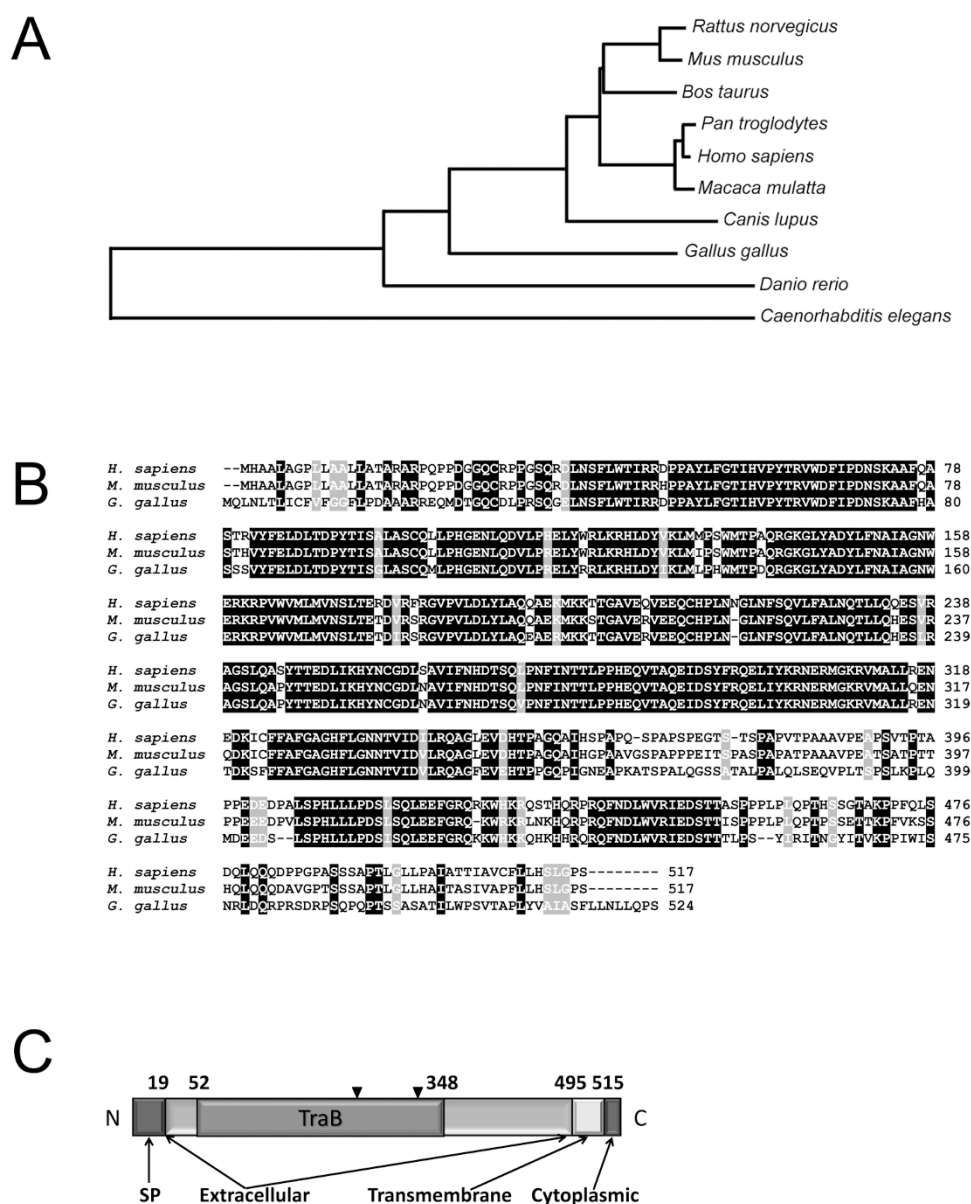
Table 1   IDs of Hkat in databases		
Name	Mouse	Human
	Hkat	HKAT
Symbol	Gm12824	LOC388630
Chromosome	4	1
Location (bp)	114079329-114287703	48226200-48462562
RefSeq	NP_001079018	NP_001181915
Ensembl	ENSMUSG00000070867	ENSG00000204018
Entrez	666048	388630
Uniprot	B1ATG9	A6NFA1

Hkat is evolutionarily conserved. A Blast search against the non-redundant protein database at National Center for Biotechnology showed that Hkat has homologues in multiple species, including *D. rerio* and *C. elegans* (Fig. 1A). Between protein sequences of

human and mouse, 85% of residues were identical, whereas between those of human and chicken, 81% of residues were identical (Fig. 1B), indicating that Hkat is highly evolutionarily conserved.

Both the human and mouse Hkat have 517 amino acids in length. The program SignalP 4.0 predicted Hkat has a signal peptide from the residue 1–19. According to CDD, a conserved domain database, Hkat harbors a conserved domain of TraB from the residue 52–348 (Fig. 1C). TraB is a protein family that is involved in production of peptide pheromones by bacteria. It is unclear what the functions of TraB are in mammalian species. In addition, Hkat also harbors a transmembrane domain from the residue 495–515. Because of the presence of a signal peptide, it is likely that the N-terminal of Hkat is in extracellular space, and the C-terminal is in the cytoplasm, connected by the transmembrane domain (Fig. 1C).

**Hkat is nutritionally regulated in fat.** To examine whether *Hkat* is nutritionally regulated, we treated mice with fasting or HFD, and then examined *Hkat* expression by qPCR analysis. In white adipose



**Figure 1 | The Hkat protein is evolutionarily conserved.** A) The evolutionary tree of Hkat orthologs. B) Alignment of Hkat protein sequences of human, mouse and chicken. Between human and mouse, 85% of residues are identical, whereas between human and chicken, 81% of residues are identical. C) Hkat has a signal peptide (SP), extracellular domain, transmembrane domain and cytoplasmic region, and it has a domain that belongs to TraB family. Triangle denotes predicted glycosylation.



tissue (WAT), 24-hour fasting reduced *Hkat* expression for more than 80% ( $P < 0.01$ ), and refeeding 4 hours following the fasting normalized its expression (Fig. 2A). In mice with 3-month of HFD treatment, *Hkat* expression in WAT was reduced for more than 70% ( $P < 0.01$ ) (Fig. 2B). In brown adipose tissue (BAT), fasting reduced *Hkat* expression for about 70% ( $P < 0.01$ ), which was normalized by refeeding (Fig. 2C). Conversely, *Hkat* expression in BAT was increased for about twofold in mice with diet induced obesity ( $P < 0.01$ ) (Fig. 2D). That is to say, fasting reduces *Hkat* expression in both WAT and BAT. HFD treatment suppressed its expression in WAT, while increased its expression in BAT. Therefore, *Hkat* expression is nutritionally regulated in fat.

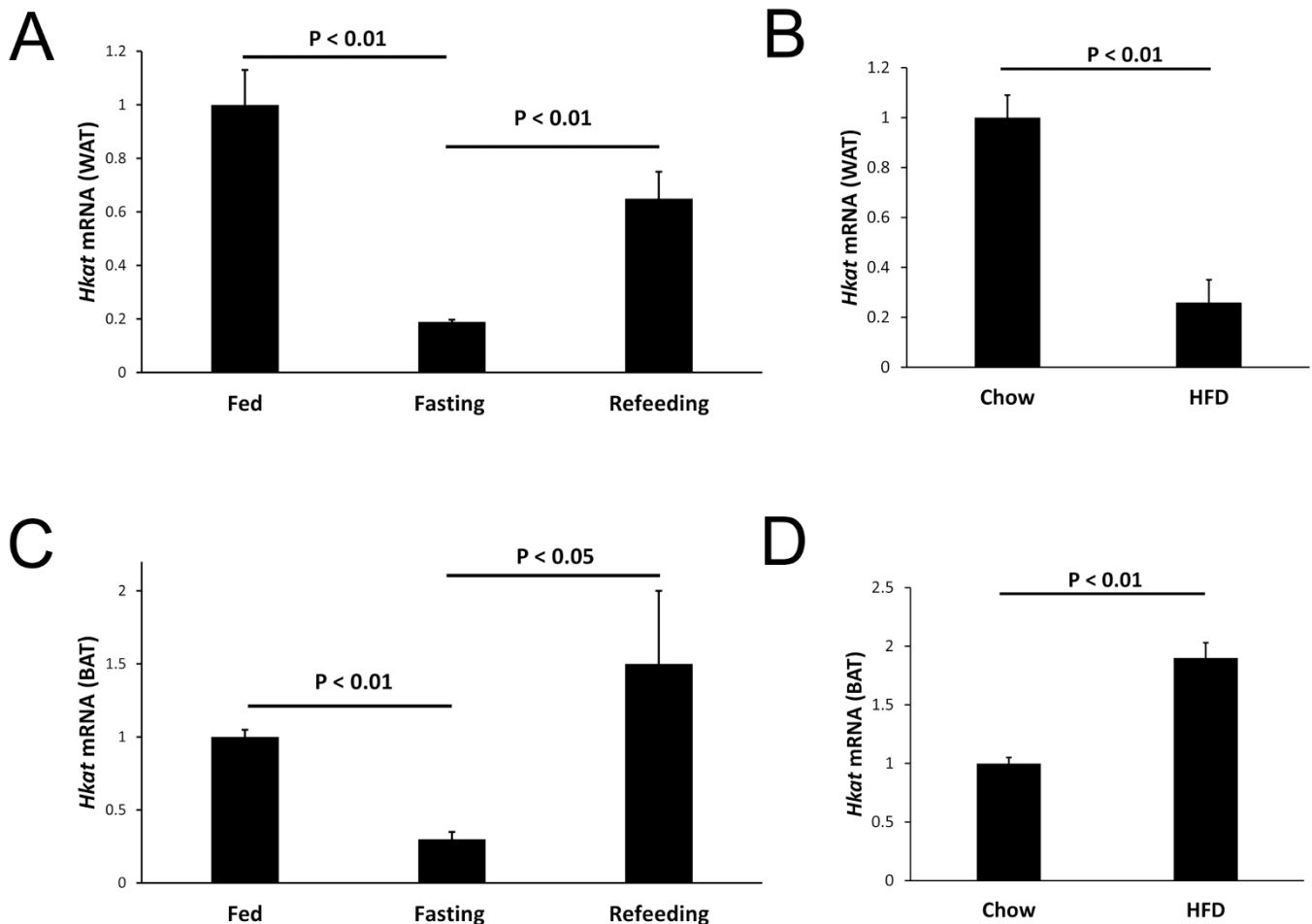
#### **Hkat is abundant in fat, but highest expressed in heart and kidney.**

Tissue expression pattern for a novel gene can be informative in revealing its functions. We therefore examined the expression pattern of *Hkat* in various mouse tissues. Three male C57B6 mice were used to dissect various tissues, including hypothalamus, cortex, stomach, small intestine, large intestine, colon, liver, heart, blood vessel, kidney, spleen, lung, muscle, urinary bladder, testis and fat. The fat tissues included epididymal fat, subcutaneous fat and brown fat. *Hkat* expression is highest in heart and kidney. In addition, *Hkat* is abundant in all fat depots, including perigonadal fat, subcutaneous fat and brown fat (Fig. 3A).

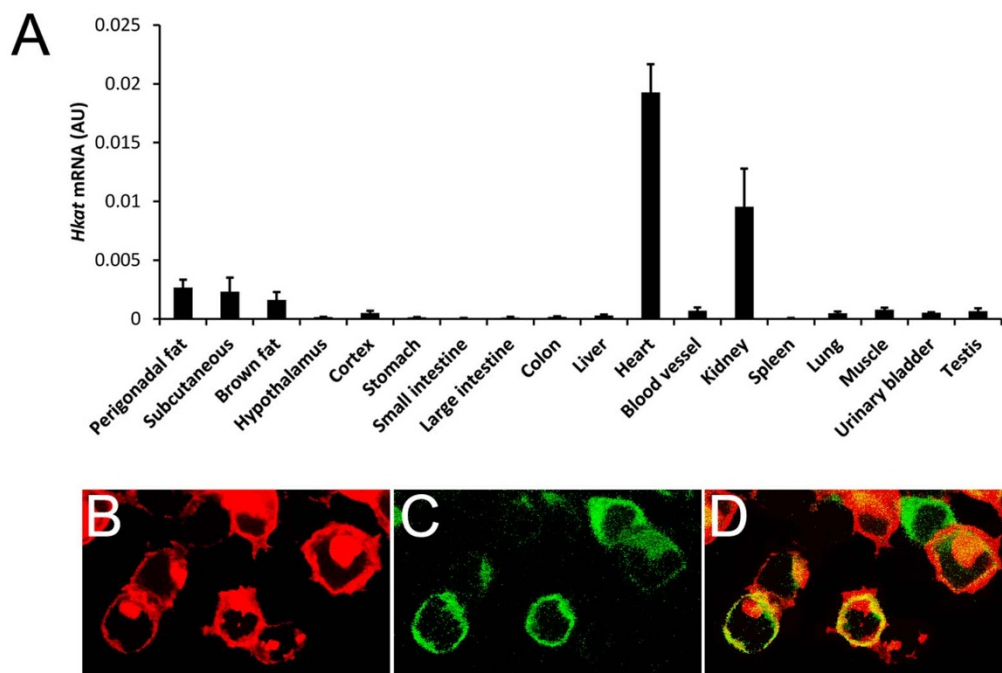
**Hkat is localized to the plasma membrane.** Proteins need to localize to specific subcellular compartments to perform functions, therefore the subcellular localization of a protein is critical to reveal its

functions. The Universal Protein Resource annotates the protein as having a transmembrane domain, from amino acid 495 to 515 (Fig. 1C). To gain experimental evidence, we examined the subcellular localization by fluorescent protein imaging. A fusion protein with a GFP at the C-terminal of *Hkat* was made by cloning the *Hkat* open reading frame into a vector containing the GFP gene. The vector encoding the fusion protein *Hkat*-GFP was then co-transfected with a vector encoding red fluorescent protein fused with LCK (lymphocyte-specific protein tyrosine kinase), which is localized to the plasma membrane. Indeed, red signals, which indicate the location of LCK, localized to the plasma membrane (Fig. 3B). Green signals, which indicate the location of *Hkat*, also localized to the plasma membrane (Fig. 3C). Note that because the vectors was transfected into the cells via transient transfection, some cells had *Hkat*-GFP only (green signals only), while some cells had LCK-RFP only (red signals only). Only cells having the two plasmids at the same time showed co-localization. Merging the two signals shows that in some cells *Hkat* and LCK co-localized (Fig. 3D), indicating that *Hkat* is localized to the plasma membrane in these cells.

**Hkat is transiently induced during adipogenesis.** *Hkat* is abundantly expressed in WAT, and its expression in WAT is sensitive to nutritional stimulation. This makes it possible that *Hkat* plays a functional role in WAT. We therefore examined whether *Hkat* is induced during adipogenesis. 3T3 L1 cells were differentiated into mature adipocytes. Two days after reaching confluence, cells were cultured in differentiation medium that contained insulin and



**Figure 2 | *Hkat* in fat is nutritionally regulated.** A) Fasting and B) HFD treatment reduced *Hkat* expression in fat. C) Fasting reduced *Hkat* expression while D) HFD treatment increased its expression in brown fat. Data are represented as mean  $\pm$  SEM.



**Figure 3** | *Hkat* tissue expression pattern and subcellular localization. **A**) *Hkat* is expressed in both white adipose tissue and brown adipose tissue, but is highest expressed in heart and kidney. **B**) *Hkat* is localized to the plasma membrane. Fluorescence imaging of HEK 293 cells transfected with plasmids encoding **B**) LCK-tRFP and **C**) *Hkat*-GFP and **D**) Merged picture.

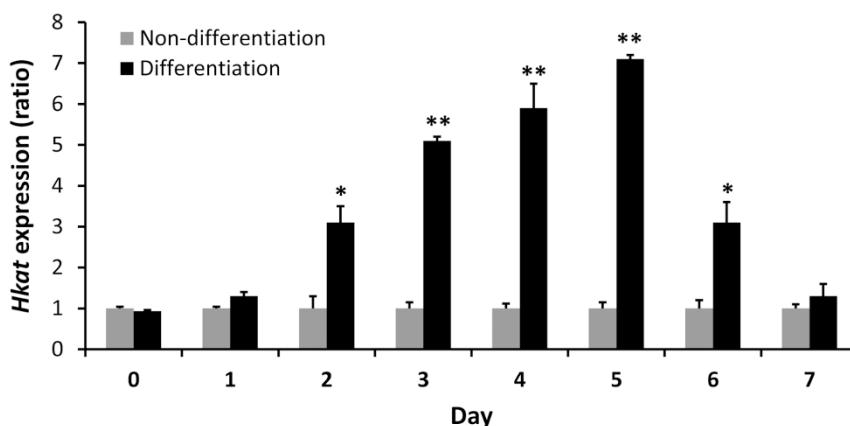
PPAR $\gamma$  agonists for 3 days to induce differentiation, followed by incubation in the regular maintenance medium for additional days. As a control, additional cells were also cultured in maintenance medium without differentiation. Ratio of *Hkat* expression between differentiated and non-differentiated cells was calculated (Fig. 4). Starting from day 2, *Hkat* had a more than 3 fold increase in differentiated cells, and reached about 7 fold on day 5. Interestingly, on day 6 and 7, *Hkat* expression was reduced (Fig. 4). Therefore, *Hkat* was transiently induced during adipogenesis.

## Discussion

We are now in the mist of a global obesity epidemic, because obesity is prevalent in both developed and developing countries, in both adults and children, and the increase in its prevalence continues<sup>34</sup>. Conclusive evidence suggests that obesity is associated with an increased risk of numerous health consequences, such as type 2 diabetes, coronary heart diseases, musculoskeletal diseases and mortality, and

Obesity is becoming one of the most significant contributors to ill health<sup>33,34</sup>. We are in urgent need to find novel therapeutic approaches to treat obesity and its complications. Proteins presented on the cell surface are accessible to various drug delivery mechanisms, therefore plasma membrane proteins are attractive drug targets<sup>35</sup>. In that regard, *Hkat* is a potentially attractive drug target because it is localized to the plasma membrane.

The microarray technique has been routinely used to examine differential expression of genes from metabolically active organs, such as the liver, white adipose tissue (WAT) and brown adipose tissue (BAT), at different nutritional states, such as diet induced obesity and fasting<sup>36,37</sup>. In contrast, RNA-seq is a newly developed sequencing based method to examine the expression of transcriptome. Compared to microarray, RNA-seq has many advantages. Two notable advantages are that RNA-seq is more sensitive, and identifies not only known genes, but also unknown transcripts<sup>38</sup>. We here used RNA-seq and identified *Hkat* as a novel nutritionally regulated gene in fat.



**Figure 4** | *Hkat* is transiently induced during adipogenesis. 3T3 L1 preadipocytes were either differentiated into adipocytes or maintained in non-differentiation medium, and *Hkat* expression was determined by qPCR in cells before (day 0) and after (day 1 to 7) starting differentiation. Shown are ratios of *Hkat* expression between cells with or without differentiation. Values for undifferentiated cells were normalized to 1. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .





There are some caveats in interpreting the results. First, although the current study focuses on the functions of Hkat in fat, *Hkat* is highest expressed in the heart and kidney. Therefore, it is likely that Hkat has important functions in the heart and kidney and these functions are not investigated in the current study. Second, the plasma membrane localization is shown by using an Hkat-GFP fusion protein expressed in HEK 293 cells, but it does not necessarily reflect its subcellular location *in vivo*, i.e., in cells of the heart, kidney and fat. To answer this question, using antibody against Hkat to do immunostaining on tissue sections will be much more informative. However, the antibody is not commercially available. Therefore, it is possible that *in vivo*, Hkat is localized to the membrane of organelles, not necessarily the plasma membrane. Third, Hkat harbors the conserved domain TraB, whose functions, however, could not be easily linked to adipocyte functions.

In summary, we have identified Hkat (Gm12824) as a novel nutritionally regulated gene in fat using RNA-seq. In WAT, both fasting and obesity dramatically reduce *Hkat* expression. In BAT, fasting reduces while obesity increases its expression. Being evolutionarily conserved, Hkat is localized to the plasma membrane and transiently induced during adipogenesis. The functional roles of Hkat and its conserved domain TraB in adipocytes, however, remain elusive.

## Methods

**Mice.** Mice were housed at 22–24°C with a 14-h light, 10-h dark cycle and provided with ad libitum water and a chow diet (6% calories from fat, 8664; Harlan Teklad, Indianapolis, IN) unless otherwise indicated. To examine nutritional stimulation induced gene expression, 10 4-week-old male C57B6 mice (Jackson laboratory, Bar Harbor, ME) were placed on either a chow diet or a high-fat, high-sucrose diet (58% kcal from fat, 26% kcal from sucrose, D-12331; Research Diets, New Brunswick, NJ) for 3 months. Five 8-week-old mice were treated with 24-hour fasting with 4 fed mice as controls. To examine the expression pattern of Hkat in various mouse tissues, 3 8-wk-old mice were used. All animal protocols were approved by the Animal Care and Use Committee of Wayne State University.

**RNA extraction and quantitative real-time PCR.** Dissected tissues were immediately placed into RNAlater solution (Ambion, Austin, TX) for subsequent RNA extraction. Total RNA was isolated from tissues with RNeasy tissue minikit with deoxyribonuclease treatment (QIAGEN, Valencia, CA). One microgram of RNA was reverse transcribed to cDNA using random hexamers (Superscript; Ambion). Relative expression levels were calculated and  $\beta$ -actin was used as an internal control. Primer sequences for *Hkat* were: forward, 5'-CCAGCCTACTTGTGGCAC-3'; reverse, 5'-GACTCGGGTATAGGAACGTG-3'. Primer sequences for  $\beta$ -actin were: forward, 5'-GTGACGTTGACATCCGTAAAGA-3'; reverse, 5'-GCCGGACTCATCGTACTCC-3'.

**Multiple alignments.** Hkat protein IDs for *Homo sapiens*, *Pan troglodytes*, *Macacamullatta*, *Canis lupus familiaris*, *Bos Taurus*, *Musmusculus*, *Rattusnorvegicus*, *Gallus gallus*, *Danioreio*, *Caenorhabditiselegans* were NP\_001181915.1, XP\_001142926.2, XP\_001101255.2, XP\_852692.2, XP\_002686455.1, NP\_001079018.1, XP\_001072500.2, XP\_422458.3, XP\_694385.5, NP\_510451.3, respectively. Hkat orthologs were based on HomoloGene Release 66, with HomoloGene ID 85034. The software MUSCLE<sup>39</sup> was used to perform multiple alignments, and Blast<sup>40</sup> was used to perform pairwise comparison between two sequences. ClustalW2<sup>41</sup> was used to generate phylogeny, and TreeDyn<sup>42</sup> was used to visualize the tree.

**Cell culture and adipocyte differentiation.** 3T3-L1 cells (American Type Cell Collection, Manassask, VA) were maintained in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS) at 37°C with 5% CO<sub>2</sub> in a humidified incubator. To differentiate 3T3-L1 cells into adipocytes, we used preadipocyte medium (Catalog number PM-1-L1, Zenbio, Research Triangle Park, NC), differentiation medium (DM-2-L1) and adipocyte maintenance medium (AM-1-L1). Briefly, 3T3 L1 cells was allowed to reach confluence in preadipocytes medium (day -2), and after 48 hours (day 0), cells medium was changed to differentiation medium for 3 days (day 3), followed by incubating cells in adipocyte maintenance medium for additional days, with feeding cells every 2 days with the same medium.

**Imaging.** To examine Hkat subcellular localization, fusion protein of Hkat-GFP was made by cloning the Hkat open reading frame (Origene, MD) into the pCMV6-AC-GFP vector. In HEK293 cells, the Hkat-GFP vector was cotransfected with a pCMV6 vector that encodes a fusion protein of LCK (lymphocyte-specific protein tyrosine kinase) and red fluorescence protein (Origene, MD) using Lipofectamine 2000 (Invitrogen, Grand Island, NY). Fluorescence Images were taken at the imaging core facility of Wayne State University with a Leica TCS SP5 Confocal Microscope.

**Statistical analysis.** Data are expressed as the mean  $\pm$  sem. Statistical significance was tested with unpaired two-tailed Student's *t* tests. The differences were considered statistically significant if  $P < 0.05$ .

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## Author contribution

RZ designed the study, performed most of the experiments, analyzed data and wrote the manuscript. All authors reviewed the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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