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Original Article

Visualizing the regulatory role of Angiopoietin-like protein 8 (ANGPTL8) in glucose and lipid metabolic pathways

Amnah Siddiqa[a,b], Elisa Cirillo[b], Samar H.K. Tareen[c], Amjad Ali[d], Martina Kutmon[b,c], Lars M.T. Eijssen[b], Jamil Ahmad[a,*], Chris T. Evelo[b,c], Susan L. Coort[b]

[a] Research Centre for Modeling and Simulation - RCMS, National University of Sciences and Technology, Pakistan
[b] Department of Bioinformatics - BiGCaT, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, The Netherlands
[c] Maastricht Centre for Systems Biology (MaCSBio), Maastricht University, The Netherlands
[d] Atta-ur-Rahman School of Applied Biosciences - ASAB, National University of Sciences and Technology, Pakistan

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ABSTRACT

ANGPTL8 (Angiopoietin-like protein 8) is a newly identified hormone emerging as a novel drug target for treatment of diabetes mellitus and dyslipidemia due to its unique metabolic nature. With increasing number of studies targeting the regulation of ANGPTL8, integration of their findings becomes indispensable. This study has been conducted with the aim to collect, analyze, integrate and visualize the available knowledge in the literature about ANGPTL8 and its regulation. We utilized this knowledge to construct a regulatory pathway of ANGPTL8 which is available at WikiPathways, an open source pathways database. It allows us to visualize ANGPTL8's regulation with respect to other genes/proteins in different pathways helping us to understand the complex interplay of novel hormones/genes/proteins in metabolic disorders. To the best of our knowledge, this is the first attempt to present an integrated pathway view of ANGPTL8's regulation and its associated pathways and is important resource for future omics-based studies.

1. Introduction

Metabolic syndrome (MetS) and diabetes mellitus (DM) are two major health concerns worldwide and their prevalence is predicted to increase significantly by the year 2030, due to global obesity epidemic [1–3]. MetS is characterized by a multiplex of clinical traits including obesity, dyslipidemia, insulin resistance, hypertension and hyperglycemia. On the other hand, DM is characterized by hyperinsulinemia and hyperglycemia often coupled with diabetic dyslipidemia. Insulin resistance and dyslipidemia are among common risk factors for developing DM, MetS and many other related disorders. These metabolic disorders share related pathological origins embedded in dysfunctional lipid and carbohydrate metabolism. Identification of the key molecular players in these pathological conditions is required in order to increase our systems wide understanding and to find better drug targets for their treatment.

In one such effort, ANGPTL8 (Angiopoietin-like protein 8) was identified as a novel hormone with unique metabolic nature through experiments performed in a collaborative effort of Genetech and Lexicon pharmaceuticals. The investigators performed a phenotypic screening of 472 secreted and membrane proteins by creating a gene knockout library of murine models in order to study their potential as a drug target [4]. In this study, ANGPTL8 was recognized as one of the genes associated to the metabolic phenotypic trait. Their data demonstrated that ANGPTL8 gene knockout mice model was associated with decreased serum triglyceride levels as compared to wild-type littermates. Further studies revealed its function in maintaining lipid homeostasis and is now characterized to be involved in both glucose and lipid metabolism. It was designated different names such as lipasin [5,6], RIFL (refeeding induced in fat and liver) [7] and betatrophin [8] in the subsequent attempts to characterize its function. The name lipasin refers to one of its functions which is to inhibit an enzyme LPL (lipoprotein lipase). LPL is a hydrolytic enzyme which is involved in trafficking of lipoproteins and their subsequent clearance [5,6]. The name RIFL refers to its expression profile because it was induced upon refeeding after a certain period of fast, predominantly in the liver and adipose tissue of the mice and humans [7]. The name betatrophin refers to its functional ability to induce the pancreatic beta cell proliferation demonstrated by Yi et al. [8]. However, this role of ANGPTL8 has remained controversial and is still being debated.

* Corresponding author at: Research Centre for Modeling and Simulation - RCMS, National University of Sciences and Technology, Sector H-12, Islamabad, Pakistan.
E-mail address: jamil.ahmad@rcms.nust.edu.pk (J. Ahmad).

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Increased serum ANGPTL8 has also been observed in several diseases including DM, MetS and NAFLD (non-alcoholic fatty liver disease) [9–12]. In this regard, inhibition of ANGPTL8 for reducing the serum triglyceride levels and its overexpression for improving glucose tolerance is being anticipated as a therapeutic strategy to lower the TG (triglycerides) levels in dyslipidemia and as a glucose lowering drug in DM, respectively [5,13-15]. However, knowledge of its physiological mechanism of action, its gene regulation and its functional characterization are prerequisites in order to exploit the full potential of ANGPTL8 in future treatment regimens for DM and/or dyslipidemia.

Different studies have investigated the molecular targets, regulators and associated signaling pathways of ANGPTL8 to decipher its physiological role. These studies partly elucidated its mechanism of action and suggested that it plays a critical role in the metabolic pathways maintaining whole-body homeostasis of glucose and fat metabolism [8,13,14,16]. It is regulated by various factors including feeding/fasting, caloric intake, thyroid hormone, insulin, glucose, irisin, insulin receptor antagonists (S961, OSI-906), SREBP (sterol regulatory element-binding protein) isoforms, LXR agonists, ChREBP (carbohydrate-responsive element-binding protein), AMPK (5′ adenosine monophosphate-activated protein kinase) and MAPK (mitogen-activated protein kinase) signaling pathway [5,7-9,16-21]. There is a consensus from observations that ANGPTL8 is a downstream signaling entity in the insulin signaling pathway [7,8,13,22]. Its activation in the presence of both glucose and insulin is suggestive of its regulation through a crosstalk mechanism mediated by glucose and insulin regulated transcription factors.

Different pathway databases such as WikiPathways [23], Reactome [24] and KEGG (Kyoto Encyclopedia of Genes and Genomes) [25] maintain the regulatory information of genes in the form of pathway diagrams. These pathway diagrams are used to represent the regulatory information of the genes, proteins, and metabolites in the order of their activation/inhibition and signaling within the cells in response to various stimuli. These pathway databases are an integral source of current analytical workflow of different genomics based data analysis [26]. Updating these pathway data repositories with new information is an essential part of omics-data based studies.

At present none of the major pathway databases including WikiPathways, Reactome and KEGG holds any interaction data regarding ANGPTL8. Therefore, the current study has been conducted with the aim to collect, analyze, integrate and visualize all the available knowledge about ANGPTL8 and its regulation from the literature. We present an updated summary of its gene structure, protein structure, regulatory roles, and regulators. We also present a literature curated pathway of ANGPTL8 regulation as a research resource which is available online through WikiPathways (Pathway Id: WP3915). The ANGPTL8 regulatory pathway was constructed using PathVisio which is a software for pathway construction and analysis [27,28]. This pathway captures all the regulatory interactions of ANGPTL8 in associated signaling pathways described in literature so far. Thus, to the best of our knowledge, this pathway presents the first instance of integrating interaction data of ANGPTL8. This pathway could aid future analytical studies using omics approaches to elucidate its precise physiological mechanism of action and unidentified interaction partners [29].

2. Methodology

The steps followed to collect, analyze, integrate and visualize knowledge related to ANGPTL8 are illustrated in Fig. 1. A systematic literature search was performed, in which research papers between the years 2012 and 2016 were obtained in order to collect information about the gene and protein structure and regulatory interactions of ANGPTL8. The keywords “ANGPTL8”, “Angiopoitein-like protein 8”, “C19orf80”, “chromosome 19 open reading frame”, “betatrophin”, “lipasin” and “RIFL” were used to select the relevant studies in Google scholar and PubMed. The selected time filter is based on the fact that functional characterization of ANGPTL8 started in the year 2012. One hundred fifty three unique studies were filtered out (Supplementary file 1) and selected in this step. However, the literature references with redundant and/or irrelevant information (as per criteria discussed above) were not cited but can be viewed in Supplementary file 1. In addition, the presence of ANGPTL8 in pathways was investigated by searching pathways databases including WikiPathways, KEGG, and Reactome [23–25]. The results of the literature search and pathways database mining were used to design the ANGPTL8 regulatory pathway. Importantly only those interactions were selected which must have been reported for liver besides other tissues/organs. The selected interactions should be experimentally validated in at least one study while taking into account only human, mouse and rat studies. The specie selection criterion is based on the following facts:

- Rodent/mice models are the most extensively and reliably used animal models to study the respective human mechanisms due to a lot of factors especially their genome similarity with humans (which is 99% [30]). However, the discussion of their potential usage and/or limitations is out of scope of this study and can be followed in [30–34].
- ANGPTL8 gene is an evolutionarily conserved gene among mammals which is indicative of its class specific conserved function. It has been reported to share 73% and 69% protein sequence similarity with its closest homologs i.e. *Mus musculus* and *Rattus norvegicus*, respectively [7,15].
- Most of the regulatory (interaction) data mined from literature has already been verified for both human and mouse/rat and is discussed in detail in the Results and discussion section.

PathVisio, a pathway editor and analysis tool, was used for the construction of ANGPTL8 regulatory pathway [27,28]. It allows the import and export of pathway files in multiple formats recognized by different pathway/network analysis softwares. It also allows linking the entities (gene, proteins or metabolites) and interactions with respective public databases. The origin of each interaction was annotated in the pathway using either pathway or literature database identifiers. This allows the users of the pathways to trace back the source data related to each interaction. Besides, the interaction present in different species are illustrated with different arrow types (illustrated in the legend of the pathway). Each entity (representing either gene, protein or metabolite) was also linked to the respective (gene, protein and metabolite) identifier in one of the public gene, protein or metabolite databases. The pathway layout was optimized after discussion among curators whereafter it was then deposited in the WikiPathways with pathway id: WP3915. The WikiPathways is an open-source pathway database maintaining pathways as dynamic models which contains links to other resources [23]. It also allows maintaining submitted pathways through updates, research community feedback and corrections while preserving previous versions.

3. Results and discussion

An extensive summary of ANGPTL8 gene structure, protein structure, regulatory roles and regulators is presented as an update. Furthermore, the ANGPTL8 regulatory pathway is described, discussing its sequence of signaling steps as captured in the pathway (WP3915) submitted in WikiPathways (Fig. 3).

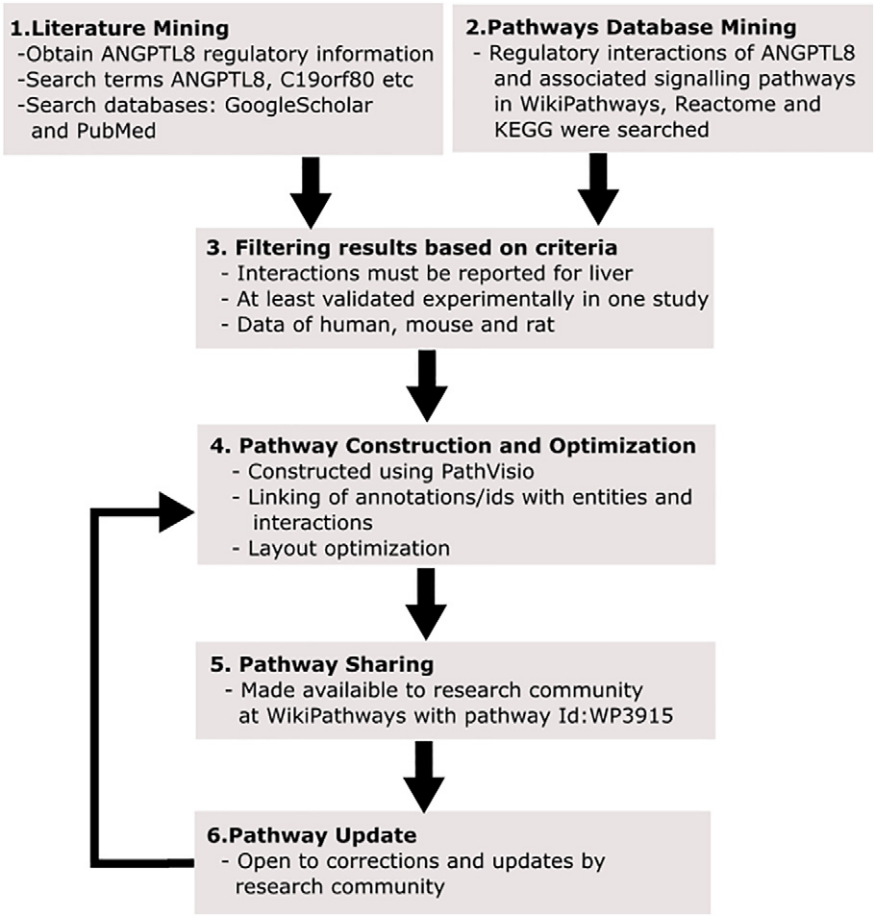

Fig. 1. Methodology workflow: Literature and pathways database mining was performed to search all the available information on ANGPTL8. Selected interactions from this information were then used to create the ANGPTL8 regulatory pathway using PathVisio. The pathway was then made available to the research community at WikiPathways (Pathway Id: WP3915). The pathway is open for feedback, corrections and updates by the research community.

3.1. ANGPTL8 gene

The ANGPTL8 gene is present in the intron of the Dedicator of Cytokinesis 6 (DOCK6) gene on chromosome 19 in humans (http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000130173) and on chromosome 9 in mouse (http://www.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000047822) It has been reported as a paralog of ANGPTL3 gene arisen due to a gene duplication event where ANGPTL3 itself is present in the intron of DOCK7 gene [17]. Quagliarini et al. [17] identified four exons in the human gene [17] and so far four transcripts of ANGPTL8 have been reported in Ensembl databank (http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000130173). The two full length transcripts encode 198 amino acids long proteins whereas the two other isoforms encode shorter forms of the protein containing 99 and 58 amino acids long respectively (Fig. 2 (A)). The shorter isoforms, lack the N-terminal amino acid residues and have been reported as a possible cause of discrepancies in the measurement of full-length ANGPTL8 transcripts in different studies in which ELISA kits based on C-terminal antibodies were used (which would measure both full-length and C-terminal fragments) [35]. Tseng et al. reported the presence of several thyroid hormone response elements (TREs) in the upstream region of ANGPTL8 gene [16]. Thyroid hormone receptors alpha (THR-α) and beta (THR-β) belong to the class of nuclear receptors which mediate the transcription of their respective target genes by binding to these TREs. Fu et al. reported the presence of a ChRE (carbohydrate response element) in the promoter region of ANGPTL8 gene which is regulated by ChREBP (carbohydrate response element binding protein) [9].

Several non-synonymous SNPs (single-nucleotide polymorphisms) exist in the ANGPTL8 gene (http://www.ensembl.org/Homo_sapiens/Gene/Variation_Gene/Table?db=core;g=ENSG00000130173). However, only two functional variant SNPs associated with ANGPTL8 have been published including rs2278426 (http://www.ensembl.org/Homo_sapiens/Variation/Explore?db=core;g=ENSG00000130173;v=rs2278426) and rs145464906 (http://www.ensembl.org/Homo_sapiens/Variation/Mappings?db=core;g=ENSG00000130173;v=rs145464906). The SNP rs2278426 corresponds to an alteration of an arginine reside to tryptophan at position 59 and its functional variant has been associated with lower HDL (high density lipoprotein) and LDL (low density lipoprotein) cholesterol plasma levels and higher fasting plasma glucose in people with different ethnicities [17,22,36-39]. The SNP rs145464906 corresponds to an alteration of a glutamine amino acid residue to stop codon at amino acid position 121 and results in a truncated form of protein [40].

3.2. Protein

ANGPTL8 belongs to ANGPTL family which consists of eight members (ANGPTL1 to ANGPTL8). ANGPTL1-ANGPTL7 share a very similar structure consisting of an amino terminal domain (coiled-coil) and a carboxy terminal (fibrinogen-like) domain attached

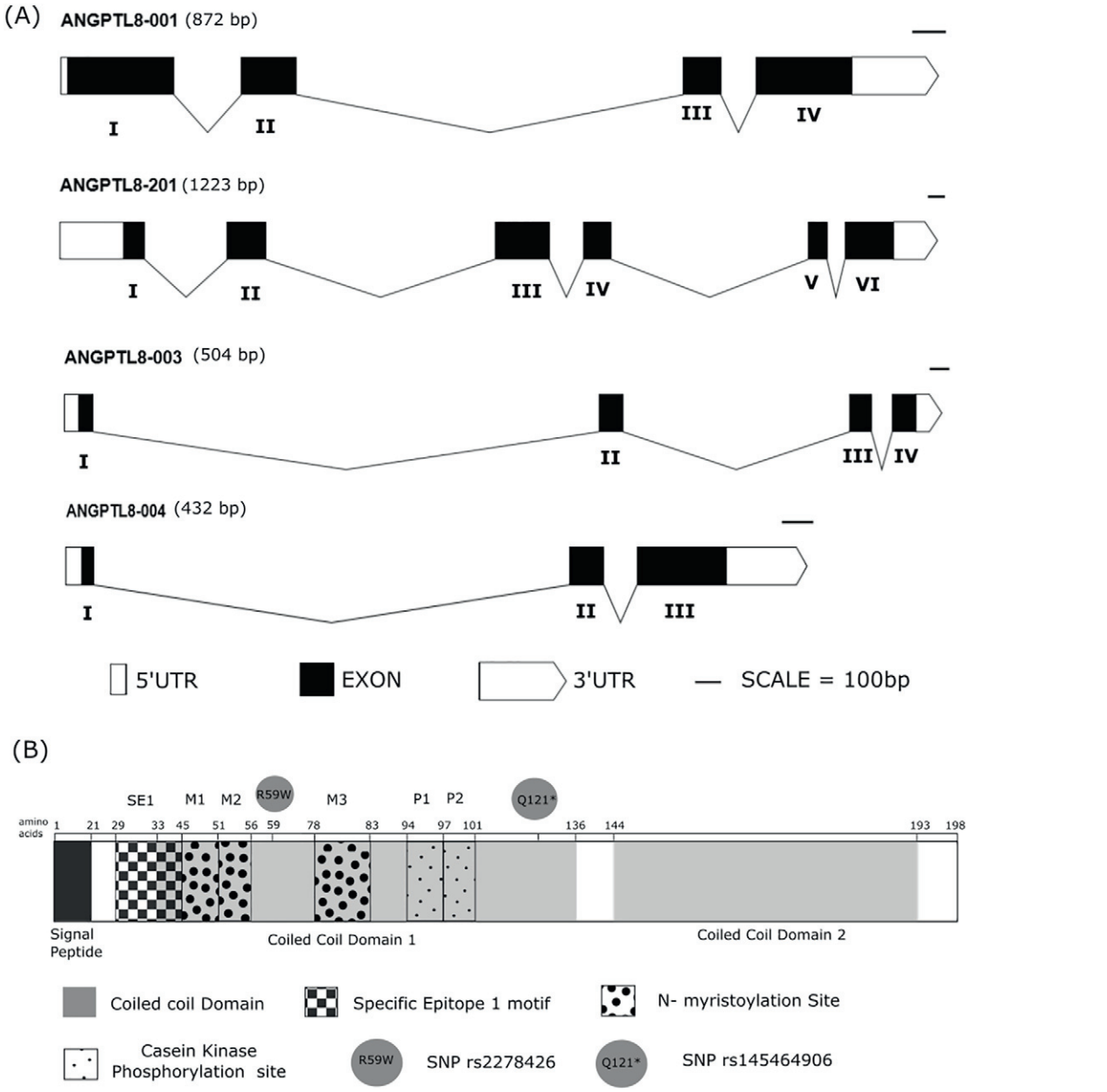

Fig. 2. (A) Gene structure of four transcripts (http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000130173) of the human ANGPTL8 gene (created using Exon-Intron Graphic Maker (http://wormweb.org/exonintron)). ANGPTL8-001, and ANGPTL8-201 transcribe protein sequences of 198 amino acids whereas ANGPTL8-003 and ANGPTL8-004 transcribe protein sequences of 99 and 58 amino acid length, respectively. (B) Protein structure of ANGPTL8. ANGPTL8 is 198 amino acids long with a predicted signal peptide, two coiled coil domains and several protein modifications. It also contains a SE1 motif which is a conserved sequence motif between ANGPTL 3, 4 and 8 and is required for inhibition of LPL.

with a linker region. ANGPTL8 has a different structure from all other protein family members as it possesses only the coiled-coil domain [17,41]. All members of ANGPTL protein family seems to serve multiple biological roles in maintaining different aspects of metabolism, angiogenesis and hematopoiesis (more details are reviewed in [41]). ANGPTLs were recognized as orphan ligands due to lack of binding with the TIE1 (tyrosine kinase with immunoglobulin-like and EGF-like domains 1) receptors unlike Angiopoietins protein family. However, ANGPTL2, ANGPTL5 and ANGPTL7 have been now reported to interact with LILRB2 (leukocyte immunoglobulin-like receptor B2) and PIRB (immunoglobulin-like) receptors present on hematopoietic stem cells (HSCs) [42]. The ability of ANGPTL8 to stimulate CD45+ hematopoietic-derived cell differentiation raises a possibility that it might also interact with the same receptors to perform this function [43].

Protein sequence analysis of ANGPTL8 showed sequence conservation across mammals indicating its class specific functional importance [8,44]. Furthermore, the protein sequence of ANGPTL8 between mouse and humans share 73% identity suggesting evolutionary conservation [5]. Phylogenetic analysis based on protein sequence alignments revealed close association of ANGPTL8 with ANGPTL3 and ANGPTL4 [5]. It shares 50% amino acid similarity with N-terminal domain of ANGPTL3 and significant sequence similarity with a stretch of 100 amino acids of ANGPTL4. ANGPTL8 also has been demonstrated to possess the conserved SE1 (Specific Epitope 1) motif which is necessary and sufficient for inhibition of LPL by

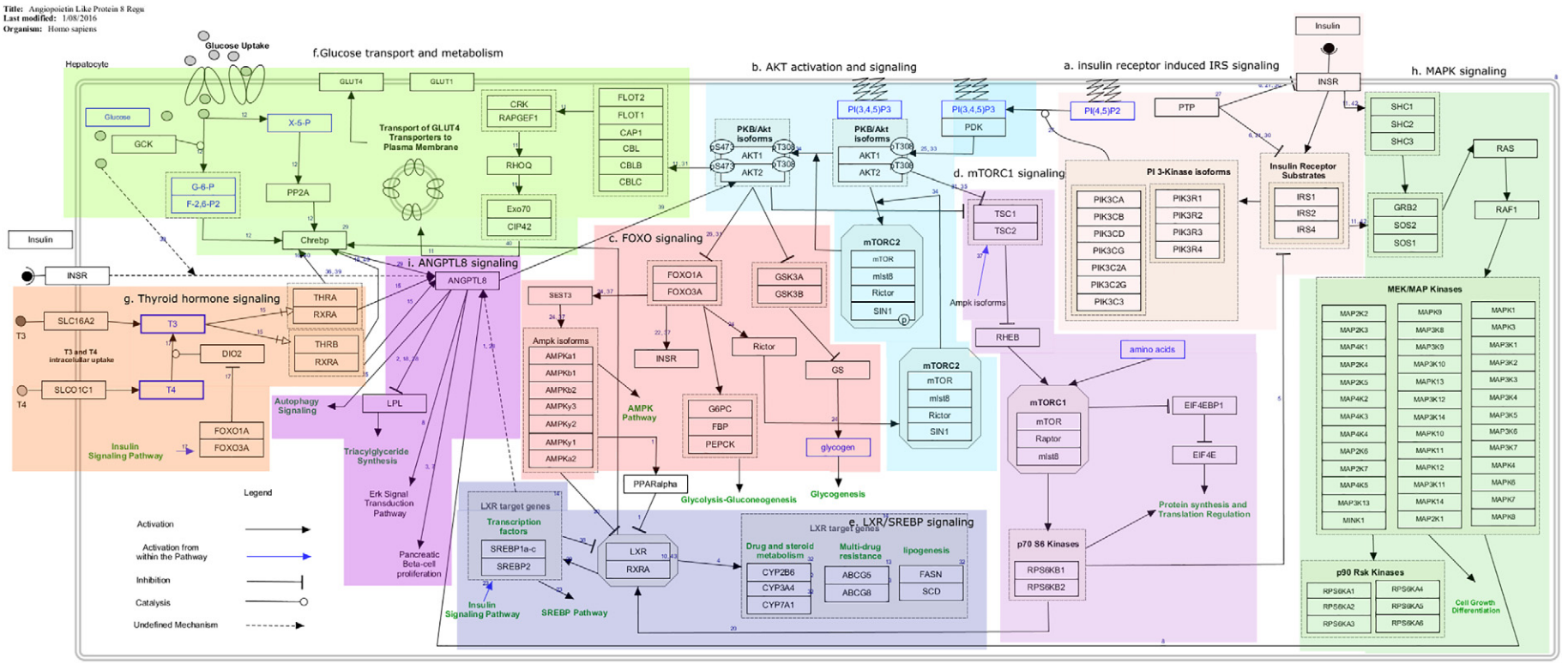

Fig. 3. The ANGPTL8 regulatory pathway (pathway id: WP3915 (http://www.wikipathways.org/index.php/Pathway:WP3915)). a) Insulin receptor induced IS signaling events; b) AKT activation and signaling; c) FOXO signaling; d) mTORC1 signaling; e) LXR/SREBP signaling; f) glucose transport and metabolic signaling; g) thyroid hormone signaling; h) MAPK signaling; i) ANGPTL8 signaling.

ANGPTL3 and ANGPTL4 demonstrated through both *in vivo* and *in vitro* experiments [45,46].

ANGPTL8 protein sequence encompasses a signal peptide (1–21 a.a) at the N-terminal region indicative of its secretory nature [8,44]. Two coiled-coil domains are also predicted to be present in the protein structure [8,45]. Several N-myristoylation sites and casein kinase phosphorylation sites on the N-terminal of ANGPTL8 are predicted in [15] (Fig. 2 (B)). These protein modifications are indicative of its membrane anchoring ability and rapid signal response, respectively.

3.3. Tissue expression and cellular localization

The metabolic tissues restricted expression profile of ANGPTL8 is suggestive of its essential role in metabolism. The tissue expression profile of ANGPTL8 demonstrated by R. Zhang showed highest expression in the liver, BAT (brown adipose tissue) and subcutaneous fat of mice out of twenty tissues tested whereas in humans only liver showed predominant expression out of forty-eight tissues tested [5]. Further experiments from this group revealed its thermoregulated nature by investigating its link with BAT [6]. Their data revealed an increase in expression of ANGPTL8 at low temperature in BAT of mice. Ren et al. demonstrated a similar expression profile for mice whereas in humans it was considerably expressed in both liver and WAT (white adipose tissue) out of the four tissues tested [7]. Data from the Quagliarini et al. shows the highest expression of ANGPTL8 in liver along with comparable expression profile in brain and WAT out of seventeen tissues tested [17]. The expression profiles evaluated using Human Protein Atlas (available from http://www.proteinatlas.org/ENSG00000130173-C19orf80/tissue) shows the protein expression of ANGPTL8 to be enriched in liver and adipose tissue whereas kidney and breast tissue also show certain expression levels (Supplementary Fig. S1) [47].

Under basal physiological conditions, ANGPTL8 expression has been demonstrated to be induced upon re-feeding and reduced upon fasting. Data of Ren et al. shows approximately 80 and 12 fold increase of the expression in WAT and liver upon re-feeding experiments conducted on fasted mice [7]. This was confirmed in several transcriptomics studies (both human and mice) in which nutritional status such as re-feeding after 6/12 h fast and re-feeding after a period of low-calorie diet was investigated [7]. Several mouse models such as that of obesity (ob/ob), diabetes (db/db), high fat fed (HFD), gestational mice and the insulin resistance mouse model treated with the insulin receptor antagonist S961 have also shown elevated expression of ANGPTL8 [5,8]. ANGPTL8 is a secretory protein as assessed due to its presence in human and mice serum along with the existence of a predicted signal peptide in its N-terminal region [8]. However, cytoplasmic ANGPTL8 has been demonstrated to exist as vesicle-like structures of various sizes around and within the lipid droplets and endoplasmic reticulum associated compartments [16]. The golgi apparatus and nucleoli are assessed as the sub-cellular locations of ANGPTL8 by using Human Protein Atlas (available from http://www.proteinatlas.org/ENSG00000130173-C19orf80/subcellular) [47].

3.4. Role in lipid metabolism

ANGPTL8 has been characterized as an important regulator of lipid metabolism but the exact mechanisms of actions still need to be delineated. Serum triglycerides level were increased in ANGPTL8 over-expressed mice models and were decreased in ANGPTL8 knock-out mice models [5,7,17,48]. Further investigations revealed that it plays a role in substrate partitioning through inhibition of LPL during fasting-feeding metabolic transition [5,48]. LPL is a rate-limiting enzyme involved in hydrolysis of dietary lipids and their subsequent uptake in peripheral tissues. The mechanism of action behind increased serum triglyceride levels in the presence of ANGPTL8 is explained by its ability to inhibit LPL and subsequently reduce the serum triglycerides clearance [5]. Wang et al. further clarified the role of ANGPTL8 in reducing the postprandial LPL activity in oxidative tissues (heart and skeletal muscles) in order to direct the serum triglycerides for uptake by adipose tissues in energy favoring conditions [48]. On the other hand, fasting increases the LPL activity in oxidative tissues due to down-regulation of ANGPTL8 and directs the triglycerides towards them. By contrast, Ren et al. demonstrated a lipogenic role of ANGPTL8 through a series of *in-vitro* and *in-vivo* experiments [7]. Their data demonstrated positive correlation of ANGPTL8 expression with the process of adipogenesis in both mice and humans whereas ANGPTL8 null mice showed reduced lipid content in adipocytes during adipogenesis. This is also in correspondence with the phenotypic triglycerides levels observed in ANGPTl8 over-expressed and null mice, respectively. However, the mechanistic protein partner of ANGPTL8 in adipogenesis and their regulation is still a quest.

In an independent study, Tseng et al. demonstrated that thyroid hormone stimulates the up-regulation of ANGPTL8 in an attempt to identify the genes which are involved in autophagy and are also regulated by T3 [16]. Their data revealed another role of ANGPTL8 in lipid metabolism which is to activate the process of autophagy by inducing catabolism of lipid droplets in the liver cells. Their experiment with over-expression and gene silencing of ANGPTL8 supports its role in the maintenance of intracellular lipid homeostasis in liver. Zhang et al. demonstrated that the Ras/c-Raf/MAPK signaling pathway regulates the expression of ANGPTL8 in hepatic, fat and pancreatic beta cells [19]. It was reported as a stress response mediated protein which can reduce the expression of ATGL (adipose triglyceride lipase) by induction of early growth response transcription factor (Egr1). This study provides another connection of ANGPTL8 in maintenance of lipid homeostasis.

Different mouse models of obesity and diabetes including db/db (diabetic), ob/ob (obese), HFD (high fat fed) and insulin resistance mouse model (using insulin receptor inhibitors S961 and OSI-906) have shown the elevated expression of ANGPTL8 in the liver [5,8,20]. Besides, altered serum levels of ANGPTL8 have also been reported in obesity, DM type 2, DM type 1, MetS and NAFLD in humans [9-12,49-51]. In an independent study the positive correlation of ANGPTL8 with atherogenic lipid profile in diabetic (type II) patients was also observed [52]. Collectively, the role of ANGPTL8 in lipid metabolism and its elevated levels in associated disorders are suggestive of its therapeutic potential in reduction of hypertriglyceridemia through its inhibition. However, more studies are required in order to identify the potential interacting partners and/or regulators of ANGPTL8 in lipid metabolism and its association with other genes/proteins/pathways to further evaluate its therapeutic potential in related disorders.

3.5. Role in glucose metabolism

Several studies have reported the role of ANGPTL8 in glucose metabolism which increases its significance as possible drug target. Interestingly, its overexpression was demonstrated to improve glucose tolerance in the livers of mice [8,13]. Fu et al. demonstrated the positive correlation between ANGPTL8 expression and glucose in diabetic setting implying the necessity and possibility of glucose regulation through ANGPTL8 [9]. This study also pointed out the possibility of ANGPTL8 to be a glucose regulated gene by describing the presence of a ChREBP binding site in its promoter region. ChREBP is a glucose mediated transcription factor and plays a significant role in both glucose and lipid metabolism. In order to further elucidate its role in glucose metabolism, Guo et al. performed a series of

experiments using transgenic human liver cell lines with ANGPTL8 over expression and knockout. Their data revealed that ANGPTL8 enhances the glucose lowering effect of the insulin signaling pathway by AKT-GSK3β and/or AKT-FOXO1 branch [13]. Overall, a model of ANGPTL8 activation via the insulin signaling pathway which helps to alleviate insulin resistance through enhancing the phosphorylation of AKT, FOXO and GSK3-β is supported [13]. Their data revealed that ANGPTL8 could phosphorylate AKT at serine 473, subsequently allowing glycogen synthesis and inhibiting gluconeogenesis. Above studies implicate the beneficial role of ANGPTL8 in improving glucose tolerance in diabetic subjects. However, its drug potential in diabetes as (glucose lowering drug) cannot be fully assessed without having the comprehensive knowledge of its gene regulation and its downstream target proteins which necessitates viewing it with a systems perspective. The systems view of ANGPTL8 would allow us to see the regulation of ANGPTL8 relative to other genes/proteins present in different pathways. It would subsequently make it easier to assess its pathogenic role by allowing us to visualize the regulatory mechanism of ANGPTL8 with respect to other genes/proteins involved in the different diseases.

3.6. Role in pancreatic beta cell proliferation

Pancreatic beta cell repopulation is one of the anticipated treatments for both diabetes type 1 and type 2 patients. In this regard, ANGPTL8 emerged as one of the possible drug targets due to its high pancreatic beta cell proliferative specificity and significant magnitude of proliferation [8]. Yi et al. demonstrated the over expression of ANGPTL8 in all of the typical models of compensatory pancreatic beta cell proliferation such as ob/ob (obesity), db/db (diabetes type II), and gestational mice along with a new insulin resistance mouse model (created by induction of insulin receptor antagonist S961). However, the expected potential of ANGPTL8 could not be successfully confirmed when applied on human islets in the transplant setting [53]. Later on, Gusarova et al. demonstrated the inability of ANGPTL8 as a stimulator of pancreatic beta cell proliferation by conducting knockdown experiments in the insulin resistance (S961 induced) mouse model [54]. Since then, the role of ANGPTL8 in pancreatic beta cell proliferation has been heavily debated. There are several studies which still support the role of ANGPTL8 in pancreatic beta cell proliferation (such as [55]) and others contradict it (such as [43]). The study by Chen et al. reported that injecting the human ANGPTL8 directly into the islets of mice increases replication of pancreatic beta cells [55]. Their data supports the role of ANGPTL8 in diabetic setting for both type 1 and type 2 patients since STZ (Streptozotocin) induced mice showed alleviation if not complete reversal of the disease. However, Cox et al. demonstrated the anti-proliferative role of ANGPTL8 along with explaining the basis of associated discrepancies [43]. Their study revealed that in mice intravenous injection of ANGPTL8 stimulated the CD45+ hematopoietic-derived cell proliferation, instead of pancreatic beta cells. They also explained that different quantification methods were used for measuring the pancreatic beta cells in previous studies investigating the role of ANGPTL8 in pancreatic beta cell proliferation which was one reason for resultant discrepancies previously. Overall, a systems view of ANGPTL8 gene regulation can clarify the contrasting results of different studies.

3.7. Regulators of ANGPTL8

A set of positive and negative regulators of ANGPTL8 have been demonstrated in different studies (Table 1). This overview is manually curated exhaustively for all of the regulators playing a role in hepatic expression of ANGPTL8 which is later used for making the interactive pathway in this study.

The expression of ANGPTL8 is nutritionally regulated in humans and mice. Refeeding increases the expression of ANGPTL8 while fasting suppresses its activation [5,7]. Metabolically active organs including liver WAT, and BAT are the predominant key expression sites in both humans and mice [5,7,17]. Besides, high fat diet induced obese mouse model and obesity in humans have also been characterized to up-regulate its expression [7,9]. The overall expression profile of ANGPTL8 has been positively associated with processes promoting energy storage processes such as regulating postprandial lipid traffic, adipogenesis, glycogen synthesis and negatively associated with processes promoting energy consumption such as gluconeogenesis [5,7,8,13,16,17]. Insulin is a key metabolic enzyme coordinating the nutritional signals to regulate these processes in liver and has also been demonstrated to increase the expression of ANGPTL8 in human liver cell lines in the presence of glucose [13]. Ren et al. also reported through their *in-vivo* and *in-vitro* experiments that insulin in the presence of glucose up-regulates its expression in human and mice fat cells [7]. Activation of ANGPTL8 specifically in the presence of both insulin and glucose is indicative of a crosstalk mechanism between insulin and glucose stimulated processes to regulate its expression. Several downstream regulators of insulin and glucose discussed below might provide clues to such a mechanism.

Carbohydrate response element-binding protein (ChREBP) is a glucose stimulated transcription factor involved in energy homeostasis. It binds to specific sequences on the promoter region of gene known as carbohydrate responsive elements (ChRE). The data of Fu et al. reveals the presence of ChRE on the ANGPTL8 promoter [9]. They also pointed out towards another study where the binding of ChREBP to the ANGPTL8 promoter in HepG2 cells was demonstrated [56]. However, further studies are required to verify this interaction for activation of ANGPTL8. Sterol regulatory element-binding proteins (SREBPs) are important integrators of nutritional and energy availability with cholesterol and fatty acid metabolism. SREBP-1a and SREBP-2 were demonstrated to modulate the expression of ANGPTL8 in livers of mice [17] whereas SREBP-1c was demonstrated to activate ANGPTL8 in human liver cell lines [18]. Liver X receptors (LXRs) are important regulators of cholesterol, fatty acid, and glucose homeostasis. LXR agonists have been reported to up-regulate the expression of ANGPTL8 in both mouse and human livers in separate studies [17,18]. Oxysterols (cholesterol metabolites) are the ligands of LXRs which belong to a class of nuclear receptors and make heterodimers with RXR to regulate gene transcription. LXR is itself under regulation of the insulin signaling pathway and indirectly regulated by glucose as well [57]. LXR is also involved in crosstalk with other nuclear receptors including thyroid hormone receptor [58]. Thyroid hormone through induction of thyroid hormone receptors was demonstrated to up-regulate the expression of ANGPTL8 in human liver cell lines [16]. It is of note that intracellular thyroid hormone activation is itself under control of nutritional stimulus dependent processes mediated by insulin signaling reported in [59]. Zhang et al. reported an activation mechanism for ANGPTL8 via the RAS-MAPK pathway upon amino acid deprivation mediated stress [19]. Their data indicates that ANGPTL8 upon activation through RAS/MAPK induces ERK expression which suppresses ATGL and plays a role in lipid metabolism. The experiments of the Melton group reported on a mouse model where S961 (an antagonist of the insulin receptor) induced expression of ANGPTL8 up to 4 fold in the liver of mice [8]. Kaestner et al. also found a similar increase up to 5 fold in the liver of mice on treatment with S961 [53]. S961 induces phenotype of insulin resistance causing hyperinsulinemia and hyperglycemia in reported mouse model. Thus S961 induced ANGPTL8 could be linked to the earlier discussion on existence of a crosstalk mechanism between insulin and glucose mediated metabolic processes for its regulation. The negative regulators of the ANGPTL8 include fasting and AMPK in liver and fasting, TNF-alpha and forskolin in adipose tissue so far [5-7,17,18]. AMPK was demonstrated to induce the suppression of ANGPTL8 through activation of PPAR-alpha [18]. This interaction

Table 1
Literature curated list of positive and negative regulators of ANGPTL8 in liver. ψ = stimulation; λ = inhibition; NE = no effect; NR = regulator not yet reported for liver.

S. no.	Name	Expression in humans	Expression in mouse	References
1	Feeding	ψ	ψ	[5,7,17]
2	Thyroid hormone	ψ	NR	[16]
3	Insulin in the presence of glucose	ψ	NR	[7,13]
4	ChREBP	ψ	NR	[49,56]
5	Srebp-1a, Srebp-2	NR	ψ	[17]
6	Srebp-1c	ψ	NE	[17,18]
7	LXR agonist	ψ	ψ	[17,18]
8	Amino acids deprivation (stress)	ψ	ψ	[19]
9	S961 (insulin receptor antagonist)	NR	ψ	[8]
10	AMPK	λ	NR	[18]
11	Fasting	λ	λ	[5,7,17]

is also well connected with nutritional regulation of ANGPTL8 where fasting was shown to suppress ANGPTL8 expression.

The pathways revealed to be relevant to the expression of ANGPTL8 include insulin signaling pathway [4,31], lipid metabolism [5,7,17,48], glucose metabolism [8,9,13], autophagy in liver [16], adipocytes differentiating pathway [7] and ERK signal transduction pathway [19].

3.8. ANGPTL8 regulatory pathway

The complete ANGPTL8 regulatory pathway designed in this study is presented in Fig. 3. The ANGPTL8 regulatory pathway is available at WikiPathways (http://www.wikipathways.org/index.php/Pathway:WP3915) making it accessible worldwide. The pathway approach used for construction of the ANGPTL8 regulatory pathway has been previously used to design the complete SREBP regulation pathway in [60]. All the regulatory interactions in the ANGPTL8 regulatory pathway are annotated with respective PubMed ids of supporting studies which allows easy access to the source data. In addition, the interactions present in only mouse/rat are depicted with a dashed line. This visualization strategy aims to clarify the distinction between human and mouse/rat interactions. The impact of ANGPTL8 in lipid and glucose metabolism seems to be under the complex regulation of the proteins working downstream of the insulin signaling (IS) pathway. Therefore, the newly designed hepatic ANGPTL8 regulatory pathway includes an updated version of the IS pathway integrated with the known regulators of ANGPTL8 (Table 1). Overall, the pathway represents an up-to-date curated interactive pathway capturing the known regulatory behavior of ANGPTL8 in glucose and lipid metabolic signaling pathways. The ANGPTL8 regulatory pathway is divided into eight subsections that are highlighted with different colors in the pathway diagram illustrated in Fig. 3. The biological evidence and literature for each subsection are explained below in more detail.

The IS pathway coordinates metabolic responses to varying cellular nutrient levels. IS pathway has two major branches involved in either metabolic programming or growth/proliferation of the cells. The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT/PKB) pathway is involved in mediating metabolic effects such as uptake of glucose, protein and glycogen synthesis, lipid storage and breakdown while the mitogen-activated protein kinases (MAPK) pathway is associated with cellular growth processes such as gene expression, cell motility, cell proliferation, cell survival and differentiation. The regulators of ANGPTL8 belong to different branches of the IS pathway which explains the complex results and findings associated with ANGPTL8 in literature so far.

3.8.1. Insulin receptor mediated insulin receptor substrate signaling

Binding of insulin to the insulin receptor (IR) initiates the IS pathway. The IR belongs to the tyrosine receptor kinase family of proteins which upon ligand (insulin) binding result in autophosphorylation of various tyrosine residues in their intracellular tyrosine kinase (TK) domain. The activated (phosphorylated) receptor has a diverse set of substrates including isoforms of Shc (p46Shc, p52Shc) and Insulin Receptor Substrates (IRS1, IRS2, IRS3, IRS4) [61]. IRS1 and IRS2 are found in most of the tissues whereas the expression of IRS4 is tissue specific (brain, liver, kidney and thymus). IRS3 is only present in rodents [62]. IRS are cytoplasmic adapter proteins which are recruited to tyrosine phosphorylated IR through their PH/PTB domains and are phosphorylated at multiple tyrosine residues in C termini [62]. The phosphorylated IRS is able to act as a signaling protein with multiple downstream effectors including phosphoinositide 3-kinase (PI3K), SHP2 and GRB.

3.8.2. AKT activation and signaling

IRS phosphorylates PI3 K upon binding which is then able to catalyze the conversion of Phosphatidylinositol (4,5)-bisphosphate (PIP2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [61]. Next, PIP3 recruits phosphoinositide-dependent kinase-1 (PDK1) and its substrate kinase AKT to the plasma membrane to facilitate the proximity required for the phosphorylation event [63]. The activation of AKT requires two independent phosphorylation events for its maximal activity, i.e. at threonine 308 (T308) within the T loop of its catalytic domain and at serine 473 (S473) within the hydrophobic motif located in a C-terminal which is a non-catalytic region of the enzyme [64]. There is a broad consensus that PDK1 [65,66] and mTORC2 [67] mediate the T308 and S473 phosphorylation of AKT, respectively. However, the sequence of events in full activation of AKT was recently demonstrated and fine-tuned in the canonical IS pathway [68]. According to this model, PDK1 phosphorylates AKT at T308 in the kinase regulatory loop which in turn phosphorylates Sin1. Sin1 is an important protein member of mTORC2 complex and is required for its activation. mTORC2 upon activation mediates a positive feedback loop to phosphorylate AKT at S473. Recently, a study by Guo et al. have reported that ANGPTL8 could also promote the phosphorylation of AKT at S473 in the presence of both insulin and glucose using HepG2 cell line [13]. Their data demonstrated the respective increase of phosphorylated forms of GSK3-β and FOXO under AKT (S473) signaling and their respective regulation of glycogen synthesis and gluconeogenesis. This interaction is also supported by the experiments of Yi in which improved glucose tolerance was observed in mice injected with ANGPTL8 as compared to control-injected mice [8]. Other regulators of AKT at S473 include DNA-dependent protein kinase (DNA-PK), ILK, tumor necrosis alpha (TNF-α) and IB kinaseϵ and TANK-binding kinase 1 (IKKϵ/TBK1) through DNA damage, inflammation and cell survival pathways, respectively [69]. However, none of these regulators have been included in the ANGPTL8 regulatory pathway in order to avoid unnecessary enrichment of the pathway.

Both phosphorylations of AKT mediate functional division in the role of AKT and affects its substrate diversity [69,70]. T308 phosphorylation of AKT is sufficient without a need for S473 to activate mTORC1 [71]. On the other hand, S473 phosphorylation of AKT is reported to mainly regulate FOXO transcription factors, GSK3-beta and glucose uptake [69].

3.8.3. FOXO signaling

FOXO proteins are the regulators of insulin mediated control of gluconeogenesis and glycogenolysis through transcription of important enzymes involved in these processes including G6Pase, PEPCK and FBP [72]. Besides, they also transcribe IR, Insulin growth factor receptors (IGFR), IRS2, rictor and sestrin3 and regulate IS [69,73,74].

3.8.4. mTORC1 signaling

AKT activates mTORC1 by inactivating tuberous sclerosis protein 2 (TSC2) and thus relieving it from inhibition of RHEB. The substrates of mTORC1 include eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), and ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (S6K1) which are involved in protein synthesis and translational regulation. S6K1 is able to inhibit the IRS and forms a negative feedback control circuit on IS [75]. Besides, it also up-regulates the expression of LXR (Liver X receptor) which transcribes several genes involved in cholesterol metabolism, lipogenesis, steroid metabolism and drugs metabolism (Fig. 3). LXR also up-regulates the expression of ANGPTL8 possibly through a SREBP dependent mechanism in liver [18]. Lee et al.reported an increased expression of ANGPTL8 upon treatment with LXR agonists palmitic acid, tunicamycin and T0901317. However, this activation was reduced upon inhibiting the SREBP1 expression through siRNA transfection [18]. Its reduced levels observed upon treatment with AICAR (an activator of AMPK) are suggestive of its inhibition to be dependent on AMPK [18]. LXR is itself negatively regulated by AMPK and SREBP-1c [76,77].

3.8.5. Glucose transport and metabolism

Glucose uptake is mediated by the CBL arm of the IS pathway [78]. Intracellular glucose transport and subsequent metabolism is regulated by multiple genes which are discussed in detail in [78]. ChREBP is a glucose responsive transcription factor and is an important regulator of hepatic lipogenesis. It is post-translationaly regulated by substrates of glycolysis (glucose breakdown) through X-5-p (xylulose 5-phosphate) and G-6-p (glucose-6-phosphate) metabolic pathways [79]. X-5-p and G-6-p are phosphometabolites which tend to remove inhibitory phosphorylation of ChREBP (through activating PP2A) and increase its nuclear translocation, respectively [79,80]. The transcriptional regulation of ChREBP is mainly demonstrated to be to be maintained by LXR and THR transcription factors [80]. Up-regulation of ANGPTL8 in presence of glucose has been pointed out in several studies [8,9,13]. In this context, ANGPTL8 was demonstrated to contain a ChREBP binding site (−398 to −382) in the promoter region [9]. In an independent study (ANGPTL8 was not the focus of the study though), ChREBP was also observed to bind to the promoter region of ANGPTL8 in human liver cell line [56]. These observations support the role of ChREBP in positive regulation of ANGPTL8.

3.8.6. Thyroid hormone signaling

Tseng et al. reported that ANGPTL8 is transactivated by T3 stimulation in human liver cell lines (HepG2 and Huh7) [16]. Their data supports a model where it is transcribed through thyroid hormone receptors heterodimerized with RXRA. Thyroid hormone receptors alpha and beta belong to a class of steroid/nuclear hormone receptors super family of ligand dependent transcription factors. These receptors recognize and bind to the specific DNA elements known as thyroid hormone response Elements (TREs) and make dimers with either themselves or with RXRA. The absence of ligand allows them to recruit co-repressor and in turn repress the expression of the respective genes. Ligand binding of these receptors allows the dissociation of co-repressors and initiates the transcription. Tseng et al. also demonstrated the presence of several potential TREs in the promoter region of the ANGPTL8 sequence [18]. In their study, the physiological function of ANGPTL8 was revealed to promote the lipid metabolism through T3 in order to regulate the autophagic flux of lipid droplets. T3 is an important regulator of energy expenditure and affects several genes involved in cholesterol and carbohydrate metabolism [81]. The expression of serum and intracellular T3 is itself under nutritional regulation through IS pathway which allows induction of DIO2 enzyme [59]. The study by Lartey et al. revealed insulin mediated activation of thyroid hormone activation by relieving the FOXO mediated repression of DIO2 in MSTO-211H cells and in the livers of LIRKO (Liver specific Insulin Receptor Knockout) and LIRFKO (Liver specific Insulin Receptor and FOXO1 Knockout) mice [59]. DIO2-mediated T3 conversion is a major pathway in the regulation of TH metabolism [59,81]. Their study demonstrated the coupling of nutrient availability with thyroid hormone activation via the PI3k-mTORC2-FOXO pathway implying important metabolic consequences.

3.8.7. MAPK signaling

Insulin signaling stimulates the cellular growth/proliferation through the MAPK pathway. It involves activation of GRB2 either through IRS and/or SHC. GRB2 upon activation recruits SOS and initiates the RAF phosphorylation signaling cascade and thereby activates RAF, MEK and ERK [78]. Yuan Zhang et al. reported that metabolic stress generated by amino acids deprivation causes the up-regulation of ANGPTL8 through the RAS/RAF/MAPK pathway [19]. They demonstrated the activation of Egr1 (Early growth response transcription factor) by ANGPTL8 which in turn inhibits the ATGL (adipose triglyceride lipase) enzyme.

3.8.8. ANGPTL8 signaling

The regulatory roles of ANGPTL8 have been discussed in detail (see Sections 3.4–3.7) and will be briefly summarized here to complete the overview of the newly designed pathway. ANGPTL8 has been demonstrated to inhibit LPL and regulate the postprandial trafficking of incoming lipids to the storage organs [5,14]. It also regulates autophagy, adipogenesis and may be pancreatic beta cell proliferation [7,8,16]. The interacting proteins from these pathways with ANGPTL8 are yet to be identified. Therefore, we have linked out the respective roles of ANGPTL8 to already existing pathways present in WikiPathways in the ANGPTL8 regulatory pathway.

The construction of the ANGPTL8 regulatory pathway (Fig. 3) is the first attempt to comprehensively integrate the current knowledge of its regulation in a pathway. ANGPTL8 is associated with several endocrine disorders such as diabetes, obesity and metabolic syndrome and is gaining strength as a potential drug target for their treatment [9,10,12,49]. In this regard, this pathway could increase the comprehension of underlying physiological and physiopathological regulation of ANGPTL8. It also increases our current understanding of ANGPTL8 regulation. For example, an increase of ANGPTL8 in the joint presence of insulin and glucose and not in the absence of either raised a possibility that a crosstalk mechanism might regulate ANGPTL8 expression [8,13]. This crosstalk is now visible in the ANGPTL8 regulatory pathway through interactions among different transcription factors including ChREBP, SREBPs, LXR and TR regulating expression of ANGPTL8 under different metabolic arms of the IS pathway. Specifically, the regulation of ChREBP and then subsequent regulation of ANGPTL8 through ChREBP requires the presence of both insulin and glucose because the transcriptional and post-translational regulation of ChREBP is itself dependent on various entities from the IS pathway. Besides this, the complex regulation of ANGPTL8 as viewed in the ANGPTL8 regulatory pathway is indicative that ANGPTL8 lies at the core of lipid and glucose homeostasis and

could act as a metabolic switch in positive energy balance conditions. Therefore, more studies are required to decipher the precise dynamics of this pathway in order to exploit the full potential of ANGPTL8 as a drug candidate in all of the related disorders.

4. Conclusion

ANGPTL8 is a novel hormone which has been reported to play role in both glucose and lipid metabolic pathways. Its unique metabolic nature allows viewing it as a potential drug target for many related endocrine disorders. The current study provided an update to understand its physiological function from literature curated findings. Besides this, the ANGPTL8 regulatory pathway has been presented as a research resource which is the first instance to map the interaction data of ANGPTL8 as interactive pathway. The pathway has been made available to the research community for usage, feedback, and updates at WikiPathways (Pathway Id: WP3915), an open source interaction database. This pathway serves to increase our current understanding of ANGPTL8 by allowing its visualization with respect to other pathways. Moreover, the pathway also allows to observe the crosstalk involved in the regulation of ANGPTL8, which was not possible before. This pathway could also assist the future omics-based investigations in finding molecular targets/regulators of ANGPTL8 as their analysis depend on the pathways data resources.

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Appendix A. Supplementary data

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