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Lipasin, thermoregulated in brown fat, is a novel but atypical member of the angiopoietin-like protein family

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ABSTRACT

Hyperlipidemia is a major contributor to cardiovascular diseases. Members of the angiopoietin-like protein family (ANGPTLs) are important determinants of blood lipid levels. Lipasin, a newly identified gene that regulates serum triglycerides, is homologous to ANGPTL3's N-terminal domain, which is sufficient and necessary for blood lipid regulation. Brown fat is critical in mediating energy homeostasis. Thermogenesis is the primary function of brown fat, in which Lipasin and some ANGPTLs are abundant; it is unknown, however, whether these genes are thermoregulated. We therefore comprehensively examined the thermoregulation of Lipasin and ANGPTLs in brown fat. Here we show that Lipasin is a novel but atypical member of the ANGPTL family because it is within the same branch as ANGPTL3 and 4 by phylogenetic analysis. The mRNA levels of Lipasin are dramatically increased in the cold environment (4 °C for 4 h) whereas those of ANGPTL4 and ANGPTL2 are suppressed. Fasting dramatically suppresses *Lipasin* but increases *ANGPTL4*. High-fat diet treatment increases *Lipasin*, but reduces *ANGPTL2*. The distinct transcriptional regulations of Lipasin, ANGPTL2 and ANGPTL4 in brown fat in response to cold exposure and nutritional stimulation suggest distinct physiological roles for ANGPTL family members in mediating thermogenesis and energy homeostasis.

1. Introduction

Hyperlipidemia, an elevation of blood lipids, such as cholesterol and triglycerides, is a major contributor to cardiovascular diseases [1–3]. Plasma triglyceride levels are determined by the balance between their production and clearance, where the former involves the synthesis of triglyceride rich lipoproteins in liver and intestine, whereas the latter involves hydrolysis of triglycerides by different lipases in peripheral tissues. Members of the angiopoietin-like protein family have received much attention as important regulators of blood lipid levels [4,5].

The angiopoietin-like protein family contains 7 members, all of which harbor one or two coiled-coil domains and a fibrinogen-like domain, similar to those in angiopoietins, hence the name angiopoietin-like proteins (ANGPTLs) [4–7] (Table 1 and 2). Among the 7 members (ANGPTL1–7), ANGPTL3 and ANGPTL4 have been demonstrated to regulate plasma triglycerides by a large body of evidence, including *in vitro* and *in vivo* studies in mice and humans [8–16]. In mice deficient for either ANGPTL3 or ANGPTL4, serum triglyceride levels are dramatically suppressed; conversely, overexpression by either recombinant protein injection or adenoviruses in mice increases serum triglycerides [8,9,11]. In humans, those with loss of function mutations in either ANGPTL3 or ANGPTL4 have low plasma triglycerides, and variations of these genes are also associated with distinct lipid profiles [12–15,17–20]. Loss of function mutations in ANGPTL5 were also found in humans with lower triglyceride levels [15]. ANGPTL2 has been found to be an important mediator of chronic adipose tissue inflammation and insulin resistance [21].

Lipasin is a newly identified gene that is involved in serum lipid regulation [22]. Lipasin, homologous to ANGPTL3's N-terminal regions that mediate serum triglyceride regulation, is highly enriched in mouse liver and brown fat [22]. Lipasin deficient mice have lower serum triglycerides [23], which are increased by adenovirus-mediated overexpression [22]. In humans Lipasin variations are associated with blood lipid profiles [17].

It is increasingly being recognized that human brown fat is critical for energy homeostasis; an imbalance between brown and white adipose tissue (BAT and WAT) functions can result in obesity. In contrast to WAT, the primary site of energy storage, BAT is mainly for energy expenditure [24,25]. While BAT is found in the interscapular region in mice, it is more widely distributed in human newborns, in areas including axillary, cervical and perirenal regions [26]. It was once thought that BAT in human adults was rare, recent discoveries, however, revealed that human adults have functional BAT, the amount of which is increased by cold and is negatively

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related to body mass index, suggesting increasing BAT is a potential therapeutic option for the management of obesity [27–29].

Thermogenesis is the primary function of BAT, in which Lipasin and some ANGPTLs are highly expressed. However, it is not known whether these genes are thermoregulated. We therefore comprehensively examined the thermoregulation of Lipasin and ANGPTLs in brown fat. Here we show that Lipasin is a novel but atypical member of the ANGPTL family, and it is within the same branch with ANGPTL3 and 4 in phylogenetic analysis using ANGPTLs' N-terminal regions. *Lipasin* is thermoregulated, and comparing to *ANGPTL4* and *ANGPTL2*, shows an opposite pattern of change by cold exposure. *ANGPTL2* in BAT is both thermoregulated and nutritionally regulated. These results suggest Lipasin and some ANGPTLs play distinct physiological roles in mediating thermogenesis and energy homeostasis.

2. Materials and methods

2.1. 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 Habor, 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-h fasting with 4 fed mice as controls. To examine the expression pattern of *Lipasin* and *ANGPTLs* in brown fat, 3 8-week-old mice were used. To examine thermoregulation, 5 mice were exposed to the 4 °C environment for 4 h, with 5 control mice at 25 °C, and then brown fat was dissected. All animal protocols were approved by the Animal Care and Use Committee of Wayne State University.

2.2. 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 β-actin was used as an internal control. Primer sequences for *Lipasin, ANGPTLs* and *β-actin* are listed in Table 3.

2.3. Multiple alignments and phylogenetic tree construction

IDs of human sequences for Lipasin AND ANGPTLs are listed in Table 1. Sequences corresponding to the fibrinogen-like domains of ANGPTLs were removed before doing multiple alignments. The software Clustal Omega was used to perform multiple alignments, and PhyML 3.0 and TreeDyn 198.3 were then used to generate the phylogenetic tree.

2.4. Statistical analysis

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

3. Results

3.1. Lipasin is a novel but atypical member of the angiopoietin-like family

The angiopoietin-like family has 7 members, ANGPTL1–7. Lipasin, a newly identified liver-enriched factor, was previously shown to be homolog of ANGPTL3's N-terminal regions that mediate triglyceride regulation [22]. However, it is not clear what the relation is among Lipasin and ANGPTL members. All ANGPTLs have a conserved structure. In addition to signaling peptide, all ANGPTLs harbor a fibrinogen-like domain at the C-terminal, and have one or two coiled-coil domains at the N-terminal (Fig. 1A) (Table 2). The two domains appear to be involved in different functions, with fibrinogen-like domain being related to angiogenesis [30] and coiled-coil domain related to lipid metabolism [9].

Lipasin, although homologous to ANGPTL3, does not have the fibrinogen-like domain, and also appears to lack the coiled-coil domain [22]. Because Lipasin appears to be mainly involved in triglyceride regulation, which is the major function of the N-terminal regions of ANGPTLs, we then performed multiple alignments, by using Clustal Omega [31], among protein sequences of Lipasin and ANGPTLs in which the fibrinogen-like domains were removed. The software PhyML [32] and TreeDyn [33] were then used to generate the phylogenetic tree. In other words, this alignment shows the relation among Lipasin and the N-terminal part of ANGPTLs.

The 8 proteins are classified into 2 branches. Lipasin and ANGPTL3 were most closely related, and shared a common ancestor with ANGPTL4. Lipasin, ANGPTL3, ANGPTL4 and ANGPTL5 are within the same branch. At the other branch, ANGPTL6 AND ANGPTL7 are mostly related, and shared a common ancestor with ANGPTL1, in addition to having ANGPTL2 within the same branch (Fig. 1B). This result suggests that Lipasin is a novel member of the angiopoietin-like protein family, but it is an atypical member, because of the lack of C-terminal fibrinogen-like domain.

3.2. Expressions of Lipasin and ANGPTLs in brown fat

To comprehensively examine and compare the expression of *Lipasin* and all *ANGPTLs*, we dissected brown and white adipose tissues from three male C57B6 mice, and performed qPCR to examine

Table 1
IDs and locations of Lipasin and ANGPTLs.

Name	Synonyms	Chromosomal Location	DNA	Protein	Uniprot	Mouse homolog	Mouse homolog ID (MGI)
ANGPTL1	ANGPT3, ANG3, angioarrestin, AngY, ARP1	1q25.2	NM_004673	NP_004664	O95841	Y	1919963
ANGPTL2	ARP2, HARP	9q34	NM_012098	NP_036230	Q9UKU9	Y	1347002
ANGPTL3	ANGPT5	1p31.1-p22.3	NM_014495	NP_055310	Q9Y5C1	Y	1353627
ANGPTL4	ARP4, FIAF, HFARP, NL2, PGAR, pp1158, HARP	19p13.3	NM_139314	NP_647475	Q9BY76	Y	1888999
ANGPTL5		11q22.2	NM_178127	NP_835228	Q86XS5	N	
ANGPTL6	AGF, ARP5	19p13.2	NM_031917	NP_114123	Q8NI99	Y	1917976
ANGPTL7	AngX, CDT6	1p36	NM_021146	NP_066969	O43827	Y	3605801
Lipasin	C19ORF80, TD26, PRO1185	19p13.2	NM_018687	NP_061157	Q6UXH0	Y	3643534

Table 2
Protein domains and modifications[a] in Lipasin and ANGPTLs.

	Protein length	Signal peptide	Coiled-coil domain	Fibrinogen-like domain	Glycosylation	Disulfide bond
ANGPTL1	491	1–23	80–168	271–491	160, 188	280 ↔ 309, 432 ↔ 445
ANGPTL2	493	1–22	76–115, 152–206	269–489	164, 192	278 ↔ 307, 430 ↔ 443
ANGPTL3	460	1–16	85–210	237–455	115, 296, 357	246 ↔ 274, 394 ↔ 408
ANGPTL4	406	1–25	100–143	179–401	177	188 ↔ 216, 341 ↔ 354
ANGPTL5	388	1–25	98–123	141–383	53, 238, 329	310 ↔ 314, 324 ↔ 338
ANGPTL6	470	1–20	59–116	251–469	58, 145	260 ↔ 287, 410 ↔ 423
ANGPTL7	346	1–26	39–119	122–343	58, 253, 267	131 ↔ 162, 285 ↔ 298
Lipasin	198	1–21	NA	NA	NA	NA

[a] Source: Uniprot.

Table 3
qPCR primer sequences.

	Forward	Reverse
Lipasin	CACTGTACGGAGACTACAAGTGC	GTGGCTCTGCTTATCAGCTCG
ANGPTL1	GGATGTGCTGTCTAGGCAGAA	TTCATGTTCCGGCTTTCCTTT
ANGPTL2	AGCCTGAGAATACCAACCGC	CCCTTGCTTATAGGTCTCCCAG
ANGPTL3	GAGGAGCAGCTAACCAACTTAAT	TCTGCATGTGCTGTTGACTTAAT
ANGPTL4	CATCCTGGGACGAGATGAACT	TGACAAGCGTTACCACAGGC
ANGPTL6	CTGGGCCGTCGTGTAGTAG	CAGTCCTCTAGGAGTATCAGCAG
ANGPTL7	TGACTGTTCTTCCCTGTACCA	CAAGGCCACTCTTACGTCTCT
ACTB	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC

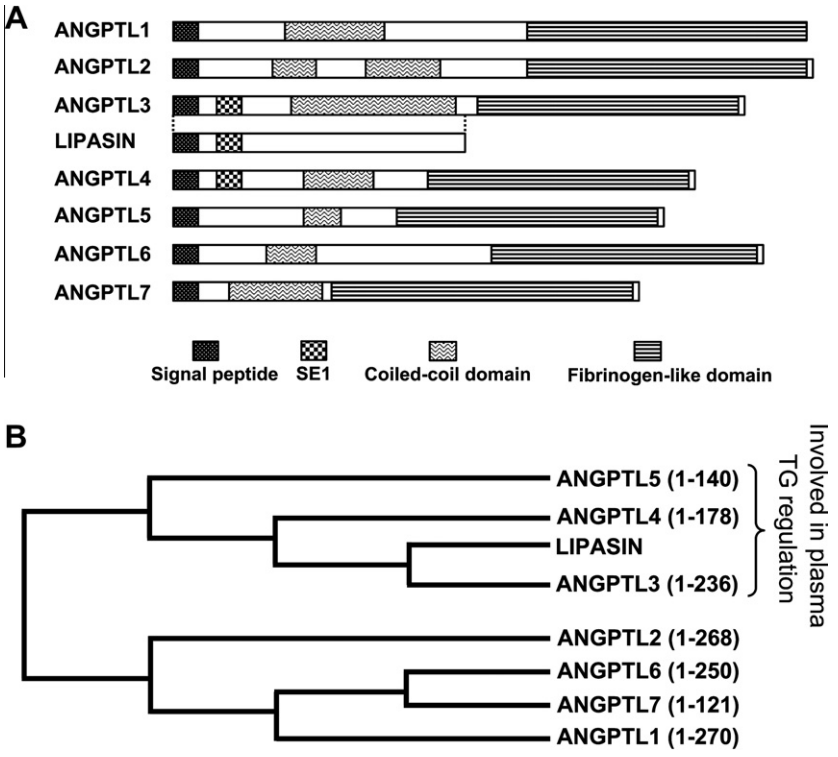

Fig. 1. Lipasin is a novel and atypical member of the angiopoietin-like protein family. (A) Protein domains of Lipasin and ANGPTLs. Figure not drawn to scale. Dotted lines denote homologous regions. SE1, a region mediating lipoprotein lipase binding. TG, triglycerides. (B) Phylogenetic tree of Lipasin and ANGPTLs without fibrinogen-like domains.

the expression of Lipasin and ANGPTLs. In both brown fat and white adipose tissue, *Lipasin*, *ANGPTL1*, *2* and *4* were abundant, while the expression of *ANGPTL3*, *6* and *7* were extremely low (Fig. 2). Two aspects for the results are noteworthy. First, *ANGPTL1* was not previously known to be expressed in BAT, in which it is in fact quite abundant. Second, although *Lipasin*, *ANGPTL 1*, *2* and *4* were expressed in both BAT and WAT, their expression in BAT were higher than in WAT, suggesting their roles in brown fat functions.

3.3. Comparison between Lipasin and ANGPTL4 expression in brown fat in response to cold exposure and nutritional stimulation

Lipasin, ANGPTL3 are ANGPTL4 are mostly related among the angiopoietin-like protein members. Because ANGPTL3 is specific to the liver, and has low expression in BAT, we compared the transcriptional regulation of *Lipasin* and *ANGPTL4*. Both *Lipasin* and *ANGPTL4* in BAT are thermoregulated (Fig. 3A). After exposed to

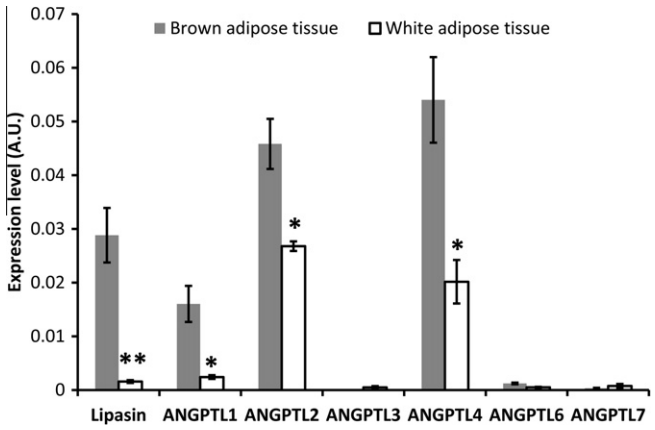

Fig. 2. Expression of *Lipasin* and *ANGPTLs* in brown and white adipose tissues. Brown and white adipose tissues were dissected from 3 male mice. $^{*}P < 0.01$; $^{**}P < 0.001$, for comparing the expression between WAT and BAT. Data are represented as mean ± SEM.

the 4 °C for 4 h, *Lipasin* in BAT was increased for more than 3-fold ($P < 0.01$). In contrast, cold exposure suppressed *ANGPTL4* expression for about 60% ($P < 0.01$). We also compared the transcriptional change of these 2 genes in response to nutritional regulation. HFD treatment of mice for 3 months increased *Lipasin* in BAT for more than 2-fold ($P < 0.05$) (Fig. 3B). No significant difference was observed for *ANGPTL4* expression following HFD treatment. We also fasted mice for 24 h and examine *Lipasin* and *ANGPTL4* expression, which showed an opposite change. Fasting grammatically suppressed *Lipasin* ($P \ll 0.01$), while increased *ANGPTL4* ($P < 0.01$) (Fig. 3C). Therefore, for cold exposure and fasting, expression of *Lipasin* and *ANGPTL4* showed opposite changes.

3.4. Expression of ANGPTL2 in brown fat in response to cold exposure and nutritional stimulation

ANGPTL2 has been linked to diabetes and obesity, and its function in white adipose tissue has been characterized [21]. However, *ANGPTL2* transcriptional regulation in BAT has not been studied. We therefore examined *ANGPTL2* expression in BAT following cold exposure and nutritional stimulation. Treating mice with 4 °C for 4 h suppressed *ANGPTL2* expression about 60% ($P < 0.01$) (Fig. 3A). HFD treatment of mice for 3 months also significant suppressed *ANGPTL2* expression and fasting suppressed *ANGPTL2* expression for about 60% ($P < 0.01$) (Fig. 3B and C). Therefore, *ANGPTL2* expression in BAT is both thermoregulated and nutritionally regulated.

Thermogenesis is the primary function of brown fat and we therefore examined the thermoregulation of *Lipasin, ANGPTL2* and *4* in brown fat of mice treated with cold exposure, which indeed significantly changed the expression of these genes, suggesting that Lipasin, ANGPTL2 and ANGPTL4 play a role in the thermogenesis of brown fat. Phylogenetic analysis of protein sequences of Lipasin and 7 ANGPTLs that lack the fibrinogen-like domains

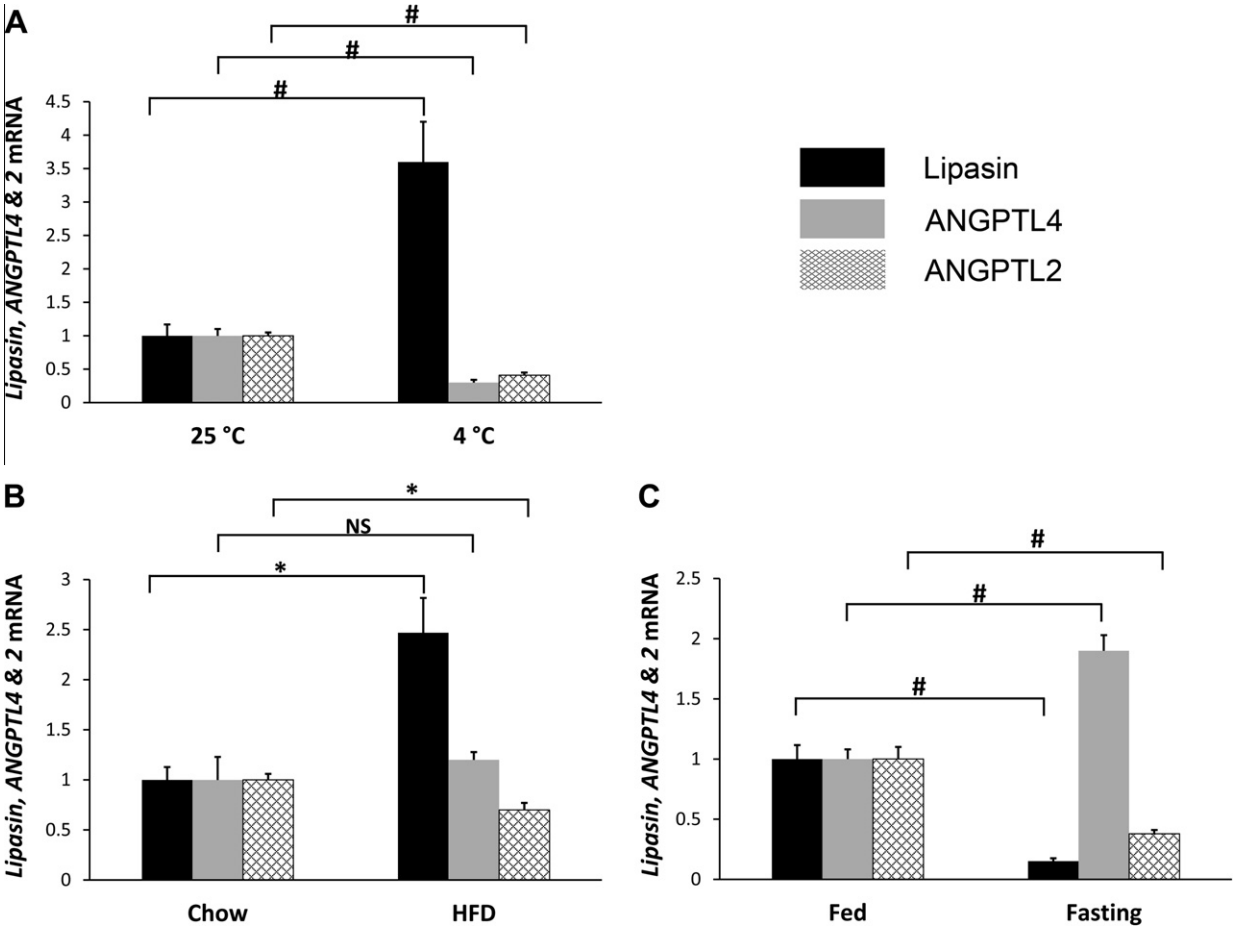

Fig. 3. Comparison of transcriptional regulation among *Lipasin*, *ANGPTL4* and *ANGPTL2* in brown fat. (A) Cold exposure (4 °C for 4 h) increases *Lipasin*, while decreases *ANGPTL4* and *ANGPTL2*. (B) HFD treatment increases *Lipasin*, but suppresses *ANGPTL2*. (C) Fasting for 24 h decreases *Lipasin* while increases *ANGPTL4*. Data are represented as mean ± SEM. $^{*}P < 0.05$; $^{\#}P < 0.01$.

showed that the 8 proteins can be classified into 2 branches, one containing Lipasin, ANGPTL3, 4 and 5, and the other one containing ANGPTL1, 2, 6 and 7. Interestingly, all members in the former branch, i.e., Lipasin, ANGPTL3, 4 and 5, are involved in human plasma triglyceride regulation, suggesting that this classification is consistent with their biological functions.

Nevertheless, it should be noted that there are many characteristics that are distinct between Lipasin and other ANGPTLs. All ANGPTLs have fibrinogen-like domains and coiled-coil domains, which are not present in Lipasin. All ANGPTLs also have numerous glycosylation sites and amino acids for forming disulfide bonds, all of which do not seem to be present in Lipasin also(Table 2). In should be emphasized that the phylogenetic analysis was not perform using full length ANGPTL sequences. It was shown that the fibrinogen-like domain and the coiled-coil domain of ANGPTL3 are mainly involved in angiogenesis and lipid regulation, respectively [30]. Therefore this classification likely reflects the functionality of ANGPTLs in regulating blood lipids. In terms of similarities, Lipasin shares homolog with ANGPTL3's and ANGPTL4's N-terminal regions that mediate LPL binding, and all the 3 genes are involved in plasma triglyceride regulation. Therefore, these characters appear to support a notion that Lipasin is a novel, but atypical member, in the ANGPTL protein family.

Lipasin is most closely related to ANGPTL 3 and 4, and it is therefore interesting to compare and contrast the 3 genes. Both ANGPTL3 and 4 need to be cleaved to remove the fibrinogen-like domain to become active, while Lipasin does not need to be cleaved. Another striking difference is the nutritional regulation. ANGPTL4 [20,34–36], but not ANGPTL3, is sensitive to nutritional regulation, and Lipasin and ANGPTL4 appear to have opposite direction of change in terms of various stimuli. In brown fat, for instance, fasting suppresses *Lipasin* while induces *ANGPTL4*, and cold exposure induces *Lipasin* while suppresses *ANGPTL4*.

In summary, we show that Lipasin is most closely related to ANGPTL3 and 4 in terms of lipid regulation functionalities. Expressions of *Lipasin* and *ANGPTL4* are thermoregulated in brown fat, and show opposite direction of change by various stimuli, including nutritionally regulation. These results support a notion that Lipasin is a novel but atypical member of the angiopoietin protein family. Expressions of Lipasin, ANGPTL3 and ANGPTL4 need to be orchestrated to regulate blood lipid profiles at different nutritional states.

4. Note added in proof

While the manuscript was in press, we noticed 2 very recent publications on Lipasin, which was referred to as RIFL (AJP Endo, 2012, 303:E334-51) and ANGPTL8 (PNAS, 2012, 109:19751–6).

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