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Regulation of glucose and lipid metabolism in health and disease

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Glucose and fatty acids are the major sources of energy for human body. Cholesterol, the most abundant sterol in mammals, is a key component of cell membranes although it does not generate ATP. The metabolisms of glucose, fatty acids and cholesterol are often intertwined and regulated. For example, glucose can be converted to fatty acids and cholesterol through *de novo* lipid biosynthesis pathways. Excessive lipids are secreted in lipoproteins or stored in lipid droplets. The metabolites of glucose and lipids are dynamically transported intercellularly and intracellularly, and then converted to other molecules in specific compartments. The disorders of glucose and lipid metabolism result in severe diseases including cardiovascular disease, diabetes and fatty liver. This review summarizes the major metabolic aspects of glucose and lipid, and their regulations in the context of physiology and diseases.

cholesterol, fatty acid, glucose, lysosome, endoplasmic reticulum, lipid droplet, metabolic disease

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Glucose and lipids are essential nutrients for humans. They provide energy and building blocks for the cell. The metabolisms of glucose and lipid are precisely regulated in healthy individuals. Defects in glucose and lipid metabolism cause many diseases, including atherosclerosis, diabetes, and fatty liver. In this comprehensive review, we discuss the regulation of cholesterol metabolism, lipid secretion, lipid storage, lipid transporters, gluconeogenesis, and human genetic diseases of metabolism.

Cholesterol metabolism and diseases

Cholesterol is a major sterol in eukaryotic cell membranes. It intercalates perpendicularly to the bilayer surface, with the 3β-hydroxyl group interacting with the polar heads and the steroid nucleus and isooctyl tail to the fatty acyl/sphingosine chains of adjacent lipids. The presence of cholesterol endows each membrane with specific biophysical property that correlates with its function. For example, 60%–90% of total cellular cholesterol resides on the plasma membrane (PM)

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which serves as a rigid, thick and semi-permeable barrier shielding the intracellular contents from extracellular milieu (Liscum and Munn, 1999). The preferential binding of cholesterol to saturated lipids drives the formation of relatively ordered domains that modulate membrane trafficking and various signaling events (Sezgin et al., 2017). By contrast, as little as 0.5%–1% of total cellular cholesterol is found in the endoplasmic reticulum (ER) where frequent insertion and secretion of proteins and lipids occur (Lange and Steck, 1997; Lange et al., 1999). Low abundance of ER cholesterol also allows local regulatory machineries, including the sterol regulatory element-binding protein (SREBP)-SREBP cleavage activating protein (SCAP) complex, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR), nuclear factor erythroid 2 related factor-1, and acyl-coenzyme A: cholesterol acyltransferases (ACATs), to sense and respond to delicate changes in membrane cholesterol levels, thereby feedback regulating cholesterol metabolism in the cell (Chang et al., 2009; Goldstein et al., 2006; Widenmaier et al., 2017).

Cholesterol is also a precursor to several biological active molecules including oxysterols, bile acids, and steroid hormones. In addition, cholesterol is engaged in the Hedgehog signaling by covalently modifying two central players of the pathway, Hedgehog and Smoothened (Porter et al., 1996; Xiao et al., 2017). Mouse embryos lacking cholesterol modification of sonic hedgehog and Smoothened demonstrate defects in limb and telencephalon patterning and cardiac morphogenesis, respectively (Huang et al., 2007; Lewis et al., 2001; Xiao et al., 2017).

Overview of cholesterol metabolism

Cholesterol biosynthesis

The 27-carbon cholesterol can be synthesized *de novo* beginning with acetyl-CoA, two molecules of which condense and react with a third acetyl-CoA to form HMG-CoA (Figure 1). The following step converts HMG-CoA to mevalonate, an obligatory precursor for sterols and nonsterol isoprenoids. This step is catalyzed by HMGCR, the rate-limiting enzyme of cholesterol biosynthesis. Mevalonate goes through a series of five reactions to yield geranyl pyrophosphate (GPP) and then farnesyl pyrophosphate (FPP). In the next two reactions, two FPP molecules join to form squalene, which is oxidized by squalene monooxygenase to 2,3-epoxysqualene. SM was recently identified as the second rate-limiting in the pathway (Gill et al., 2011). FPP can also condense with isoprenyl pyrophosphate that is generated prior to GPP to form geranylgeranyl pyrophosphate (GGPP). Subsequent cyclization of 2,3-epoxysqualene produces lanosterol, the first intermediate bearing the steroid nucleus. Lanosterol then proceeds through the Bloch pathway, the Kandutsch-Russell pathway, or a hybrid pathway involving both (Mitsche et al., 2015), finally becoming cholesterol.

Cholesterol uptake

Apart from *de novo* biosynthesis, the cell can acquire exogenous cholesterol from the blood and diets. Cholesterol in circulation exists in various forms of lipoproteins that differ in sizes, protein and lipid contents (therefore densities), and apolipoprotein types. Very low-density lipoproteins (VLDLs) are produced by the liver and are rich in triacylglycerol (TAG) and cholesterol. After lipid hydrolysis and exchange, VLDLs are converted to intermediate-density lipoproteins and then to low-density lipoproteins (LDLs), which are highly enriched in cholesterol and cholesteryl esters (CEs) and specifically carry apolipoprotein B (APOB). LDL is taken up by the LDL receptor (LDLR) on the cell surface involving autosomal recessive hypercholesterolemia (ARH; also known as LDLRAP1) in hepatocytes and lymphocytes and Disabled homolog 2 (DAB2) in fibroblasts (Figure 1). These two clathrin adaptor proteins recognize the canonical endocytic sorting signal NPxY sequence in the cytoplasmic tail of LDLR, and further recruit adaptor protein-2 and clathrin to initiate endocytosis in coated vesicles. As the endosomal pH decreases, LDLR switches from an open conformation to a closed one and releases the bound LDL. It is then sorted by the COMMD/CCDC22/CCDC93 and Wiskott-Aldrich syndrome protein and SCAR homolog complex and recycles back the surface (Bartuzi et al., 2016; Fedoseienko et al., 2018). LDL, on the other hand, is delivered towards late endosomes/lysosomes where the carried CEs are hydrolyzed to free cholesterol. By coordinated actions of Niemann-Pick type C (NPC) 1, 2 and lysosome-associated membrane glycoprotein 2, cholesterol in the lysosomal lumen is inserted onto the limiting membrane (Kwon et al., 2009; Li and Pfeffer, 2016; Liao et al., 2018), followed by transport to downstream organelles, primarily the PM and the ER (Das et al., 2014; Pfisterer et al., 2016; Wang et al., 2019), which may require lysosome-peroxisome-ER membrane contacts (Chu et al., 2015; Xiao et al., 2019; Yang, 2019).

Unlike LDLR-mediated uptake of plasma LDL that occurs in most cells, cholesterol in the diets is largely (>85%) unesterified and selectively absorbed by NPC1-like 1 (NPC1L1) protein on the apical surface of enterocytes (Figure 1) (Wang, 2007; Wang and Song, 2012). Upon arriving at the small intestine, dietary cholesterol is solubilized by bile salts and passes through the unstirred water layer in the form of mixed micelles (Wang, 2007). It is then picked up by the N-terminal domain of NPC1L1 (Ge et al., 2011; Kwon et al., 2011; Zhang et al., 2011), which, as a consequence, triggers the dissociation of C-terminal tail from the PM (Li et al., 2014a). This exposes the YVNxxF sequence to NUMB, another clathrin adaptor protein that belongs to the phosphotyrosine-binding domain family as ARH and DAB2 do.

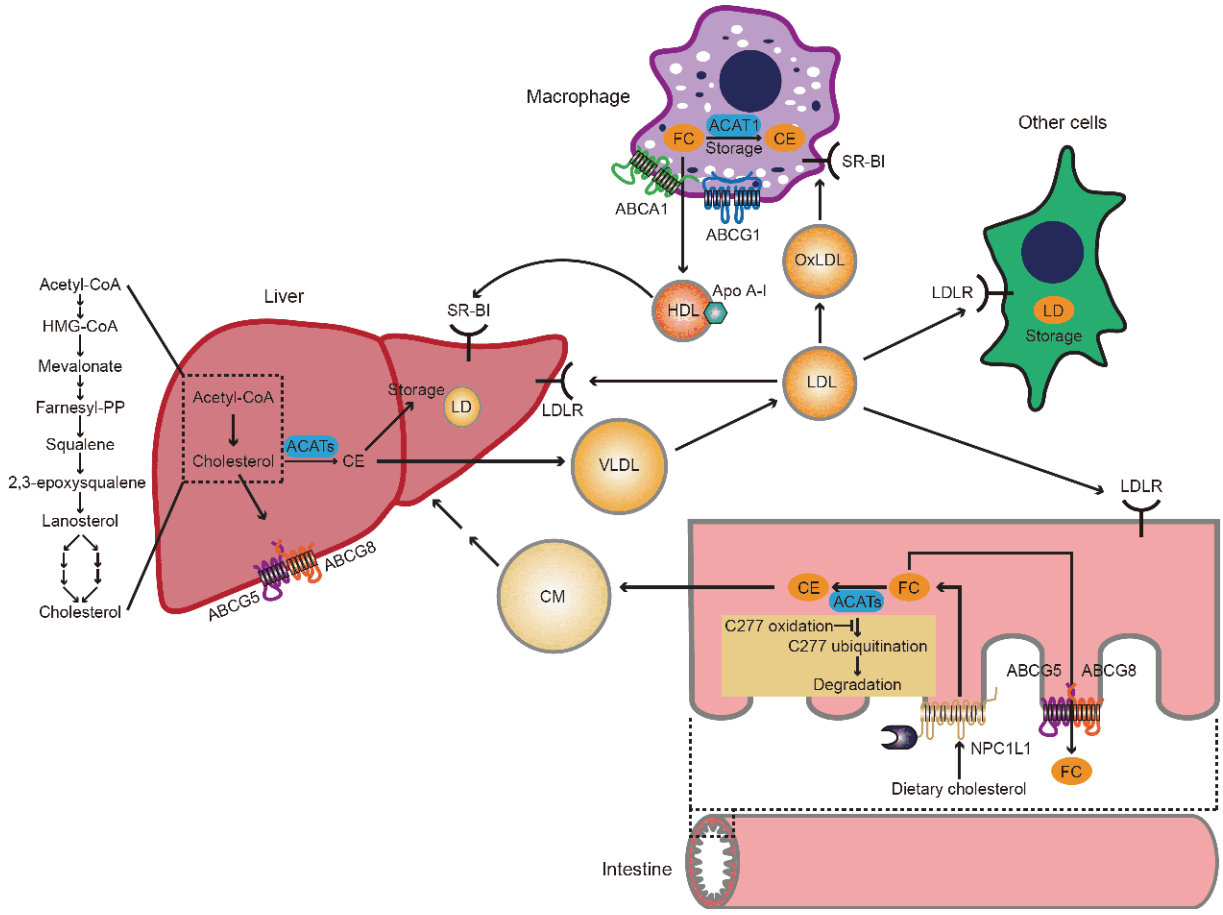

Figure 1 Overview of cholesterol metabolism in different tissues. Cholesterol is mainly synthesized in the liver but all mammalian cells are able to make cholesterol. It is transported as a component of lipoproteins in the blood. Peripheral cells take up cholesterol through low-density lipoprotein (LDL) receptor (LDLR)-mediated endocytosis. The transfer of LDL cholesterol from the liver to other tissues is called forward cholesterol transport. Macrophages acquire cholesterol from oxidized LDL (oxLDL) via scavenger receptor class B type I (SR-BI), and excrete cholesterol to high-density lipoprotein (HDL) via ABCA1 and ABCG1. HDL then carries cholesterol back to the liver in a process called reverse cholesterol transport. In the intestine, NPC1L1 takes up cholesterol from the intestinal lumen by the vesicular endocytic mechanism. Cholesterol and fatty acid are converted to cholesteryl ester (CE) by ACAT2, which is stabilized at cysteine 277 by lipid overload-induced oxidization (Wang et al., 2017b). CE constitutes the hydrophobic core of lipoproteins including chylomicron (CM), very low-density lipoprotein (VLDL), LDL, and HDL. ABCG5 and ABCG8 form a heterodimer which transports cholesterol into the intestinal lumen.

However, ARH and DAB2 are not involved in the internalization of NPC1L1 and neither is NUMB in LDLR (Wei et al., 2014). The presence of such distinct endocytic mechanisms may correspond to differential expression of adaptor proteins in specific tissues as well as allow LDL uptake by LDLR and free cholesterol uptake by NPC1L1 to be independently modulated for the optimal input levels. The vesicles carrying endocytosed cholesterol migrate along the microfilaments, reaching the endocytic recycling compartment and eventually the ER (Ge et al., 2008; Xie et al., 2011). It is noteworthy that NPC1L1 is also highly expressed in human liver and facilitates re-absorption of biliary cholesterol excreted by ATP-binding cassette (ABC) subfamily G (ABCG) member 5/ABCG8 heterodimer (Temel et al., 2007; Wang et al., 2011; Xie et al., 2012) (see below).

Cholesterol efflux

Cholesterol in excess induces cytotoxicity (Tabas, 2002) and must be exported out of the cell. In enterocytes, surplus cholesterol is secreted back into the intestinal lumen via the ABCG5/G8 on the apical membrane, or be packed with CEs into nascent chylomicrons, which, after further maturation at the Golgi, are released from the basolateral side into the lymph system and then the circulation (Figure 1). Alternatively, free cholesterol is effluxed with phospholipids by ABC subfamily A member 1 (ABCA1) to extracellular APOA-I directly or indirectly (Phillips, 2014), forming nascent high-density lipoprotein (HDL) (preβ-HDL) particles that enter the blood as well (Brunham et al., 2006). Like enterocytes, hepatocytes express ABCG5/G8 at the apical (canalicular) membrane for biliary cholesterol secretion and ABCA1 at the basolateral (sinusoidal) membrane for HDL biogenesis. Both hepatocytes and enterocytes are the major sites for APOA-I production. Pancreas can generate APOA-I as well. Chylomicrons and HDLs then travel through the vasculature to the rest of the body, with the former providing

dietary fatty acids to and the latter removing excess cholesterol from peripheral cells. In cholesterol-laden macrophages (foam cells), ABCA1, ABCG1 and scavenger receptor class B type I (SR-BI) facilitate most of the cholesterol release (Phillips, 2014), with ABCA1-mediated cholesterol efflux generating preβ-HDL that, under the activity of lecithin: cholesterol acyltransferase, converts to mature HDL for accepting cholesterol from ABCG1 (Gelissen et al., 2006). HDLs are transported back to the liver where CEs or holoparticles are selectively taken up by SR-BI and other mechanisms (Linton et al., 2017; Zanoni et al., 2018). The cholesterol hydrolyzed from CEs is either utilized by the hepatocytes, or excreted into the bile directly or after conversion to bile acids followed by ultimate fecal elimination. In addition to reverse cholesterol transport and hepatobiliary secretion, transintestinal cholesterol excretion has gained increasing attention as a significant route for cholesterol removal from the body as well (Reeskamp et al., 2018).

Cholesterol esterification

Another strategy for the cell to combat cholesterol accumulation is condensing it with fatty acyl-CoA. This reaction is catalyzed by two ER-localized isoenzymes, ACAT1 throughout the body and ACAT2 in enterocytes and hepatocytes (Figure 1). ACATs are activated by cholesterol and utilize cholesterol or other sterols and sterol-like molecules bearing a 3β-hydroxyl group as the substrates (Chang et al., 2009). The resultant CEs are either stored in lipid droplets (LDs) or secreted in the hydrophobic core of lipoproteins. ACAT2-mediated CE production is enhanced at high cellular lipid levels, where reactive oxygen species (ROS)-induced oxidation competitively blocks ubiquitination and degradation of the enzyme, thereby ameliorating lipotoxicity caused by lipid overload (Wang et al., 2017b). However, the presence of CEs in macrophages of the artery walls confers a foamy appearance characteristic of early stage atherosclerosis (Jerome, 2010).

Cholesterol metabolism disorders

Given that cholesterol is a double-edged sword whose levels need to be tightly maintained within the physiological range, dysregulation of cholesterol biosynthesis, uptake, efflux and esterification can result in a number of human diseases.

Smith-Lemli-Opitz syndrome (SLOS): insufficient cholesterol production

SLOS (OMIM #270400) is an autosomal recessive disorder caused by a deficiency of 7-dehydrocholesterol reductase, the last enzyme of the Kandutsch-Russell pathway that converts 7-dehydrocholesterol to cholesterol (Fitzky et al., 1998; Wassif et al., 1998; Waterham et al., 1998). It is relatively common in Caucasians (1:20,000 to 1:70,000 live births) but rare in Africans and Asians (Porter, 2008). Patients with SLOS display cognitive problems and various malformations including microcephaly, ptosis, cleft palate, polydactyly, syndactyly of the 2nd and 3rd toe, (Nowaczyk and Irons, 2012). Biochemically, plasma and tissue levels of 7-dehydrocholesterol and its isomer 8-dehydrocholesterol are markedly elevated in SLOS patients, whereas plasma cholesterol levels can be low or low-normal. It remains unclear whether the toxicity from 7-dehydrocholesterol or the derivatives or a loss of cholesterol results in the clinical manifestations of SLOS (Porter and Herman, 2011). Dietary cholesterol supplementation is a logically sound strategy since it can restore cholesterol levels and block 7-dehydrocholesterol biosynthesis via downregulation of HMGCR (Svoboda et al., 2012). However, the beneficial effects of cholesterol supplementation on treating SLOS are far from certain.

Schnyder corneal dystrophy (SCD): cholesterol accumulation in the cornea

SCD (OMIM #121800) is a rare autosomal dominant disease characterized by corneal opacification due to deposition of excess free cholesterol. The causative gene for SCD is UbiA prenyltransferase domain containing 1 (*UBIAD1*) (Orr et al., 2007; Weiss et al., 2007), which encodes a prenyltransferase utilizing FPP and GGPP to synthesize coenzyme Q10 and vitamin K2, respectively (Mugoni et al., 2013; Nakagawa et al., 2010). The wild-type UBIAD1 continuously shuttles between the ER and Golgi in the presence of GGPP (Schumacher et al., 2015; Schumacher et al., 2016). SCD-associated mutations confer resistance to GGPP-induced translocation, sequestering UBIAD1 in the ER so that it binds HMGCR and prevents its degradation by sterols (Jiang et al., 2019; Jo et al., 2019). This increases HMGCR levels and, consequently, cholesterol production. However, why UBIAD1 mutations specifically result in cholesterol accumulation in the cornea but no other tissue remains unknown. The only treatment for SCD is corneal transplant surgery. It would be interesting to test whether cholesterol mobilizers (e.g., 2-hydroxypropyl-β-cyclodextrin) or HMGCR degraders (e.g., HMG499 (Jiang et al., 2018)) could serve as potential therapeutic appoaches as well.

Familial hypercholesterolemia (FH): impaired LDLR pathway

FH (OMIM #143890) is one of the most common inherited metabolic disorders, with a prevalence estimated to be 1 in 500 or higher for heterozygous form and 1 in 300,000–1,000,000 for homozygous form (Foody and Vishwanath, 2016). The genetic causes of FH include mutations in the *LDLR*, *APOB*, proprotein convertase subtilisin/kexin type 9 (*PCSK9*) (gain-of-function), as well as *ARH*, which contributes to a rarer, recessive form of FH (Henderson et al.,

2016; Soutar and Naoumova, 2007). Notably, there are FH patients that do not carry mutations in any of these identified genes (Abul-Husn et al., 2016; Futema et al., 2014). The roles of LDLR, APOB and ARH in the LDLR pathway have been mentioned earlier. PCSK9 is a secreted protein that binds the ectodomain of LDLR and targets it for lysosomal degradation (Lagace, 2014). The decreased LDLR-mediated LDL uptake resulting from disrupted LDL-LDLR binding or reduced surface LDLR numbers accounts for markedly elevated plasma LDL cholesterol (LDL-C) levels that predispose to premature coronary heart disease (Brown and Goldstein, 1986).

Statins can compete with HMG-CoA for binding the catalytic domain of HMGCR and are widely prescribed to FH patients and those with atherosclerotic cardiovascular disease. However, reduced production of cholesterol and other products of the mevalonate pathway following statin treatment mitigates normal feedback inhibition of HMGCR in a multivalent manner (Brown and Goldstein, 1980; Goldstein and Brown, 1990), causing compensatory increases in HMGCR protein that may limit the maximal effectiveness of the drug. Statin intolerance and statin-associated side effects have been reported as well (Adhyaru and Jacobson, 2018). This prompts the needs for new drugs that may overcome statin limitations or target plasma LDL-C levels by different mechanisms. Recently, a potent HMGCR degrader HMG499 was found to prevent statin-induced HMGCR increment in mice (Jiang et al., 2018). A combined treatment of statins and ezetimbe, a well-known cholesterol absorption inhibitor targeting NPC1L1 (Garcia-Calvo et al., 2005), further lowers LDL-C levels in FH heterozygotes (Grundy et al., 2019). The anti-PCSK9 monoclonal antibodies alone or synergistically with statins have demonstrated truly promising effectiveness with excellent safety profiles in FH patients or those intolerant to statins (Sabatine, 2019).

NPC disease: lysosomal cholesterol accumulation

NPC disease is a rare autosomal recessive disorder that can affect infants, children or adults (Vanier, 2010). It is caused by mutations in *NPC1* (95% of cases, OMIM #257220), which encodes a large (1,278 amino acids) lysosomal transmembrane protein, or *NPC2* (5% of cases, OMIM #607625), which encodes a small (132 amino acids) soluble protein. Both NPC1 and NPC2 are able to bind cholesterol, with the former recognizing the 3β-hydroxyl group via the N-terminal domain (Kwon et al., 2009) and the latter burying the isooctyl tail deeply within a hydrophobic pocket (Xu et al., 2007). Cholesterol is suggested to transfer between NPC2 and NPC1 through a hydrophobic handoff mechanism (Gong et al., 2016; Kwon et al., 2009; Wang et al., 2010a). According to this model, NPC2 docks onto the luminal domains of NPC1 and delivers the molecule to NPC1 for insertion into the membrane. Hence, deficiency in NPC1 or NPC2 protein results in excess storage of cholesterol as well as sphingolipids and other lipids in late endosomes/lysosomes of nearly all cell types. The increased levels of lipids in the brain and in the liver and spleen lead to neurological (e.g., progressive neurodegeneration) and visceral (e.g., hepatosplenomegaly) symptoms featured by NPC disease. Miglustat (N-butyldeoxynojirimycin), an inhibitor of glucosylceramide synthase, is the only approved therapy for NPC patients in European Union and other countries but not in the US (Perez-Poyato and Pineda, 2011). 2-hydroxypropyl-β-cyclodextrin injections and adeno-associated virus-mediated *NPC1* gene delivery effectively ameliorate cerebellar and liver dysfunctions and prolong lifespans in murine and feline models of NPC1 disease (Davidson et al., 2009; Liu et al., 2009; Vite et al., 2015; Xie et al., 2017).

Tangier disease: defective macrophage cholesterol egress

Tangier disease (OMIM #205400) is a very rare inborn error of cholesterol metabolism characterized by extremely low levels of HDL and APOA-I in plasma accompanied by massive deposition of CEs in macrophage-rich tissues throughout the body (Kolovou et al., 2006). It results from mutations in the *ABCA1* gene (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999), which encodes a multiple membrane-spanning protein mediating the export of cholesterol and phospholipids to lipid-poor APOA-I particles. This is the first step of RCT pathway whose deficiency may accelerate the development of atherosclerosis and increase risk for coronary artery disease (CAD). The mechanisms underlying ABCA1-mediated cholesterol efflux are still unclear. However, a recent cryo-EM structural analysis of human ABCA1 reveals an enclosed hydrophobic tunnel formed by two large extracellular domains at the very top of the structure that may accommodate cholesterol and phospholipids (Qian et al., 2017). The ATPase activity of ABCA1 is required for conformational change and lipid storage in the extracellular domains (Ishigami et al., 2018; Nagao et al., 2012). Indeed, about two thirds of disease-causing missense mutations are present in the extracellular domains and nucleotide-binding domains of the ABCA1 protein (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=ABCA1). Unfortunately, drugs that can increase HDL levels in unaffected individuals are ineffective in patients with Tangier disease, and the specific treatment is yet to be established (Assmann et al., 2001; Puntoni et al., 2012).

Sitosterolemia: increased intestinal absorption and decreased biliary excretion of sterols

Sitosterolemia (OMIM #210250) is another very rare disease characterized by substantially elevated plasma and tissue levels of sterols, particularly plant sterols such as sitosterol and campesterol (Berge et al., 2000). It is caused by mutations in the *ABCG5* or *ABCG8* gene and inherited in an

autosomal recessive pattern. ABCG5 and ABCG8 belong to the ABC transporter superfamily as ABCA1 does. However, they are half-size exporters that contact each other at the transmembrane domains and the cytoplasmic end of nucleotide-binding domains (Lee et al., 2016). The ABCG5/G8 heterodimer is exclusively localized at the apical surface of enterocytes and hepatocytes and promotes the excretion of cholesterol and plant sterols into the gut lumen and bile, respectively (Graf et al., 2003). Moreover, unlike ABCA1 that utilizes APOA-I as the extracellular acceptor, cholesterol secretion by ABCG5 and G8 is induced in the presence of bile salts (Vrins et al., 2007). In healthy individuals, about 30%–80% of cholesterol and 5% of plant sterols consumed are estimated to be absorbed by the body (Bosner et al., 1999; Izar et al., 2011). Accumulating sterols in patients with sitosterolemia can lead to xanthomas, hypercholesterolemia and even premature CAD. The recommended treatments for sitosterolemia include low-plant-sterol diets, ezetimbe and bile acid sequestrants (Yoo, 2016).

Conclusions and perspectives

Cholesterol metabolism is intensely governed by numerous regulators and multivalent mechanisms that act in a concerted manner in response to metabolic needs. The importance of cholesterol homeostasis is signified by severe pathological consequences that, in addition to those mentioned above, also include peroxisomal disorders in which cholesterol transport is impaired (Chu et al., 2015), Alzheimer's disease whose pathogenesis might be attributable to CE accumulation (Di Paolo and Kim, 2011), various cancers that are associated with elevated cholesterol levels (Moon et al., 2019; Wang et al., 2018a; Yue et al., 2014) and many others. Although the therapeutic strategies may not be available for some diseases as of now, the advanced understanding of cholesterol metabolism together with emerging technical approaches will surely help to identify more viable drug targets and new therapeutics.

Cholesterol homeostasis regulated by the cellular secretory pathway

Among the many intricate biological processes, cholesterol metabolism represents the first example of a biological feedback system, initially reported by the German physician Rudolf Schoenheimer (Brown and Goldstein, 2009). A delicate balance of systemic and cellular cholesterol levels is maintained by specific tissues, and relies largely on the cellular secretory pathway for such control (Brown and Goldstein, 2009). The eukaryotic secretory pathway is a series of steps to transport biomolecules to specific destinations in and out of the cells (Barlowe and Miller, 2013), constituting the primary means by which a specific cell communicates and influences beyond itself. The interplay between the cellular secretory pathway and lipid/cholesterol homeostasis illustrates the contribution of fundamental cellular mechanism(s) in specific physiology process.

As discussed in Cholesterol metabolism and diseases, a sufficient supply of cholesterol is required to sustain growth and to maintain multiple cellular functions (Espenshade and Hughes, 2007), whereas excess cholesterol in the circulation is associated with the development of atherosclerosis, coronary heart disease, and stroke (Durrington, 2003; Ou et al., 2018). Circulating cholesterol levels are maintained primarily secretion of endogenously produced cholesterol (liver) or exogenously absorbed cholesterol (small intestine) in the form of lipoproteins, as well as by receptor-mediated cholesterol uptake from plasma lipoproteins (Goldstein and Brown, 2015). Genetic studies on FH patients have identified *LDLR*, *APOB*, and *PCSK9* as three autosomal dominant hypercholesterolemia genes (ADH) in humans that prominently regulate cholesterol homeostasis through lipoprotein metabolism. In brief, mutations that disrupt the function of LDLR cause hypercholesterolemia and dramatically increase the risk for cardiovascular disease (CVD) (Brown and Goldstein, 1986). In contrast, loss-of-function mutations of APOB, a core protein component of lipoproteins including chylomicrons produced from the small intestine and the hepatic VLDL, lead to reduced plasma cholesterol levels as a result of decreased lipoprotein secretion into the circulation (Schonfeld, 2003). Furthermore, loss-of-function mutations in PCSK9, a secreted factor that binds to the LDLR and induces its degradation, result in low plasma cholesterol levels by increasing lipoproteins clearance from the circulation (Horton et al., 2009).

Remarkably, the above three ADH genes all encode products entering the secretory pathway, and represent three major classes of cargos: the transmembrane protein LDLR and the soluble secretory protein PCSK9, whereas APOB assembles unique carrier for bulk lipids (Figure 2). These close connections with the secretory pathway indeed reflect the dynamic nature in lipid/cholesterol regulation. In this section, we will summary the current understanding on the cellular transport of these key factors in cholesterol metabolism, as well as the master transcription factor SREBP, focusing on their entrance into the secretory pathway upon exiting from the ER, *en route* to the Golgi apparatus.

Entering the secretory pathway from the ER mediated by the Coatmer Complex II (COPII) complex

About one third of the eukaryotic genome encodes proteins that are sorted into the secretory pathway, following their synthesis and translocation into the ER lumen. To exit ER, most of these cargos are packaged into specialized transport

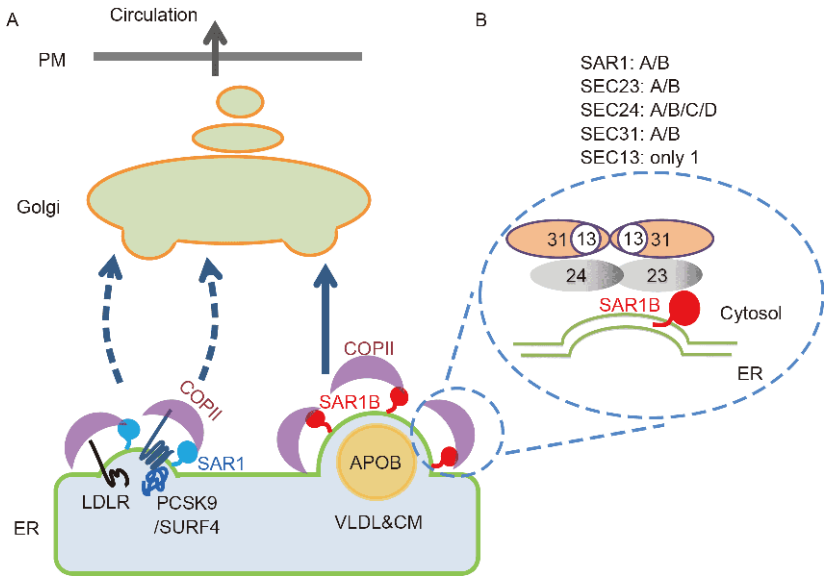

Figure 2 Delivery of the products encoded by the three ADH (autosomal dominant hypercholesterolemia) genes by the secretory pathway, initially via transport vesicles produced by the COPII complex. A, The transmembrane LDLR directly interacts with the COPII complex while the soluble proprotein convertase subtilisin/kexin type 9 (PCSK9) may access COPII via the transmembrane "cargo receptor" surfeit locus protein 4 (SURF4). APOB-containing lipoproteins selectively rely on SAR1B in COPII-mediated ER exit. B, The organization of the COPII complex during vesicle budding. Upper: paralogs of different COPII subunits in mammals.

vesicles that are generated by the COPII complex at the so-called ER exit sites (Zanetti et al., 2011). The assembly of the COPII complex is initiated by a small GTPase named SAR1, upon its activation by its guanine nucleotide exchange factor on the ER surface. Activated SAR1 exposes the N-terminal amphipathic helix and inserts it into the ER membrane, and subsequently recruits a hetero-dimer made of SEC23 and SEC24 (the inner coat complex) to form the pre-budding complex. This is followed by the recruitment by a hetero-tetramer made of two copies of SEC31 and SEC13 (the outer coat complex). The fully assembled COPII complex thus forms caged vesicles, and subsequently inactivates SAR1 to complete scission of the transport vesicles, *en route* to the Golgi apparatus.

Mammals express multiple paralogs of the COPII genes, including two SAR1 GTPases (SAR1A/B), two SEC23s (SEC23A/B), four SEC24s (SECA-D), and two SEC31 (SEC31A/B) (Gillon et al., 2012), potentially expanding the repertoire of COPII coat structures (Figure 2). Although deletions of SAR1, SEC23, or SEC24 are all lethal in yeast (Lee et al., 2004), mutations in the genes encoding several mammalian COPII components have been associated with a diverse array of unique symptoms, suggesting that the COPII complex may regulate specific processes in physiological settings. Of note, mutations in human *SAR1B* result in chylomicron retention disease (or Anderson disease), a distinct defect in fat absorption due to reduced chylomicron assembly and secretion by intestinal enterocytes (Annesi et al., 2007; Jones et al., 2003). In contrast, missense mutations in human *SEC23A* result in malformations in the craniofacial skeleton (Boyadjiev et al., 2006; Boyadjiev et al., 2010), with similar skeletal abnormalities observed in SEC23A-deficient zebrafish (Lang et al., 2006). Mutations in human *SEC23B* result in the autosomal recessive disorder congenital dyserythropoietic anemia type II, with abnormalities restricted to the hematopoietic erythroid compartment (Bianchi et al., 2009; Schwarz et al., 2009). Surprisingly, although red blood cells appear grossly normal in SEC23B-deficient mice, these animals die at birth due to dramatic destruction of the pancreas (Tao et al., 2012), likely reflecting that relative expressions levels determine the tissue-specific function of SEC23 (Khoriaty et al., 2018).

SEC24 plays a central role in cargo recognition and recruitment during COPII-mediated transport. Biochemical and structural studies have identified multiple cargo recognition sites on SEC24 (Bi et al., 2002; Bickford et al., 2004; Gürkan et al., 2006; Mancias and Goldberg, 2008; Miller et al., 2003). The four mammalian SEC24 paralogs can be divided into two subfamilies, SEC24A/B and SEC24C/D, sharing ~60% sequence identity within but only ~25% identity across subfamilies (Mancias and Goldberg, 2008; Zanetti et al., 2011). Mammalian SEC24A/B exhibits ~30% sequence identity to yeast SEC24p, compared to ~25% for SEC24C/D. The latter share ~30% identity with LST1p or ISS1p, the non-essential SEC24 paralogs in yeast (Peng et al., 2000; Roberg et al., 1999; Shimoni et al., 2000). A number of sorting signals that occupy specific sites on SEC24 have been characterized (Bickford et al., 2004; Mancias and Goldberg, 2007, 2008; Mossessova et al., 2003), most of which are short stretches of 2–4 amino acids.

While simple, short sorting motifs may provide flexibility by allowing more cargos to be accommodated by COPII (Barlowe, 2003; Barlowe and Helenius, 2016), additional mechanism may be necessary to ensure the specificity of their selection by SEC24.

COPII-mediated transport of LDLR and PCSK9, a negative regulator of LDLR

LDLR is a Type 1 transmembrane protein that mediates the clearance of LDL from the circulation (Goldstein and Brown, 2009). The mature receptor contains an N-terminal ligand-binding domain, an epidermal growth factor like repeat domain (EGF-A), an O-linked carbohydrate-rich domain in its extracellular part, followed by a transmembrane domain and a short cytosolic tail. The newly synthesized LDLR exits ER in COPII vesicles, via a di-acidic motif located within the cytosolic tail that directly interacts with the SEC24 subunit (Strøm et al., 2011). Upon arrival at the Golgi apparatus, LDLR undergoes extensive modifications of N-linked glycosylation and elongation of O-linked glycosylation, resulting in an increase of the molecular weight from ~120 kD (ER form) to 160 kD (post-Golgi form) (Goldstein et al., 1985). PM localized LDLR binds and internalizes LDL, in a classic process named as receptor-mediated endocytosis (Goldstein and Brown, 2009), involving the clathrin coat adaptor protein ARH (Garcia et al., 2001; He et al., 2002). The internalized receptor is then freed and recycled back to cell surface. However, recent studies have identified a soluble secretory protein named PCSK9, which binds LDLR and escorts the receptor to the lysosome for degradation.

PCSK9 is an atypical member of the proprotein convertase family (Horton et al., 2007). It contains a signal sequence (1–30 amino acids) which directs the newly synthesized Pro-PCSK9 into the ER lumen. The mature protein contains a pro-domain (amino acids 31–152), a catalytic domain (amino acids 153–425) and a C-terminal domain (amino acids 426–694) (Horton et al., 2007). The pro-domain is auto-cleaved in the ER at the VFAQ152↓SIP (Horton et al., 2009) site by the catalytic domain, but remains associated with the rest of the PCSK9 and is secreted together as a complex (Figure 2). Secreted PCSK9 interacts with the EGF-A domain of LDLR and triggers the endocytosis of LDLR in a clathrin- and ARH-dependent manner (Zhang et al., 2008). Through yet-to-be identified mechanisms, PCSK9 bound LDLR is directed to the lysosome for degradation after endocytosis (Costet et al., 2008). Hence, inhibiting PCSK9 represents a promising strategy for raising LDLR levels on cell surface, thereby achieving cholesterol lowering (Costet et al., 2008; Young and Fong, 2012). A number of neutralizing antibodies have been successfully developed to block circulating PCSK9, nevertheless, decreasing the production or secretion of PCSK9 offers an alternative strategy to inhibit the negative regulator of LDLR and to achieve cholesterol lowering in the circulation (Cohen and Hobbs, 2013).

The first lines of evidence that PCSK9 secretion may involve specific regulation came from human genetic studies, which found that several PCSK9 loss-of-function mutations prevent the mutant protein from exiting ER (Horton et al., 2007). Genetic ablation of the four SEC24 paralogs in mice offers interesting insights in PCSK9 secretion (Chen et al., 2013). While mice deficient of SEC24B/C/D are all lethal albeit at different developmental stages, the SEC24A knockout (KO) mice are 100% viable but have lower circulation cholesterol levels due to decreased PCSK9 secretion. Furthermore, haplo-insufficiency of SEC24B on top of SEC24A deficiency further reduces plasma cholesterol, while SEC24C or D fails to do so, even though SEC24C or SEC24D KO mice display an earlier and more severe lethal phenotype than SEC24B or SEC24A KO mice. Biochemically, SEC24A, and SEC24B to a lesser extent, mediates the budding of PCSK9 into COPII vesicles. Interestingly, these two paralogs, but not SEC24C/D, could be detected with PCSK9 immuno-precipitation, implying a physical association likely involving yet-to-be identified transmembrane "cargo receptor(s)". Recently, CRISPR screen has identified a protein named SURF4 as the COPII cargo receptor that promotes the cellular secretion of PCSK9 (Emmer et al., 2018), though the physiological function of SURF4 in cholesterol regulation remains to be tested *in vivo*.

Bulk lipid secretion via APOB-containing lipoproteins

Delivery of bulk lipids including TAG and cholesterol are mediated primarily by APOB-containing lipoprotein particles secreted from the small intestine (chylomicron) and the liver (VLDL) (Davidson and Shelness, 2000). APOB, the major structural protein of these lipoproteins, is a large amphipathic protein with 4,536 amino acids in humans. A smaller form of APOB, known as APOB-48, is produced by RNA editing mechanism that produced a truncated protein with ~48% portion of the full length protein (APOB-100) (Davidson and Shelness, 2000). The APOB-100 protein consists of five domains with alternating amphipathic α-helices and amphipathic β-strands, designated as βα1-β1-α2-β2-α3 which could form hydrophobic interactions with various lipids such as TAGs, CEs and phospholipids (Schonfeld et al., 2005).

The assembly of APOB-containing lipoproteins starts with the synthesis of APOB at the ER surface, which is co-translational translocated into the ER lumen (Boren et al., 1992). The translocation requires the signal sequence which spans amino acids 1–27 in APOB, and occurs through SEC61 translocon channel in the ER membrane (Young, 1990). However, this lipid-poor APOB polypeptide is not

stable and is subjected to degradation (Davidson and Shelness, 2000). The initial lipid loading onto APOB requires the microsomal triglyceride transfer protein (MTP), which forms a heterodimer with protein disulfide isomerase (PDI) likely as a chaperone (Gordon et al., 1995). Lipid transfer by MTP/PDI is vital for lipoprotein assembly and secretion, as loss-of-function mutations (Ohashi et al., 2000; Rehberg et al., 1996) or pharmacological inhibition of MTP lipid transfer activity (Wetterau et al., 1998) reduces APOB secretion.

With the initial loading of TAGs, phospholipids, and cholesterol onto APOB in the lumen of the ER, the primordial lipoprotein particles are destined for ER exit and initially transport to the Golgi apparatus for further lipid loading (Brodsky and Fisher, 2008). The ER-to-Golgi transport of APOB-containing lipoproteins also relies on the COPII vesicles (Figure 2) (Gillon et al., 2012). However, as the primordial APOB-containing particles may exceed the typical size of COPII vesicles (50–80 nm), it has been proposed that the COPII needs to expand to accommodate these unique cargos (Gillon et al., 2012). Interestingly, several factors implicated in the ER exit of procollagen, a prototype of "super-sized" secretory cargos (Malhotra et al., 2015), also play important roles in APOB-containing lipoprotein secretion. These include TANGO1 (the reported transmembrane cargo receptor for procollagen), TALI/Mea6 (a TANGO1 like protein generated from a chimeric transcript between Mia2 and cTAGE5), and KLHL12 (an E3 ubiquitin ligase that regulates the shape of COPII coats) (Butkinaree et al., 2014; Santos et al., 2016; Wang et al., 2016b). Although the model of size control of COPII vesicle in lipoprotein secretion has not been formally established, these studies indeed imply that APOB-containing lipoproteins may require unique factors that facilitate their exit from the ER, in a manner involving nutritional signals (Liu et al., 2019). Furthermore, mutations in SAR1B, one of the two SAR1 GTPases that assemble COPII, cause chylomicron secretion defects in humans (Jones et al., 2003). Consistent with this hint from human genetics, we have also found that genetic ablation of hepatic SAR1B disrupts VLDL secretion (Wang and Chen, unpublished data). Intriguingly, loss of hepatic SAR1A fails to affect VLDL secretion, despite >90% sequence identity between the two paralogs (Fromme et al., 2007). Hence, the specific mechanism responsible for the ER exit of APOB-containing lipoproteins remains to be fully elucidated.

Regulated ER-to-Golgi transport of SREBPs, master regulators of cholesterol homeostasis

One of the best understood examples of COPII-mediated transport in physiology is the delivery of SREBP from the ER to the Golgi apparatus. SREBPs are the master transcription factors that control both biosynthesis and secretion of lipids from the liver, as well as receptor-mediated cholesterol uptake of plasma lipoproteins (Brown and Goldstein, 1986), thereby functioning at the center of cholesterol homeostasis (Brown and Goldstein, 1997; Horton et al., 2002). SREBPs (SREBP-1 and -2) contain an N-terminal trans-activating domain that belongs to a family of basic helix-loop-helix-leucine zipper transcription factors, a transmembrane segment that integrates into the ER membrane, and a C-terminal regulatory domain (Brown et al., 2000). Despite adopting similar structures and exhibiting some overlapping function upon overexpression, SREBP-1 primarily activates genes involved in fatty acid metabolism such as fatty acid synthetase (*FAS*), whereas SREBP-2 favors genes responsible for cholesterol synthesis and uptake, including *HMGCR* and *LDLR* (Horton et al., 2002). While fatty acid synthesis is only partially determined by SREBPs, cholesterol synthesis is almost entirely dependent on the actions of these proteins (Horton et al., 2002).

Upon deprivation of sterols, activation of SREBPs is achieved by relocation of the precursor protein from ER membranes to the Golgi apparatus, where proteolytic cleavage of SREBPs takes place, releasing the N-terminal trans-activating domain for nuclear localization (Brown and Goldstein, 1997). This compartmentalized proteolytic process is sequentially catalyzed by two Golgi-localized proteases, S1P and S2P, and requires SCAP to interact with the C-terminal domain of SREBPs (Hua et al., 1996; Sakai et al., 1996). The cholesterol-regulated processing is understood in the case of SREBP-2 (Horton et al., 2002). Notably, SCAP, aided by another class of sterol binding proteins Insig1/2, senses changes in sterol levels that signal the delivery of SREBP-2 to the Golgi for cleavage-dependent activation (Brown and Goldstein, 2009). In particular, high content of cellular cholesterol renders SCAP in a conformation that fails to interact with the COPII complex and halts ER-to-Golgi transport of SREBP-2 (Brown et al., 2002), thus preventing the latter's access to S1P and S2P and essentially diminishing the action of the transcription factor. Conversely, deprivation of sterols induces SREBP-2 Golgi relocation, increasing the active form of the protein that eventually up-regulates cholesterol production (Nohturfft et al., 2000). Therefore, ER-to-Golgi transport of SREBP represents a central step in the feedback regulation of cholesterol homeostasis, in which sterols directly influence the activity of the master regulator SREBP in cholesterol metabolism (Brown et al., 2018).

Studies have identified the COPII sorting signal in SCAP as an MELADL sequence located in the cytosolic loop next to the transmembrane domain 6 of SCAP, which directly interacts with the SEC24C subunit of COPII (Sun et al., 2005; Sun et al., 2007). Moreover, excess sterols, via binding to SCAP or Insigs, causes conformational changes in SCAP that block the access of COPII proteins to the MELADL

sorting signal, effectively down-regulating SREBP activation and thus cholesterol production (Radhakrishnan et al., 2008; Radhakrishnan et al., 2004). Therefore, modulating ER-to-Golgi transport of the SCAP/SREBP complex represents a possible target to regulate SREBP activity and cholesterol metabolism (Schekman, 2007). Indeed, the transcriptional coactivator CRTC2 decreases the ER export of SREBP by disrupting COPII vesicle formation (Han et al., 2015), whereas the ER/LD-associated protein CIDEB promotes sterol-dependent SREBP activation by facilitating the loading of SREBP/SCAP into COPII vesicles at the ER exit sites (Su et al., 2019).

Conclusions and perspectives

In summary, while a great deal of knowledge has been gained on both the secretory pathway and lipid regulation, respectively, the "marriage" of the fundamental cellular process and the pivotal physiological event create numerous new questions awaiting investigation. In particular, the specificity, efficiency, and means to respond to selective regulatory cues remain to be discovered for the transport of key factors in lipid metabolism in vesicles. Meanwhile, non-vesicular form delivery of lipids and ions, via membrane contact sites between different cellular organelles, is increasingly appreciated in metabolic regulation (Gatta and Levine, 2017). Investigation on these dynamic yet complex transport events will undoubtedly unravel fundamental mechanisms in both cellular organization and metabolic control.

The storage and usage of neutral lipids

Neutral lipids, in particular TAG, are the major energy reservoir of cells. The cells store neutral lipids in LDs, an unusual type of organelle with a hydrophobic lipid core and a monolayer phospholipid surface coated with LD-resident proteins (Gao et al., 2019; Henne et al., 2018; Olzmann and Carvalho, 2019; Walther et al., 2017). LDs actively engage with many other cellular organelles, in particular the ER and mitochondria, in the processes of LD biogenesis, LD growth, lipid exchange and lipid breakdown (Figure 3).

Besides their major energy storage role, LDs also execute many other important cellular functions *in vivo*. Free fatty acids, which can be peroxidated by ROS, often lead to detrimental effects in cells. The partitioning of excess fatty acids into TAG and the storage of TAG in LDs protect cells from lipotoxicity (Lee et al., 1994; Listenberger et al., 2003). The anti-oxidative role of LDs is important in the crosstalk between glial cells and neurons or neuroblasts. Cell-autonomous elevations of ROS, fatty acids, or lipid peroxidation generate lipotoxic insults to neurons and neuroblasts, causing neurodegeneration and proliferation arrest, respectively. By channeling neuronal free fatty acids to TAG, and through intercellular transportation of lipoprotein particles for storage in glial cell LDs, neurons or neuroblasts are protected from such lipotoxic insults (Bailey et al., 2015; Liu et al., 2017b; Liu et al., 2015a). Despite the presence of resident proteins, the surface of LDs also acts as a transient harbor for other proteins. The most well-known and interesting ones are histones. In *Drosophila* embryos, massive amounts of the histones H2A, H2Av and H2B were found to be anchored on LDs through a previously uncharacterized protein, Jabba. The storage of histones on LDs enhances the antibacterial response of the developing embryo and provides a histone pool to ensure rapid growth during embryogenesis (Anand et al., 2012; Cermelli et al., 2006; Johnson et al., 2018; Li et al., 2014b; Li et al., 2012). LDs are also important for the replication of some viruses, including hepatitis C, dengue, and rotaviruses (Roingeard and Melo, 2017). Hepatitis C virus core protein is associated with LDs and recruits nonstructural proteins and replication complexes to LD-associated membranes to promote virus assembly (Miyanari et al., 2007).

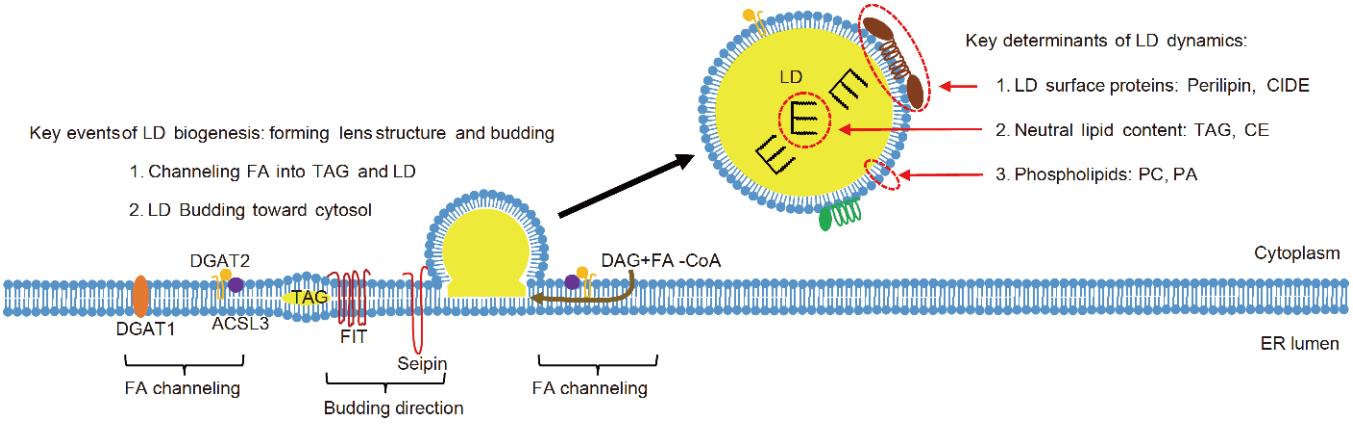

Figure 3 Key events and determinants of lipid droplet (LD) biogenesis and dynamics. The incorporation of fatty acid (FA) into triacylglycerol (TAG) is important for both forming lens structure and growing of LDs prone to budding. The LD surface protein, the content of neutral lipid core and the composition of the phospholipid monolayer are three major determinants of LD dynamics. ACSL, Acyl-coA synthetase long chain family member 3; DGAT, diacylglycerol acyltransferase.

LD biogenesis and dynamics

LD biogenesis

Based on current knowledge, LDs arise by *de novo* biogenesis from the ER (Henne et al., 2018; Walther et al., 2017). The diacylglycerol acyltransferases DGAT1 and DGAT2, two final-step TAG biosynthetic enzymes, are mainly localized in the ER. Accumulation of endogenously synthesized TAG between the two membrane leaflets in the ER, forming a lens structure, is presumed to be the first step in LD biogenesis (Choudhary et al., 2015). The stabilization of the lens structure, and its growth into a nascent LD that buds off from the ER, have been intensively studied in recent years (Figure 3). So far, FIT2 and Seipin are the two most important players identified and characterized in detail in these processes.

FIT2, also known as FITM2, is a conserved ER-resident membrane protein with two members in mammals, and was first discovered to be required for fat storage in both mice and zebrafish (Kadereit et al., 2008). Further electron microscopy analysis revealed that when FIT2 protein is lost, nascent LDs fail to bud from the ER and continue to grow in the ER membrane, which highlights an important role of FIT2 in LD biogenesis (Choudhary et al., 2015). Mechanistically, FIT2 has been postulated to possess lipid binding and enzymatic activity. It binds to TAG, and partial loss-of-function FIT2 mutations have reduced TAG binding ability (Gross et al., 2011). However, it is not fully clear whether FIT2 has an enzymatic role and whether such a role is essential for LD biogenesis.

Another important player is Seipin, a two-transmembrane domain containing ER protein (Figure 3). Seipin is mutated in the most severe lipodystrophy, BSCL2 (OMIM #269700) (Fei et al., 2011a; Gao et al., 2019; Magré et al., 2001). Similar to FIT2, Seipin is found at ER-LD junctions. The role of Seipin in LDs was first revealed in a yeast whole-genome screen for regulators of LD morphology. Loss of Seipin results in irregular clustered LDs that are closely associated with the ER, probably due to a defect in LD assembly. Loss of Seipin also results in giant LDs, which might be due to a defect in a later step in LD homeostasis and is likely caused by elevated phosphatidic acid (PA) levels (Fei et al., 2008; Szymanski et al., 2007). Evidence for the multifunctional nature of Seipin comes from several different studies. Deleting its N-terminal region affects its role in delaying LD initiation, while deleting its C-terminal region affects its putative role in phospholipid regulation (Cartwright et al., 2015; Tian et al., 2011). Similar to *FIT2* mutants, nascent LDs accumulate near the ER and most often fail to grow in *Drosophila* and human *SEIPIN* mutant cells (Wang et al., 2016a). Interestingly, a recent study indicates that in ER-LD contacts, Seipin forms a neck-like structure and acute depletion of Seipin *in vivo* affects ordered TAG partitioning, which indicates that Seipin facilitates TAG flow to LDs (Salo et al., 2019). Recent structural studies of human and fly Seipin reveal that Seipin forms a ring-shape oligomer and contains a lumenal lipid binding domain (Sui et al., 2018; Yan et al., 2018). Currently, it is not known how to reconcile the structural information with the potential multifunctional nature of Seipin.

Besides FIT2 and Seipin, several other factors localized in ER-LD contacts also participate in LD biogenesis or growth, such as the fatty acyl-CoA ligase ACSL3, and the sorting nexin family protein MDM1/SNX14, probably by activating and channeling fatty acids into TAG and LDs (Figure 3) (Datta et al., 2019; Hariri et al., 2019). Rab18, a LD component identified for a long time, has recently been shown involved in the formation of ER-LD contact to promote LD growth (Ozeki et al., 2005; Xu et al., 2018). Rab18 acts through interacting with ER-associated NRZ tethering complex and a set of SNAREs. PEX30, a membrane-shaping protein, cooperates with Seipin in both LD biogenesis and peroxisome biogenesis, processes which happen in the same subdomain of the ER and are modulated by phospholipids (Joshi et al., 2018; Wang et al., 2018b).

LD surface proteins and LD dynamics

Mature LDs undergo remarkable dynamic changes under *in vivo* physiological conditions (Yu and Li, 2017). LD-resident proteins play critical roles in this process (Figure 3). Perilipin, the most abundant LD-resident protein, was discovered back in 1991 (Greenberg et al., 1991). Mammals have five Perilipin family proteins, which modulate the accessibility of LDs and regulate the activity of lipases (Kimmel and Sztalryd, 2016). Perilipin 1 (*Plin1*)-deficient mice are lean and deprivation of PLIN1 also reduced the obesity of *db/db* mice (Martinez-Botas et al., 2000). PLIN1 deficiency in humans causes autosomal dominant partial lipodystrophy (OMIM #613877) (Gandotra et al., 2011). Perilipin is also involved in atypical LD fusion mediated by Fsp27 protein, a CIDE family LD-associated protein. The Fsp27-mediated LD growth process involves a directional net lipid transfer from smaller to larger LDs at LD-LD contact sites (Gong et al., 2011). PLIN1 interacts with the CIDE-N domain of Fsp27 and significantly promotes Fsp27 activity in lipid transfer in adipocytes (Sun et al., 2013).

PLIN proteins also take part in other cellular processes involving LDs. For example, in *Drosophila*, PLIN2, also known as LSD2, together with Halo and the motor coordinator Klar, is important for specific LD movements during embryogenesis (Welte et al., 2005). Besides cultured cells, *in vivo* LD movements have only been characterized during *Drosophila* and zebrafish embryogenesis, where the transparent eggs are helpful for *in vivo* observation. Using *Drosophila* embryo LDs as an *in vivo* model, an unexpected property of intracellular motor-mediated transport was re-

vealed: LD transport is largely unaffected by variation of the number of motors attached to the cargo (Shubeita et al., 2008).

Besides LD-resident proteins, many LD-associated proteins identified by isolation-proteomic studies are summarized, including most organisms studied so far (Zhang and Liu, 2019). Several mechanisms of protein localization to LDs have been revealed, including hydrophobic hairpin motifs, amphipathic helices, and protein-protein interactions (Kory et al., 2016). Unlike other organelles, LDs have a limited capacity for protein binding. Therefore, crowding of LD-associated proteins which have different LD-binding affinities largely determines LD protein composition, in particular under lipolysis conditions when the LD is shrinking and has a reduced surface area (Kory et al., 2015).

Membrane lipids and LD dynamics

LD dynamics can also be directly affected by membrane lipids and lipid metabolic enzymes on LDs, including CCT, the rate-limiting enzyme for PC synthesis (Figure 3). CCT1 and CCT2 were first found in a screen for genes affecting LD morphology and distribution (Guo et al., 2008). Targeting of CCT1 to LDs provides more PC during LD expansion, which likely acts as a surfactant to prevent LD coalescence (Krahmer et al., 2011). This finding is consistent with a previous LD lipidomics study that LD phospholipid is enriched with PC and lysoPC (Bartz et al., 2007). Besides contributing to LD growth/expansion and the neutral lipid core, phospholipids in the bilayer affect membrane surface tension, which is a key parameter of LD budding. Decreasing the surface tension favors the egress of neutral lipids from the phospholipid bilayer and LD budding (Ben M'barek et al., 2017). Interestingly, a recent study indicates that asymmetric coverage of the monolayer surface, determined by both phospholipids and proteins, controls the directionality of LD budding (Chorlay et al., 2019). Whether the asymmetry is directly linked to, or even the cause of, membrane surface tension is not known. In yeast, several lipid metabolic enzymes, including Pah1, are found in the inner nuclear membrane, a membrane compartment which is not known to exhibit metabolic activity. These enzymes promote lipid storage and formation of nuclear LDs, which sequestrate a transcription factor and regulate genome transcription (Romanauska and Köhler, 2018). In a yeast screen, mutants with elevated PA contain large LDs, and further *in vitro* assays support the idea that the PA level is positively correlated with LD size (Fei et al., 2011b).

The TAG biosynthetic pathway and regulation of its enzymes

Although CEs and other neutral lipids such as retinyl esters can be found in LD cores, TAG is the most studied neutral lipid in LDs. Therefore, it is reasonable to look at the source of TAG and the regulation of TAG synthetic enzymes (Figure 4).

Source of fatty acids

Fatty acids are the major components of TAG. Acetyl-CoA is the building block for the *de novo* synthesis of fatty acids. In lipogenic tissues (cells), acetyl-CoA can originate from many nutrient sources, including sugars, proteins, and dietary fats. Mitochondria have a central function in converting different nutrient sources to acetyl-CoA (Ding et al., 2018). The mitochondrial TCA cycle generates citric acid, a key intermediate for shuttling acetyl-CoA out of mitochondria for fatty acid and cholesterol synthesis. Besides the activity of mitochondria, genes in lipogenesis pathway also affect the production of fatty acids. Acetyl-CoA carboxylase (ACC) and FAS, two key enzymes in *de novo* lipogenesis, are transcriptionally regulated by SREBP-1a and overexpression of SREBP-1a results in overproduction of fatty acids along with enlargement of the liver in mice (Shimano et al., 1996).

TAG synthetic pathway

The sequential action of glycerol-3-phosphate acyltransferase (GPAT), acylglycerol-3-phosphate acyltransferase, lipin phosphatidic acid phosphatase, and DGAT enzymes generates TAG from fatty acids and glycerol (Wang et al., 2017a). Most of the enzymes are localized in the ER, while some may be in mitochondria, the inner nuclear membrane or the LD surface for local lipid synthesis. Although this is the core pathway of TAG synthesis, there is also a monoacylglycerol acyltransferase (MOGAT) pathway for TAG production, which adds acyl-CoA to existing monoacylglycerol and diacylglycerol (DAG). The contribution of different pathways to the final TAG content of LDs may vary in different cells/tissues and is not well defined.

Regulation of TAG synthetic enzymes

Both transcriptional and post-transcriptional regulations play important roles in controlling TAG synthetic enzymes. Transcriptional control of the TAG synthetic pathway has been studied quite extensively. Key transcriptional factors such as SREBP, PPARα, ChREBP, C/EBPα, C/EBPβ, and ERR are all positive regulators, while miRNAs and HDAC are negative regulators on some occasions (Wang et al., 2017a).

At the post-transcriptional level, the spatiotemporal activity of these enzymes is regulated by protein subcellular localization, stability, modification, and specific binding partners. The subcellular localization of these enzymes also contributes significantly to LD dynamics (Figure 4). GPAT4, which produces lysophosphatidic acid, and other enzymes in TAG synthesis, including DGAT2, are localized in some growing LDs, to provide phospholipids and TAG

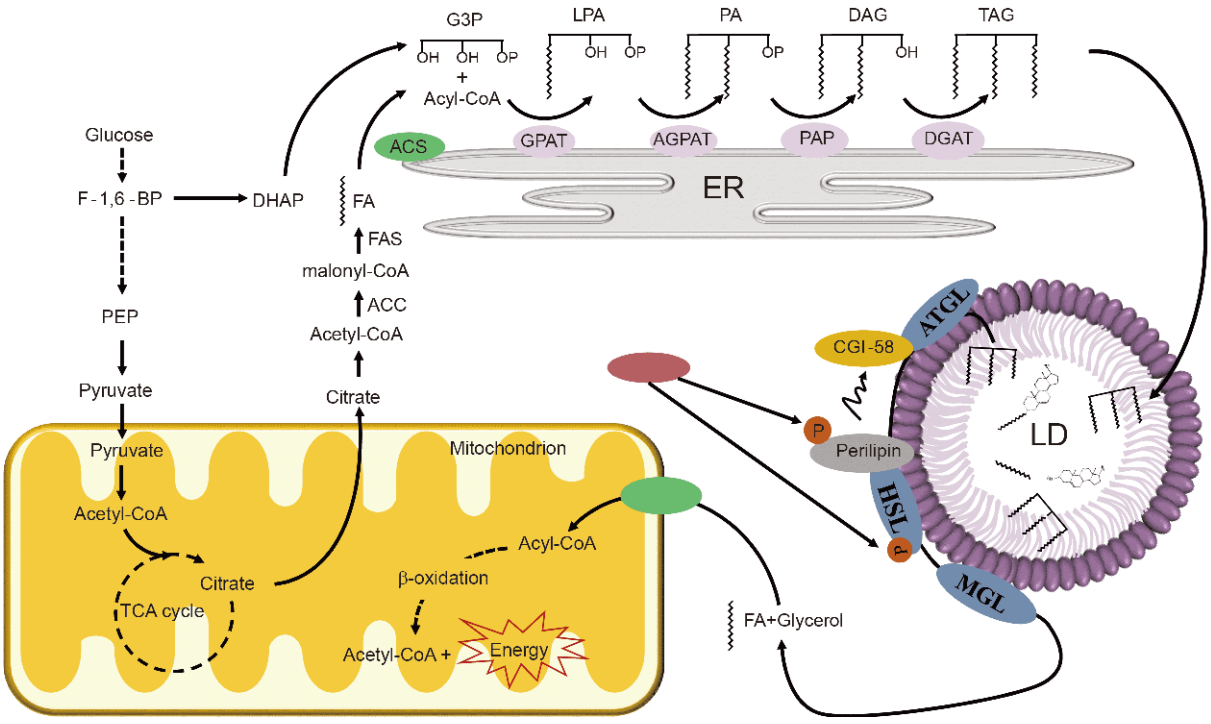

Figure 4 The biosynthesis and breakdown of TAG. FA and glycerol backbone are two substrates for *de novo* TAG synthesis by a cascade of ER-localizing enzymes, including glycerol-3-phosphate acyltransferase (GPAT), acylglycerol-3-phosphate acyltransferase (AGPAT), lipin phosphatidic acid phosphatase (PAP) and DGAT. FA is derived from glucose via glycolysis and TCA cycle, which shuttles acetyl-CoA from mitochondria to cytosol in the form of citrate. The TAG in LD is broken down by three lipases: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) and the process is also known as lipolysis. The activities of lipases are regulated by protein kinase A (PKA), Perilipin and CGI-58. FA released from lipolysis is activated and imported into mitochondria for further breakdown via β-oxidation. ACS, acetyl-CoA synthetase.

for LD growth (Wilfling et al., 2013). It was recently found that the levels of GPAT3, MOGAT2, and DGAT2 are reduced by ER-associated degradation (ERAD). AIDA, a C2-domain-containing protein, is essential for the activity of the ERAD E3-ligase HRD1 in this process (Luo et al., 2018). Phosphorylation is a classic protein modification, and it is not surprising that it regulates the activities of several TAG synthetic enzymes (Wang et al., 2017a). Besides phosphorylation, other types of regulation, in particular acetylation, have recently been reported in several cases. Tip60-dependent acetylation of Lipin 1 promotes its translocation from cytosol to the ER where it enhances TAG synthesis (Li et al., 2018). Calcineurin B homologous protein 1 (CHP1), when N-myristoylated, binds and activates GPAT4. When CHP1 is absent, peroxisomal glycerolipid synthesis compensates for the loss of ER lipid synthesis, thus adding new regulatory layers to glycerolipid synthesis (Zhu et al., 2019).

Lipid breakdown

The breakdown of lipids, in particular of the neutral lipid TAG, is known as lipolysis (Zechner et al., 2017). Besides the well-studied cytosolic lipolysis, organelle-mediated lipolysis has also been explored recently, including lipophagy and chaperone-mediated autophagy (CMA). The end-products of lipolysis, mainly fatty acids, have several destinations/functions. The major one is generation of ATP and acetyl-coA through mitochondrial β-oxidation, but other paths also exist, including signaling and re-esterification (Figure 4).

Cytosolic lipolysis

Three enzymes mediate cytosolic lipolysis, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) (Figure 4). At first, HSL was postulated to be the key rate-limiting enzyme in TAG breakdown (Egan et al., 1992). However, the subtle adipocyte phenotype of *Hsl*$^{-/-}$ mice (Osuga et al., 2000), indicated that an unidentified rate-limiting lipase exists, which was discovered in 2004 as ATGL (Zimmermann et al., 2004). ATGL is highly expressed in adipose tissue and is associated with LDs. Follow-up studies in ATGL-deficient mice and other species including *Drosophila* all support the conclusion that ATGL is the *bona fide* rate-limiting TAG hydrolase. ATGL-deficient mice exhibit increased adipose mass, defective cold adaptation, elevated TAG accumulation in the heart, and premature death. All these phenotypes can be attributed to defective TAG hydrolysis (Haemmerle et al., 2006).

Regulation of cytosolic lipolysis

Since cytosolic lipases were discovered, the regulation of these enzymes has attracted much attention. A classic example is the regulation of HSL subcellular location by extracellular signals. Upon lipolytic activation by β-adrenergic receptor, cAMP activates protein kinase A (PKA), which phosphorylates HSL and PLIN1, thus promoting the translocation of HSL from cytosol to LDs (Egan et al., 1992; Sztalryd et al., 2003). However, the full meaning of the phosphorylation of PLIN1 was only revealed after the identification of ATGL regulators.

Loss of ATGL in humans causes neutral lipid storage disease (OMIM #610717), with a phenotype of TAG accumulation and myopathy (Fischer et al., 2007). Chanarin-Dorfman syndrome (CDS, OMIM #275630) is another neutral lipid storage disease. The cloning and functional characterization of the CDS mutant gene *CGI-58* revealed that CGI-58 interacts with ATGL and stimulates its TAG hydrolase activity up to 20-fold (Lass et al., 2006). Importantly, subsequent studies found that PLIN1 interacts with CGI-58 and phosphorylation of PLIN1 terminates this interaction to promote the interaction between CGI-58 and ATGL (Granneman et al., 2009). Therefore, the lipolytic activity of ATGL and HSL is highly coordinated by PKA-mediated protein phosphorylation. Besides this positive regulation of ATGL, negative regulators have also been identified. G0S2, G(0)/G(1) switch gene 2, named after its role in the cell cycle, interacts with ATGL and inhibits its TAG hydrolase activity (Yang et al., 2010). HIG2, containing a hydrophobic domain highly similar to the ATGL inhibitory domain of G0S2, also inhibits ATGL activity (Zhang et al., 2017).

ATGL is also regulated by protein stability and transcription. For example, mTOR1 suppresses the transcription of ATGL and HSL (Chakrabarti et al., 2010). COP1, an E3 ubiquitin ligase, binds to ATGL and promotes its degradation through polyubiquitination. Accordingly, acute depletion of COP1 ameliorates high-fat diet-induced liver steatosis, a phenotype similar to ATGL overexpression (Ghosh et al., 2016). Other modifications of ATGL have not yet been reported.

Regulation of lipolysis by autophagy

Other than cytosolic factors, autophagosome-lysosome activities also regulate lipolysis in different ways. CMA is a process that selectively recognizes and degrades specific cytosolic proteins in lysosomes. The LD-resident proteins PLIN2 and PLIN3 are CMA substrates, and during starvation CMA-mediated degradation of PLIN2 and PLIN3 is enhanced, while the level of ATGL is elevated. In contrast, *ex vivo* or *in vivo* blockage of CMA or expression of a CMA-resistant PLIN2 reduces the association of ATGL with LDs, subsequently leading to lipid oxidation along with accumulation of LDs (Kaushik and Cuervo, 2015).

Besides the indirect regulation of LDs by CMA, autophagy also directly acts on LDs. Inhibition of autophagy by 3-methyladenine increases TAG storage (Singh et al., 2009). During nutrient deprivation, LDs and autophagic components become associated, and LDs are moved into lysosomes. It is not surprising that lysosomal lipase plays a key role in degrading TAG; however, the regulatory details of this process are not fully clear.

Pathways involving fatty acids

The main products of LD breakdown are fatty acids, which have several destinies, including channeling into mitochondria for β-oxidation or re-esterification into TAG (Figure 4). The re-esterification remodels the fatty acid composition of TAG. The function and regulation of this remodeling is barely understood. As we can see from the previous section, mitochondria have a key role in providing substrates for fatty acid biosynthesis and TAG, and in energy-spending tissues, fatty acid β-oxidation happens in mitochondria. Therefore, it is not surprising that mitochondria fulfill a multi-faceted role in lipid metabolism via both lipogenesis and lipolysis. The exact contribution of mitochondria depends on the cell state. When cells are starved, fatty acids, released from LDs, move into highly fused mitochondria near LDs. Autophagy, in turn, replenishes LDs with fatty acids during the starvation process (Rambold et al., 2015). Later on, the re-esterification of fatty acids into TAG, and storage of TAG in LDs, acts as a lipid buffering system that protects mitochondria from a lipotoxic flux of fatty acids released by autophagic degradation during a prolonged period of nutrient deprivation (Nguyen et al., 2017).

In lipolytic cells such as brown adipocytes, which generate heat using fatty acids, mitochondria associate with LDs, and are named peridroplet mitochondria (PDM). Interestingly and unexpectedly, PDMs have reduced β-oxidation and separate from LDs upon activation of brown adipose tissue (BAT), which suggests a role of PDMs in lipid production instead of lipid breakdown. Indeed, increasing the number of PDMs by PLIN5 overexpression leads to enhanced TAG synthesis (Benador et al., 2019; Benador et al., 2018).

The released fatty acids, and probably their lipolytic derivatives, may also act as signals or lipid modifiers. *Atgl* deficiency in mice decreases the mRNA levels of PPARα, PPARδ and PGC1α target genes. The hydrolytic activity of ATGL likely produces fatty acids and probably their derivatives as lipid ligands for PPAR activation (Haemmerle et al., 2011). The specificity and regulation of lipolysis-generated fatty acid-derived lipid signals and lipid modifiers need to be further explored.

Conclusions and perspectives

LD biology has blossomed into an exciting field. Many new

frontiers are waiting to be explored and fundamental questions need to be addressed. Just a few are listed here. How do environmental or developmental changes influence LD and lipid dynamics? Is there a cross-talk between lipid storage and lipolysis during remodeling and plasticity of lipid metabolism? Extending from LD biogenesis to physiology, can we better understand LD-related diseases, and provide novel insights to combat them?

In addition to general questions, there are several more specific areas that are interesting as well. What are the physiological consequences of disturbing LD dynamics? How can we explain the different physiological consequences of LD-related mutations in different tissues or organisms? Can we reveal general or cell type-specific regulatory mechanisms of LD biology? Are there specific machineries to regulate the storage of TAGs, CEs or other neutral lipids? The development of new biophysical approaches and imaging techniques for studying LD biology, including the recently used cryo-EM and *in vitro* adiposomes, open up many opportunities to reveal the regulation of LD dynamics and the *in vivo* dynamics of LDs during physiological processes, such as development and aging, as well as during pathological processes.

Transporters and lipid metabolism

Transporters are a group of cell gatekeepers with essential effects on influx and efflux of xenobiotics and metabolites. As an active system, cells need to absorb nutrients and excrete wastes through these membrane-bound proteins to maintain their homeostasis. Transporters consist of water channels, ion channels, ATP-dependent transporters (ATP-binding cassettes and ATP-driven pumps), and solute carriers (SLCs). As the largest group of transporters (Hediger et al., 2013), SLCs control the exchange of metabolites between cellular and extracellular environment, participate in fundamental physiological functions, and are involved in pathological processes on certain conditions. The latest SLC tables (http://www.bioparadigms.org/slc/intro.htm) and recent reports show that there are 65 subfamilies and 456 SLC members in this superfamily (César-Razquin et al., 2015; Perland and Fredriksson, 2017). SLCs are mainly divided into four transport types, including cotransporter, exchanger, facilitated transporter, and orphan transporter (Zhang et al., 2019). The predominant substrates transported by SLCs are diverse and complex, including glucose, amino acids, fatty acids, vitamins, metal ion and many drugs. These SLCs are localized on the cell surface and subcellular organelle membranes, and some have high abundance or specific expression in different tissues or cell types (César-Razquin et al., 2015). It has been shown that SLCs are associated with human diseases, such as type 2 diabetes mellitus (T2DM), depression, gout, cancer and other diseases (Dupuis et al., 2010; Flannick et al., 2014; Flynn et al., 2013; Hyde et al., 2016; Rusu et al., 2017; Veglia et al., 2019). Abnormal lipid metabolism leads to obesity, nonalcoholic fatty liver disease (NAFLD), CVD, insulin resistance, and T2DM. These

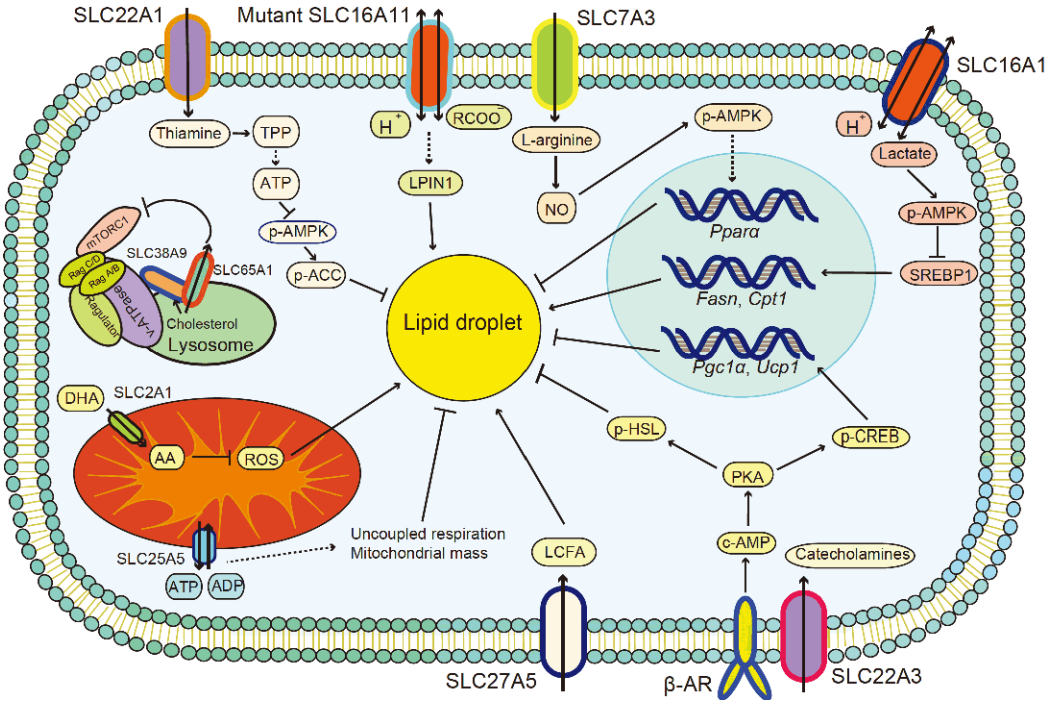

Figure 5 Illustration of SLC members involved in lipid metabolism. Thick unidirectional or bidirectional solid black arrows indicate substrate transportation direction. Thin unidirectional solid black arrows, dashed black arrows and black stop symbols indicate the flow of molecules.

chronic metabolic diseases not only negatively impact people but also consume a lot of medical resources. It is therefore essential to explore exact pathogenesis and new therapeutic targets. Recently, some reports indicate that SLCs, such as SLC2A1, SLC22A1 and SLC22A3, may be the candidate targets of these abnormal lipid metabolism related diseases (Chen et al., 2014; Song et al., 2019; Vazquez-Chantada et al., 2013). Here we review some of the reported SLCs participating in lipid metabolism (Figure 5).

Glucose transporters and lipid metabolism

The glucose transport (GLUT) family possesses 14 members, all of which are encoded by SLC2 genes. GLUT proteins have about 500 amino acid residues and 12 transmembrane segments. They are extensively expressed in every type of human cells, albeit in a tissue-specific manner. The predominant substrates of GLUT proteins include glucose, galactose, mannose, and fructose (Mueckler and Thorens, 2013). Single nucleotide polymorphisms (SNPs) of *SLC2A1* (GLUT1) are implicated in the development of NAFLD, and *SLC2A1* expression was low in NAFLD patients compared with the controls (Vazquez-Chantada et al., 2013). THLE2 human liver cell line lacking *SLC2A1* showed differentially expressed genes related to lipid metabolism, storage and transport, even hepatic steatosis and apoptosis. Silencing of *SLC2A1* significantly induced ROS level and enhanced the accumulation of LDs in THLE2 cells treated with oleic acid. Dehydroascorbic acid (DHA), an oxidized form of ascorbic acid (AA), is one of the substrates transported by SLC2A1. It is speculated that SLC2A1 protects NAFLD progression by allowing the entry of DHA into mitochondria. Dysfunction of SLC2A1 prevents DHA from getting into mitochondria, thereby attenuating the level of AA, an important antioxidant against ROS, and exacerbating NAFLD progression (Kc et al., 2005).

Hyperglycemia in T2DM patients is linked to insulin resistance and defective glucose absorption. *SLC2A4* (GLUT4) is predominantly expressed in adipose tissues, and skeletal and cardiac muscles (Huang and Czech, 2007). In most T2DM patients, *SLC2A4* expression is low in adipose tissues, and its function is impaired in the skeletal muscle (Shepherd and Kahn, 1999). Co-depletion of *Slc2a4* in mouse adipose tissues and skeletal muscle impairs glucose uptake in both organs. However, *de novo* lipogenesis and VLDL-TAG secretion are elevated as a consequence of enhanced glucose uptake in the liver, as evidenced by the upregulation of hepatic glucokinase and the key lipogenic enzyme ACC and SREBP-1c expression (Kotani et al., 2004). Thus, *Slc2a4* dysfunction in adipose tissues and skeletal muscle may promote glucose uptake and lipid synthesis in the liver, and hepatic lipid secretion offers another energy form to cover the glucose shortage.

Cationic amino acid transporters and lipid metabolism

Amino acids are essential for all living cells. SLC7 family is one of the groups that transport amino acids in and out of the cell. SLC7A1-4 and SLC7A14 are the cationic amino acid transporters which play a critical role during the process of nitric oxide (NO) synthesis by delivering L-arginine (Fotiadis et al., 2013). NO can stimulate mitochondrial biogenesis and trigger AMP-activated protein kinase (AMPK) to induce the expression of PGC1α, which cooperates with PPARα to promote the expression of genes involved in fatty acid oxidation (Lira et al., 2010; Nisoli et al., 2003).

Nutrition limitation or starving can lead to lipid accumulation in the liver of animal models. Genetic ablation of *Slc7a3a* or knockdown of *Slc7a3* promotes hepatic lipid accumulation in zebrafish starved for 1 week or in mice starved for 24 h. Downregulation of *SLC7A3* in human liver L02 cells results in hepatic lipid accumulation during glucose starvation by decreasing arginine delivery and blunting the NO-AMPK-PPARα-signaling pathway (Gu et al., 2014).

Organic cation transporters and lipid metabolism

Organic cation transporter 1 (OCT1, *Slc22a1*) belongs to the SLC22 family that participates in the transportation of organic cations, anions and zwitterions. This family is responsible for the absorption and excretion of drugs, endogenous and exogenous compounds (Koepsell, 2013). *Oct1* deficiency alleviates hepatic steatosis by enhancing β-oxidation of fatty acid and activating phosphorylation of AMPK. *OCT1* deletion inhibits the absorption of thiamine, a new OCT1 substrate, thereby causing a shortage of thiamine pyrophosphate and attenuating glycolysis. The changes in hepatic energy status stimulate catabolism initiated by the AMPK pathway and consequently suppress lipid accumulation in the liver. Furthermore, metformin as an established substrate of OCT1 can competitively block thiamine uptake by OCT1 (Chen et al., 2014).

Increasing energy expenditure by activating BAT and the beige adipocytes in white adipose tissue (WAT) is a promising therapeutic direction to improve metabolic health (Bartelt and Heeren, 2014; Harms and Seale, 2013). Although BAT is the ideal adipose tissue to combat obesity, obese and elder people have reduced BAT amount with limited capacity (Cypess et al., 2009). Therefore, the beiging of WAT seems to be a more effective approach to defeat metabolic diseases. Under cold challenge, the release of catecholamines, including norepinephrine (NE), stimulate β3-adrenergic receptors to induce lipolysis and thermogenesis in WAT (Bartelt and Heeren, 2014; Nguyen et al., 2011). OCT3 (*Slc22a3*), another SLC22 family member, was recently reported to modulate WAT beiging in mice (Song et al., 2019). Adipose-specific *Oct3* KO mice had elevated

thermogenesis, lipolysis and β-adrenergic signaling in WAT. Further research showed that ablation of *Oct3* promotes WAT beiging and mitochondria biogenesis, as well as alters energy metabolism via the enhanced NE/β-AR/PKA signaling pathway. Lipolysis induced by NE was increased in the differentiated primary human AT-derived mesenchymal stem cells following OCT3 inhibition. The study identifies a new clearance route of NE in adipose tissue via OCT3, and suggests that lowering catecholamine concentrations in adipose tissue may be a new way to treat obesity.

Fatty acid transporter and lipid metabolism

Fatty acids are a class of energy source, and can esterify with glycerol to generate TAG for storage mainly in adipose tissues. Excessive or ectopic deposition of TAG in adipose tissue, liver and muscle leads to a series of metabolic diseases, such as obesity, NAFLD and insulin resistance (Rasouli et al., 2007). Absorption of long-chain fatty acid (LCFA) into cells is modulated by several membrane proteins, including SLC27 family and fatty acid translocase (FAT)/CD36 (Anderson and Stahl, 2013).

SLC27 family encompasses six fatty acid transport proteins (FATP1-6), all of which share a highly conserved FATP sequence and an AMP binding domain. While FATPs are mainly located in the PM, FATP4 contains an ER localization domain and is present in the ER of enterocytes (Milger et al., 2006). FATPs not only transport LCFA, but also display acyl-CoA synthetase activity to activate LCFA. Many loss-of-function animal studies of SLC27 family members demonstrate that these FATPs are essential for maintaining lipid homeostasis (Anderson and Stahl, 2013). FATP5 (*Sc27a5*) is expressed exclusively in the PM of hepatocytes. Knockdown of *Fatp5* decreases LCFA uptake in hepatocytes, while overexpression of *FATP5* in HeLa cells enhances fatty acid absorption. *Fatp5* KO mice showed lower TAG levels with altered lipid profiles in the liver compared with control mice. Neutral lipids accumulated in the central veins instead of distributed evenly in *Fatp5* KO livers after 48 h fasting (Doege et al., 2006). Furthermore, AAV-mediated knockdown of *Fatp5* before or after diet-induced NAFLD improved NAFLD and whole-body glucose homeostasis (Doege et al., 2008). Meanwhile, *Fatp5* depletion disrupted bile acid conjugation in gallbladder. Reduced food intake and increased energy expenditure conferred resistance to high-fat diet (HFD)-induced obesity (Hubbard et al., 2006). Hence, *Fatp5* may be a potential target for the treatment of NAFLD and obesity.

More recently, a study demonstrated that changing lipid metabolism in PMN-MDSCs, pathologically activated neutrophils related to many diseases, contributes to its function on immunosuppression. FATP2 is engaged in this process by facilitating transport of arachidonic acid and synthesis of prostaglandin E2 so as to promote cancer progression in PMN-MDSCs (Veglia et al., 2019).

Monocarboxylate transporters (MCTs) and lipid metabolism

MCTs have 14 members encoded by the SLC16 gene family. MCT1–4 are proton-dependent, *bona fide* transporters of monocarboxylates. They are also responsible for the influx or efflux of lactate, pyruvate, ketone bodies and some drugs (Halestrap, 2013). These substrates play critical roles during cell energy homeostasis. MCT1 is broadly expressed in many tissues and functions as the major MCT for lactic acid efflux or uptake depending on the metabolic status of a cell. MCT4, despite a lower affinity for pyruvate compared to MCT1, has a higher expression level in glycolytic cells, where it helps prevent the efflux of pyruvate, as the conversion of pyruvate to L-lactate is crucial for energy metabolism in these cells (Halestrap and Wilson, 2012).

To understand the physiological role of MCT1 (*Slc16a1*), a genetically modified mouse model was established. While $Mct1^{-/-}$ mice died early during embryonic development, $Mct1^{+/-}$ mice grew normally and, owing to reduced accumulation of lipids in the liver and adipose tissues, were less prone to HFD-induced glucose intolerance and insulin resistance compared with $Mct1^{+/+}$ mice. Food consumption and intestinal absorption were lower in $Mct1^{+/-}$ mice, whereas metabolic rate was higher. Moreover, the genes involved in hepatic lipid metabolism were reduced in $Mct1^{+/-}$ mice on HFD (Lengacher et al., 2013). A more recent work revealed the molecular mechanism by which MCT1 insufficiency protects against hepatic steatosis. In fact, deficiency of MCT1 activates AMPK constitutively as a consequence of attenuated hepatic lactate metabolism, thereby increasing the accumulation of precursor SREBP-1 in the cytoplasm and suppressing the expression of genes involved in lipid metabolism (Carneiro et al., 2017).

There are four missense SNPs and one silent mutation in *SLC16A11*, which is considered as a new risk factor for T2DM in Mexicans and Latin Americans. The ER localization and liver expression of SLC16A11 suggest its role in hepatic lipid metabolism (Williams et al., 2014). Knockdown of *SLC16A11* caused accumulation of acylcarnitines, DAGs, and TAGs in primary human hepatocytes (Rusu et al., 2017). *Slc16a11* KO mice on chow or HFD showed normal phenotype. However, overexpression of a gain-of-function mutant *Slc16a11* in the liver of *Slc16a11* KO mice on HFD resulted in abnormal lipid accumulation and insulin resistance. Further studies reveal that mutant SLC16A11 promotes lipin 1 expression and accelerates TAG deposition in the liver (Zhao et al., 2019). These results suggest that, instead of high or low expression of genes, abnormal protein products produced by mutated base sequences may cause

human diseases and can serve as another potential therapeutic target.

Mitochondrial carrier and lipid metabolism

Some transporters are localized in subcellular organelle membranes, such as mitochondrion and lysosome. A series of studies shows that lysosomal transmembrane protein SLC38A9 is necessary for mTORC1 activation by amino acids and cholesterol (Castellano et al., 2017; Rebsamen et al., 2015; Wang et al., 2015; Wyant et al., 2017). SLC38A9 senses cholesterol and activates mTORC1 via its cholesterol-responsive motifs in the lysosome, whereas SLC65A1, which exports cholesterol from the lysosome, binds SLC38A9 and inhibits mTORC1 activation (Castellano et al., 2017). Mitochondrial carriers, namely SLC25 transporter family, participate in many important metabolic processes, such as oxidative phosphorylation, fatty acid oxidation, and amino acid degradation (Palmieri, 2013). SLC25A1 is a citrate carrier responsible for the outflow of citrate from mitochondria. Disabled SLC25A1 affects the transportation of citrate, causing abnormal distribution of citrate between cytosol and mitochondrial matrix that may underlie some diseases (Fernandez et al., 2018; Iacobazzi and Infantino, 2014; Infantino et al., 2014). *Slc25a7* (UCP1) is known for its role in BAT thermogenesis in mice and humans, and is considered as a potentially powerful treatment for obesity and metabolic diseases (Betz and Enerbäck, 2018; Chouchani et al., 2019).

Depletion of mitochondrial ATP transporter *Ant2* (*Slc25a5*) has been reported to damage erythropoiesis and B lymphopoiesis and lead to mouse death (Cho et al., 2015). However, liver-specific *Ant2* KO mice survived normally without any dysfunction in the liver. These mice were resistant to liver steatosis and obesity induced by HFD. Uncoupled with respiration, mitochondrial ADP/ATP exchange activity was severely decreased whilst mitochondrial contents were significantly increased in the liver-specific *Ant2* KO mice. They also had decreased serum levels of glucose, cholesterol and insulin, and increased levels of ketone bodies, suggestive of enhanced energy consumption and fatty acid oxidation (Cho et al., 2017). These results indicate that *Ant2* may be a potential target against liver steatosis and obesity through the interference of energy metabolism.

Conclusions and perspectives

The above studies provide many pieces of evidence for the relationship between transporters and lipid metabolism, and indicate these transporters are potential therapeutic targets for lipid metabolic diseases. An increasing number of new members and substrates will help us further understand the biological function and potential value of SLCs. As SLCs can control the entry of drugs into human cells, analysis of SLC structures and substrate conformations will also benefit rational drugs design and efficient drugs delivery. However, challenges remain for transporter studies, such as the technical barriers between research and qualified biological reagents. So far, it is still difficult to screen SLC substrates, to characterize their biochemical and biophysical properties, and to identify their transmembrane structures. New methods are needed to uncover the mysterious and complex connection between SLCs and lipid metabolism.

Regulation of hepatic gluconeogenesis

T2DM is a common and frequently encountered metabolic disease caused by multiple pathogenic factors. In the world, nearly 9% of the adult population suffers from this disease, which causes at least 1.5 million deaths daily (Rines et al., 2016). Moreover, diabetes significantly increases the risk of several other chronic diseases, including CVD, kidney disease, skin infections and sexual dysfunction, which severely affect people's health (Hossain et al., 2007; Wang et al., 2018c).

T2DM is characterized by hyperglycemia and insulin resistance (Saini, 2010). In normal individuals, increased insulin levels following feeding promote the uptake of blood glucose into peripheral tissues, such as skeletal muscle and adipose tissue. Subsequently, in the fasted state, gluconeogenesis and glycogenolysis contribute to maintain euglycemia and provide a sustained glucose supply to different cell types, such as neurons, red blood cells and renal medullary cells (Rizza, 2010). In T2DM, decreased insulin responsiveness leads to increased glucose production and decreased glucose consumption, thereby resulting in elevated blood glucose levels. Therefore, the maintenance of glucose homeostasis requires the correct balance between glucose utilization and production.

The liver is the major organ responsible for endogenous glucose production, especially by gluconeogenesis (with the kidney and the gut contributing a little) and glycogenolysis (Cano, 2002; Ekberg et al., 1999; Meyer et al., 2002; Mithieux et al., 2009; Moore et al., 2012; Radziuk and Pye, 2001). Excessive gluconeogenesis is an early event that induces high fasting blood glucose levels in individuals with T2DM (Consoli et al., 1989). Diverse mechanisms work together to regulate hepatic gluconeogenesis, including allosteric control by metabolites, endocrine control by hormones such as insulin, glucagon and corticosteroids, and redox control (Lin and Accili, 2011; Petersen et al., 2017). In this review, we mainly focus on discussing the hormonal regulation of hepatic gluconeogenesis and assess the current and potential drug targets for T2DM.

Overview of gluconeogenesis

Gluconeogenesis is a metabolic pathway contributing to the synthesis of glucose from diverse substrates, such as pyruvate, lactate, amino acids and glycerol during fasting (Figure 6) (Weber et al., 1964). In mammals, gluconeogenesis occurs mainly in the liver, which accounts for about 90% of the endogenously synthesized glucose, and the kidney, which accounts for about 10% (Ekberg et al., 1999; Lin and Accili, 2011; Meyer et al., 2002; Moore et al., 2012; Petersen et al., 2017). Gluconeogenesis is controlled by three rate-limiting enzymes: phosphoenolpyruvate carboxykinase (PEPCK), which catalyzes the decarboxylation and phosphorylation of oxaloacetate to generate phosphoenolpyruvate; fructose 1,6-bisphosphatase (FBPase), which converts fructose 1,6-bisphosphate to fructose 6-phosphate; and glucose 6-phosphatase (G6Pase), which generates glucose from glucose 6-phosphate (Figure 6) (Hers and Hue, 1983; Pilkis and Claus, 1991). The expression levels of PEPCK and G6Pase reflect the capacity of gluconeogenesis at the molecular level (Perry et al., 2018; Wang et al., 2010b). At the cellular level, glucose production can be measured directly (Wang et al., 2010b; Wang et al., 2012; Wang et al., 2009), while the pyruvate tolerance test and hyperinsulinemic-euglycemic clamps are effective methods for determining the ability of animals to produce glucose (Ayala et al., 2011; Han et al., 2015; Li et al., 2011; Zhang et al., 2018a).

Hormonal control of hepatic gluconeogenesis

Various hormonal stimuli can alter hepatic gluconeogenic flux (Figure 7). Insulin, secreted by pancreatic beta cells after feeding, can suppress gluconeogenesis by direct and indirect mechanisms (Hatting et al., 2018). In pancreatic alpha cells, insulin inhibits the secretion of glucagon, resulting in decreased expression of gluconeogenic genes in the liver (Altarejos and Montminy, 2011; Fisher and Kahn, 2003; Ravier and Rutter, 2005). Moreover, insulin suppresses lipolysis in white adipose tissue and proteolysis in the skeletal muscle, leading to reduced release of free fatty acids and amino acids respectively, thereby resulting in the decreased supply of gluconeogenic substrates (Girard, 2006; Sharabi et al., 2015).

Insulin can modulate hepatic gluconeogenesis through transcriptional regulation of gluconeogenic genes, including *Pepck* and *G6pase* (Barthel and Schmoll, 2003) (Figure 7). PI3K-AKT is the major intracellular pathway involved in the actions of insulin on hepatic gluconeogenesis (Lizcano and Alessi, 2002). Binding of insulin to its receptor results in the recruitment of PI3K to the PM, where it phosphorylates PIP2 to generate PIP3. Inhibition of PI3K by LY294002 and wortmannin were found to abolish the suppressive effect of insulin on *Pepck* and *G6pase* expression (Agati et al., 1998), which indicates that PI3K plays an important role in the insulin-induced suppression of gluconeogenesis. PIP3 increases the activity of PDK1, which can phosphorylate AKT at Ser473, leading to its activation (Hemmings and Restuccia, 2012; Lizcano and Alessi, 2002). Among the three AKT family members, AKT2 seems to play the major role in regulating gluconeogenesis. *Akt2* KO mice develop insulin resistance and other T2DM-like symptoms (Gonzalez and McGraw, 2009). AKT phosphorylates FOXO1, a transcrip-

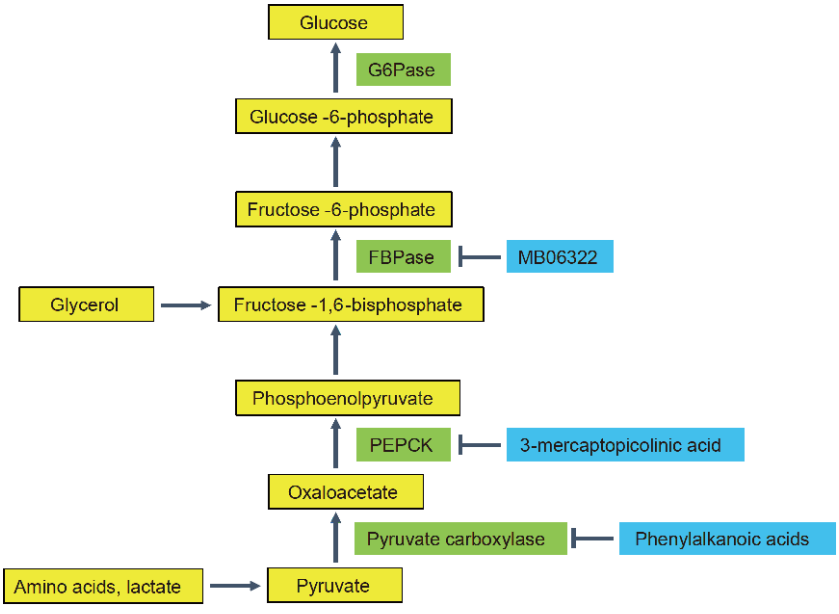

Figure 6 Key steps of gluconeogenesis. In gluconeogenesis, substrates like amino acids, lactate and glycerol can be converted to pyruvate or other intermediates (yellow boxes). Inhibitors (blue boxes) that target different enzymes (green boxes) attenuate gluconeogenesis and may have potential for treating T2DM.

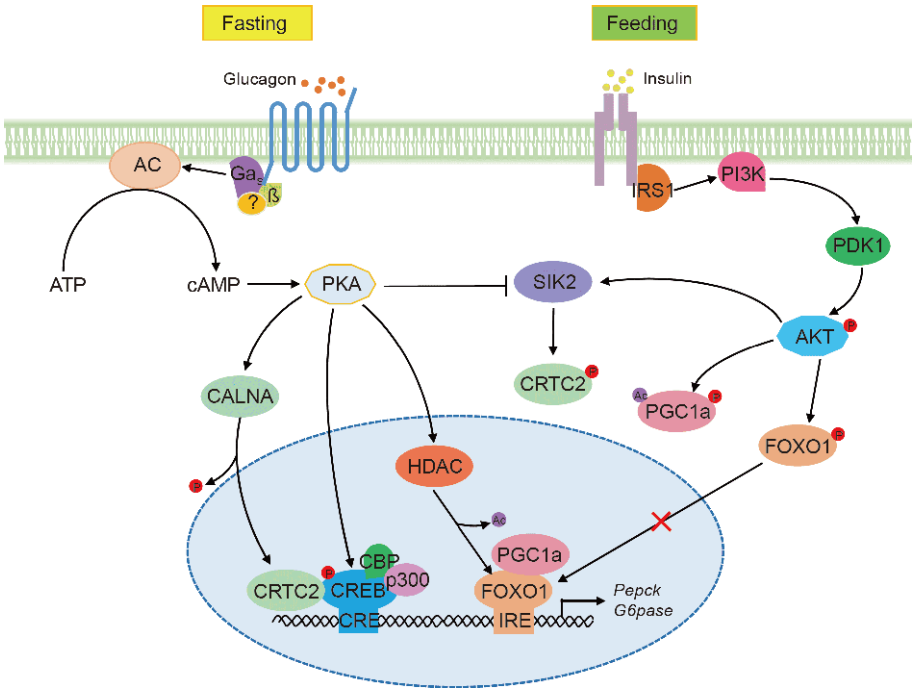

Figure 7 Transcriptional regulation of gluconeogenesis under feeding and fasting conditions. In the fasted state, glucagon binds to glucagon receptor, leading to the activation of $G\alpha_s$ and adenylate cyclase. Adenylate cyclase catalyzes the formation of cyclic AMP and the subsequent activation of PKA. Activated PKA can phosphorylate CREB directly at Ser133, promoting the recruitment of CREB with its co-activator CBP/p300 onto chromatin. In addition, activation of PKA leads to inhibition of SIK2 and the activation of Calcineurin, which results in dephosphorylation and nuclear translocation of CRTC2, thus enhancing the association of CREB with CRTC2 and the expression of gluconeogenic genes. Furthermore, following PKA activation, dephosphorylated HDACs translocate into the nucleus and deacetylate FOXO1, thereby enhancing its transcriptional activity. In the fed state, insulin binds to insulin receptor and activates the PI3K/AKT pathway. Activated AKT phosphorylates FOXO1, leading to its nuclear export and inactivation. Meanwhile, activation of AKT leads to the phosphorylation and acetylation of PGC1α, and phosphorylation of CRTC2, thereby suppressing the expression of *Pepck* and *G6pase*. AC, adenylate cyclase; CALNA, Calcineurin; IRS1, insulin receptor substrate 1; CRE, cAMP response element; IRE, insulin response element.

tion factor, at Thr24, Ser253 and Ser316, leading to its nuclear export and inactivation, with subsequent suppression of gluconeogenesis (Lu et al., 2012; Tikhanovich et al., 2013). Mechanistically, FOXO binds the insulin-responsive elements of the *Pepck* and *G6pase* promoters *in vitro* and enhances the expression of the associated genes (Ayala et al., 1999; Hall et al., 2000). Liver-specific *Foxo1* deletion leads to reduced gluconeogenic gene expression and fasting glucose levels (Matsumoto et al., 2007). In addition, phosphorylation of FOXO1 attenuates its interaction with PGC1α, which is a transcriptional coactivator shown to enhance the activity of FOXO1 and induce the expression of gluconeogenic genes in mice (Puigserver et al., 2003). Insulin can also modulate the activity of PGC1α by altering its acetylation and phosphorylation status. In mice, insulin activates the CDK4 complex, which promotes the acetylation of PGC1α by GCN5 acetyltransferase, thereby suppressing hepatic glucose production (Lee et al., 2014). Moreover, AKT can phosphorylate PGC1α at Ser570, which results in the decreased expression of gluconeogenic genes (Li et al., 2007).

Glucagon is a counterregulatory hormone of insulin. It is secreted by pancreatic alpha cells and promotes gluconeogenesis during fasting. Administration of small doses of glucagon is sufficient to increase blood glucose levels (Myers et al., 1991), and glucagon-neutralizing antibodies disrupt the action of glucagon on glucose production (Tan et al., 1985). Glucagon-mediated regulation starts with the activation of the glucagon receptor, followed by activation of Gαs. The activated Gαs increases the activity of adenylate cyclase, which subsequently increases intracellular cAMP levels and PKA activation (Lin and Accili, 2011) (Figure 7). Activated PKA can phosphorylate cAMP response element-binding (CREB) protein directly at Ser133 (Gonzalez and Montminy, 1989), which promotes the recruitment of CREB with its co-activator CBP/p300 onto chromatin (Arias et al., 1994; Chrivia et al., 1993; Kwok et al., 1994). CREB was found to induce the expression of gluconeogenic genes by binding to its target CRE sites in the gene promoters. Inhibition of CREB activity leads to reduced mRNA levels of *Pepck* and *G6pase*, and also to decreased blood glucose levels *in vivo* (Erion et al., 2009; Herzig et al., 2001). The transcriptional activity of CREB can be enhanced through binding with its co-activators, such as CBP/p300 and CREB-regulated transcription coactivator 2 (CRTC2). Under feeding conditions, CRTC2 is sequestered in the cytoplasm through phosphorylation-dependent interactions with 14-3-3 proteins (Altarejos and Montminy, 2011). Salt-inducible ki-

nases (SIKs) and other Ser/Thr kinases can phosphorylate hepatic CRTC2 at Ser171, Ser275 and Ser307 (Jansson et al., 2008; Koo et al., 2005; Saberi et al., 2009; Screaton et al., 2004). In response to fasting, increased glucagon leads to PKA activation and subsequent inhibition of SIK2 (Altarejos and Montminy, 2011). Meanwhile, PKA-dependent phosphorylation contributes to increased intracellular Ca^{2+} levels, which activate the CRTC2 phosphatase calcineurin, leading to subsequent dephosphorylation and nuclear translocation of CRTC2 (Wang et al., 2012). Enhanced association of CRTC2 with CREB seems to be sufficient to induce the expression of gluconeogenic genes. *Crtc2* KO mice with diet-induced obesity have fasting hypoglycemia and improved insulin sensitivity, while mice with constitutively active CRTC2 develop hyperglycemia (Hogan et al., 2015; Wang et al., 2010b). CBP/p300 seems to be essential in CREB-mediated regulation. CBP/p300 can be phosphorylated and thereby inactivated by SIK (Dentin et al., 2007). Insulin triggers phosphorylation of CBP at Ser436, which attenuates its interaction with CREB. Additionally, activated CBP/p300 acetylates CRTC2 at Lys628, which can stabilize CRTC2 and increase its activity (Liu et al., 2008).

During the early phase of fasting, CREB/CRTC2 are the central modulators of gluconeogenesis. Activation of CREB can also induce *Pgc1α* expression, and PGC1α, coupled with FOXO1, plays the major role in regulating hepatic gluconeogenesis during the later phase of fasting. In the liver, phosphorylation of Class IIa HDACs (HDAC4, 5 and 7) by AMPK family kinases causes them to be localized in the cytoplasm. In response to fasting, HDACs are rapidly dephosphorylated, then translocate into the nucleus and deacetylate FOXO1, thereby enhancing its transcriptional activity (Frescas et al., 2005; Mihaylova et al., 2011; Wang et al., 2014). Suppression of HDACs in diabetic mice leads to decreased fasting blood glucose levels and improved glucose tolerance, which suggests that Class IIa HDACs are required in glucagon-induced hyperglycemia (Mihaylova et al., 2011).

Glucagon can also regulate the activity of key enzymes involved in gluconeogenesis. Fructose-2,6-bisphosphate (F-2,6-P2) is an allosteric inhibitor of FBPase-1. Upon glucagon stimulation, activated PKA phosphorylates Ser36 of fructose-2,6-bisphosphatase (FBPase-2). This activates FBPase-2, resulting in decreased intracellular levels of F-2,6-P2, which in turn enhance the activity of FBPase-1 and promote gluconeogenesis (Kurland and Pilkis, 1995; Okar and Lange, 1999; Pilkis et al., 1982). Furthermore, glucagon has been shown to increase the activity of glucose-6-phosphatase, thus promoting hepatic gluconeogenesis (Band and Jones, 1980; Striffler et al., 1984).

Gluconeogenesis can be stimulated by other diabetogenic hormones, such as growth hormones, epinephrine and glucocorticoids. Both glucagon and epinephrine can promote gluconeogenesis by increasing the intracellular cAMP levels. However, glucagon is about 100-fold more effective than epinephrine (Exton and Park, 1968). Glucocorticoid activity has been associated with insulin resistance (Andrews and Walker, 1999). Upon fasting, glucocorticoids bind and activate glucocorticoid receptor, which induces the transcription of gluconeogenic genes (Zinker et al., 2007). Recent studies have discovered that hormones secreted by muscle or adipose tissue have profound effects on gluconeogenesis. Irisin, identified as an "exercise hormone" by Spiegelman's group, inhibits gluconeogenesis in the liver (Boström et al., 2012; Liu et al., 2015b). Another newly discovered hormone named asprosin, which is secreted by white adipose tissue in response to fasting, promotes gluconeogenesis through the OLFR734-mediated cAMP-PKA pathway (Li et al., 2019; Romere et al., 2016).

Targeting gluconeogenesis for the treatment of T2DM

Screening and designing inhibitors that target rate-limiting enzymes may be an effective strategy to reduce blood glucose levels. Indeed, phenylalkanoic acids inhibit pyruvate carboxylase, suppress hepatic gluconeogenesis and attenuate hyperglycemia in rats with streptozocin-induced diabetes, while inhibition of PEPCK by 3-mercaptopicolinic acid results in hypoglycemia (Bahl et al., 1997; DiTullio et al., 1974) (Figure 6). MB06322 (also known as CS-917) acts as an AMP mimetic which can inhibit FBPase-1 and attenuate hyperglycemia (van Poelje et al., 2006). However, phenylalkanoic acids lack tissue specificity and may inhibit insulin secretion, and some patients experienced nausea and vomiting after MB07803 treatment. Since the levels of F-2,6-P2 can potently affect blood glucose levels and insulin sensitivity, activation of PFK-2/FBPase-2 by specific molecules may be a good choice to treat T2DM (Wu et al., 2001, 2002).

Searching for receptor antagonists, agonists and antibodies to combat hyperglycemia, and developing them for eventual clinical use, is a research hotspot. MK-0893, a selective and reversible glucagon receptor antagonist, decreased glucagon-induced blood glucose levels in diet-induced obese mice (Xiong et al., 2012). Several glucagon receptor antagonists have been tested in clinical trials, including MK-0893. LGD-6972 decreased fasting blood glucose in normal and diabetic models, and a phase II clinical trial is underway to test its safety and efficacy. In addition, PF-06291874 and LY2409021 can decrease both fasting and postprandial glucose levels in patients (Kazda et al., 2016; Kazierad et al., 2016). Unfortunately, both PF-06291874 and MK-0893 can increase LDL-C levels, and the later clinical trials of MK-0893 were shut down. Agonists of insulin receptor, such as insulin glargine and insulin detemir, are widely used in patients with T2DM. Despite its high efficacy, insulin is difficult to preserve, and patients need to bear the pain of

injections. Recent work has shown that small-molecule agonists like DB03909 and 4548-G05 may have potential therapeutic use (Eliahu et al., 2010; Qiang et al., 2014).

Targeting transcriptional factors and co-activators seem to be an effective method for treating T2DM. Although it is difficult to selectively inhibit gluconeogenesis without altering other metabolic processes, some progress has been made. For example, a small molecule named ZLN005 can inhibit the transcriptional activity of PGC1α in the liver, resulting in decreased *Pepck* and *G6pase* expression and improved pyruvate tolerance, while mitochondrial function remains unchanged (Zhang et al., 2013). AS1842856 and AS1708727 have been shown to block FOXO1 activation, and were successful in decreasing blood glucose levels in *db/db* mice (Nagashima et al., 2010; Tanaka et al., 2010). Another small molecule named KG-501 can disrupt CREB-CBP interaction, thereby attenuating cAMP-responsive gene expression (Best et al., 2004). Manipulation of nuclear receptors may also be beneficial for treating T2DM. Treatment with SR9009, an agonist of REV-ERBα, leads to decreased blood glucose and body weight in diet-induced obese mice. The same effects have been seen when using SR3335, a RORα inverse agonist (Kumar et al., 2011; Solt et al., 2012). Additionally, glucocorticoid receptor antagonists, such as mifepristone and A-348441, have been shown to ameliorate diabetes (Zinker et al., 2007).

Metformin is the most commonly used therapy for T2DM and is known to inhibit hepatic gluconeogenesis. The precise mechanisms underlying its effects have not been well defined. It is possible that metformin leads to the phosphorylation and activation of AMPK, thereby reducing the expression of gluconeogenic genes via modulation of the CREB-CRTC2 complex (Shaw et al., 2005; Zhou et al., 2001). In addition, metformin may also inhibit the activity of mitochondrial complex I, resulting in an increase in the AMP/ATP ratio, which can activate AMPK and inhibit the activity of FBPase (Hardie et al., 2012; Shaw et al., 2005). It was reported that metformin suppresses hepatic gluconeogenesis by inhibiting glycerophosphate dehydrogenase (Madiraju et al., 2014). However, in *Ampk* KO mice, metformin continues to inhibit glucose production (Foretz et al., 2010). Therefore, the functional mechanism by which metformin inhibits gluconeogenesis is complicated and needs further investigation.

Conclusions and perspectives

T2DM is an epidemic disease, which seriously affects the health and the quality of life of many patients. Targeting hepatic gluconeogenesis is an effective strategy to ameliorate hyperglycemia in diabetes. Despite the existence of several antidiabetic drugs, there is still a need to develop new therapeutic reagents that specifically inhibit hepatic glucose production. More studies are necessary to figure out the regulatory mechanisms of gluconeogenesis and improve our understanding of the pathogenesis of T2DM. Key aims for future research include the discovery of new hormones and the development of drugs with tissue selectivity and clinical safety that target specific regulators involved in different pathways. Success in these areas will bring new hopes for the prevention and treatment of T2DM.

Human genetics and lipid metabolism

Blood lipid levels are a major contributor to the development of atherosclerotic cardiovascular diseases, fatty liver and insulin resistance (Kannel et al., 1961). Environmental factors such as western diet feeding and sedentary life styles accelerate hyperlipidemia. However, individual persons or different populations have variable susceptibility to those environmental factors. Current evidence suggests that the heritability for blood lipids is high, with 40%–50% for LDL-C, 40%–60% for HDL cholesterol (HDL-C), and 35%–48% for TAG (Kathiresan et al., 2007; Weiss et al., 2006).

Before the completion of the human genome project, family-based linkage analysis and candidate gene approaches are traditional ways to identify the genetic mutations related with certain phenotypes. After the human genome project is completed, genome-wide association study (GWAS) becomes the driving force in identifying the mutations associated with a variety of diseases, from dyslipidemia, diabetes, autism diseases to body weight, body height, etc.

Era before the human genome project

FH is a typical genetic-driven dyslipidemia that is inherited as autosomal dominant trait. It was first described as “inborn error of metabolism” by Dr. Carl Muller in 1938 (Muller, 1938). In the 1960s, Dr. Khachadurian found that there were two forms of FH, heterozygotes and homozygotes, and the pedigree structures were consistent with the dominant inheritance of a single gene (Khachadurian, 1964). Recent population studies suggest a worldwide prevalence of approximately 1 in 300–500 people for the heterozygous form of FH and 1 out of every 1,000,000 individuals for the homozygous form of FH (Goldberg et al., 2011).

The genetic defect in FH remains unknown until the 1970s, when Drs. Michael S. Brown and Joseph L. Goldstein started tackling this question at the University of Texas Southwestern Medical Center in Dallas (Goldstein and Brown, 2009). Combining cell biology and biochemistry, they first purposed the existence of LDLR, whose defect causes FH (Figure 8A) (Goldstein and Brown, 1974). Later on, they cloned the *LDLR* gene through sophisticated procedures and found over hundreds of mutations in the *LDLR* gene that

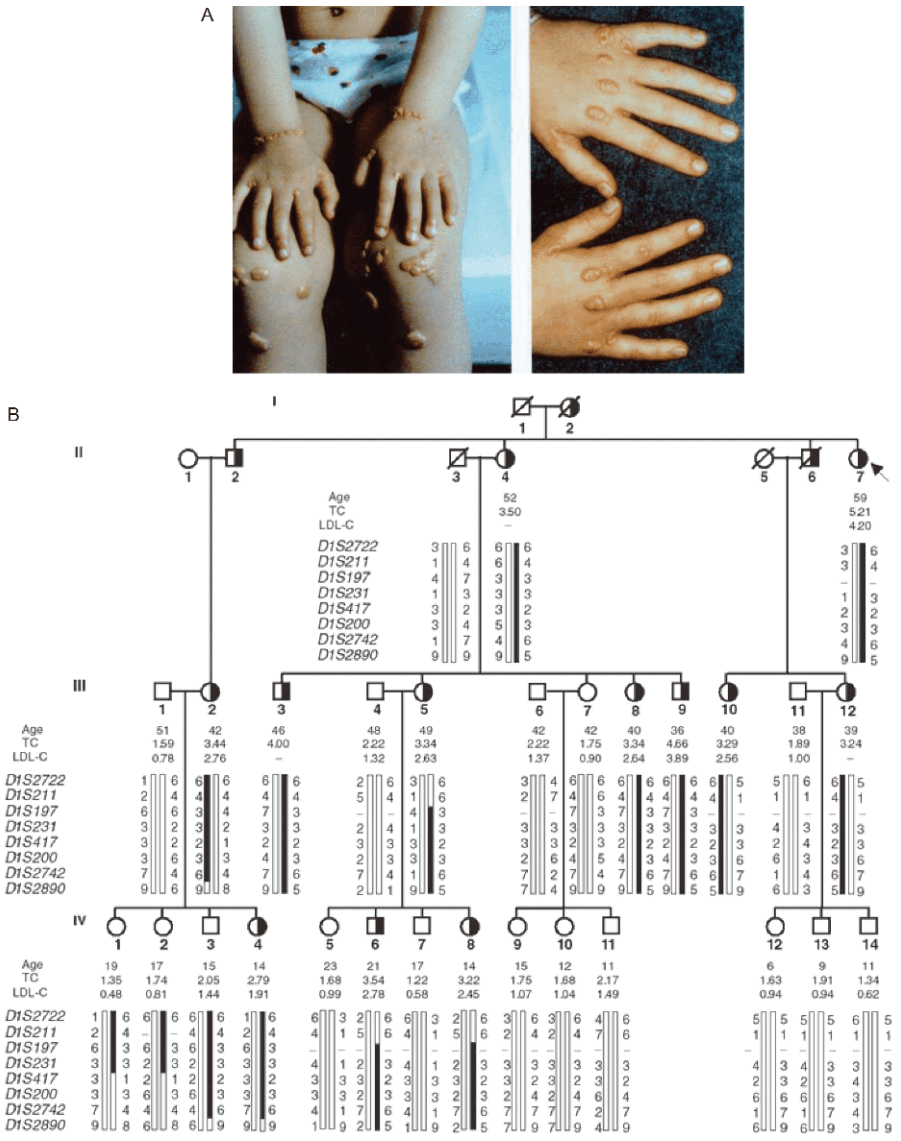

Figure 8 Characterization of FH. A, a 10-year-old girl with homozygous FH with mutations in *LDLR*. Adapted from Goldstein and Brown, 2009 with permission. B, Pedigree of family with *PCSK9* mutations. Adapted from Abifadel et al., 2003 with permission.

causes FH (Hobbs et al., 1992; Russell et al., 1983). Now more than 1,700 mutations in the *LDLR* gene have been reported, of which 79% display hypercholesterolemia phenotype (Usifo et al., 2012). Defects in *LDLR* account for more than 90% of FH cases (Austin et al., 2004).

The second FH gene is *APOB*, which encodes the major protein on LDL particle and functions as the ligand for LDLR. The first FH mutation of *APOB* was identified through candidate based gene sequencing (Soria et al., 1989). In contrast to the large number of variants identified in the *LDLR* gene, only a few variants have been characterized in the *APOB* gene so far. However, some of those variants, like *R3500Q*, have high prevalence, with 1 in every 1,250 persons. Defects in *APOB* account for about 5% of FH cases (Austin et al., 2004).

The third FH gene is *PCSK9*, which encodes a secretory protein that interacts with and targets LDLR to lysosome for degradation (Horton et al., 2007; Seidah et al., 2003). The earliest reported mutations of *PCSK9* were gain-of-function mutations, and they were identified through family-based linkage analysis (Figure 8B) (Abifadel et al., 2003). The gain-of-function PCSK9 mutants accelerate LDLR degradation and cause hypercholesterolemia. Later on, loss-of-function mutations for PCSK9 were discovered with target sequencing in hypocholesterolemia population (Cohen et al., 2005; Cohen et al., 2006). Those loss-of-function mutations protect against coronary heart disease by increasing LDLR level and accelerating LDL-C clearance, which lays the foundation for the development of PCSK9 inhibitors and will be promising LDL-C lowering drugs (Gouni-Berthold et al., 2016). Now, over 20 different mutations have been detected in *PCSK9* (Nordestgaard et al., 2013).

Autosomal recessive hypercholesterolemia is a type of hypercholesterolemia that is inherited recessively. Heterozygotes have similar levels of blood lipids to the general population, and the homozygotes have an intermediate between those of FH heterozygotes and homozygotes (Arca et al., 2002; Soutar et al., 2003). In 1973, Drs. Khachadurian and Uthman first described what is now called "autosomal recessive hypercholesterolemia" (Khachadurian and Uthman, 1973; Zuliani et al., 1999). Family-based linkage analysis and gene mapping lead to the identification of the *ARH* gene, which is localized to chromosome 1p35 (Garcia et al., 2001). *ARH* codes for a 308 amino acids adaptor protein that interacts with clathrin and the NPxY motif of LDLR. These interactions enable ARH to function as the endocytic adaptor for the clathrin-mediated LDLR in the liver (Garcia et al., 2001).

Sitosterolemia is another rare recessive hypercholesterolemic condition that is characterized by increased intestinal absorption and decreased biliary excretion of dietary sterols, hypercholesterolemia and premature coronary heart disease. It was first reported in the 1970s (Bhattacharyya and Connor, 1974). Candidate gene cloning and family-based linkage analysis lead to the identification of sitosterolemia genes adenosine triphosphate-binding cassette transporters *ABCG5* and *ABCG8* (Berge et al., 2000; Lu et al., 2001). *ABCG5* and *ABCG8* code for the ABC half-transporters that function as a complex and limit sterols absorption in the intestine and accelerates the sterols biliary excretion in the liver (Berge et al., 2000; Lu et al., 2001).

Family-based linkage analysis and candidate gene approaches are powerful ways to find the rare mutations with a large effect on blood lipid levels. However, those disease-related families are very rare, and heavily dependent on clinical diagnosis. Since the familiar hypercholesterolemia was first described as "inborn error of metabolism" by Dr. Carl Muller in 1938 (Muller, 1938), the inheritance of dyslipidemia has been extensively studied in European-ancestry population and other ancestry with large population, such as Chinese Han and East-Asian people. Some minority or people living in rural areas hold great promise to find novel rare mutations in dyslipidemia with family-based study. Recently, Zhang et al. analyzed a Chinese Kazakh family with a low level of LDL-C. Through whole-exon sequencing of these family members, they identified that mutations of the *LIMA1* gene decrease intestinal cholesterol absorption and cause low LDL-C (Zhang et al., 2018b).

Era after the human genome project

In the wake of the human genome project, strong expectations have been set for the discovery of new genetic mutations in a variety of human diseases. In contrast to the candidate-based gene sequencing and linkage studies that are well suited to identify rare variants with profound effects, GWAS studies are designed to ascertain the contribution of common genetic variants in population-wide diseases variability. For each GWAS, researchers scan and compare the whole genomes of people with and without a particular disorder (Figure 9A), which will point to SNPs or gene copy number variation that is strongly associated with the phenotype (Figure 9B).

The first GWAS was performed on genetic susceptibility to myocardial infarction in 2002 (Ozaki et al., 2002), right after the completion of the first human genome draft in 2000. The emergence of next-generation DNA sequencing technology and the cost-effective genome-wide genotyping arrays, together with increased sample size, have made the GWAS widely used in almost all types of human diseases, from dyslipidemia, CVD, diabetes to body weight, height, intelligence quotient, etc.

The first several GWAS studies of lipids were conducted on samples enriched with diabetic populations (Saxena et al., 2007; Heid et al., 2008; Kathiresan et al., 2008; Kooner et al., 2008; Sandhu et al., 2008; Wallace et al., 2008; Willer et al., 2008). Those studies identified a total of 19 loci associated with HDL-C, LDL-C and TAG. Those loci include some of the well-known genes that play key roles in lipid metabolism, for example, *ABCA1*, *APOB*, *CETP*, *HMGCR*, *LDLR*, and *PCSK9*, which confirmed GWAS as a valid approach to identify lipid-associated loci. In 2009, a comprehensive GWAS study was conducted on all four major serum lipid classes including HDL-C, LDL-C, TC and TAG. It covered 20,000 persons from 16 population-based cohorts. This study established 22 loci significantly associated with serum lipid levels, including 16 known loci and 6 newly identified loci (Aulchenko et al., 2009). In 2010, Teslovich et al. performed a meta-analysis of 46 lipid GWASs with totally more than 100,000 individuals of European descent. This study identified 95 loci with 59 showing genome-wide significance for the first time. Three of the novel genes *GALNT2*, *PPP1R3B*, and *TTC39B* were experimentally validated in mouse models (Teslovich et al., 2010). Three years later in 2013, the Global Lipids Genetics Consortium reported GWAS of lipid in over 180,000 European-ancestry individuals and over 7,000 non-European-ancestry individuals. This study identified 157 loci with genome-wide significance, with 62 found for the first time (Willer et al., 2013). Following this, a number of other GWAS of blood lipids were conducted with different population and larger cohorts, which totally identified more than 200 loci that showed genome-wide significant association (Dewey et al., 2016; Hoffmann et al., 2018; Klarin et al., 2018; Lange et al., 2014; Liu et al., 2017a; Lu et al., 2016a; Lu et al., 2016b; Lu et al., 2017; Surakka et al., 2015). GWAS also identified 69 miRNAs in physical proximity to SNPs associated with abnormal levels of circulating lipids and several of these miRNAs have been shown to control the

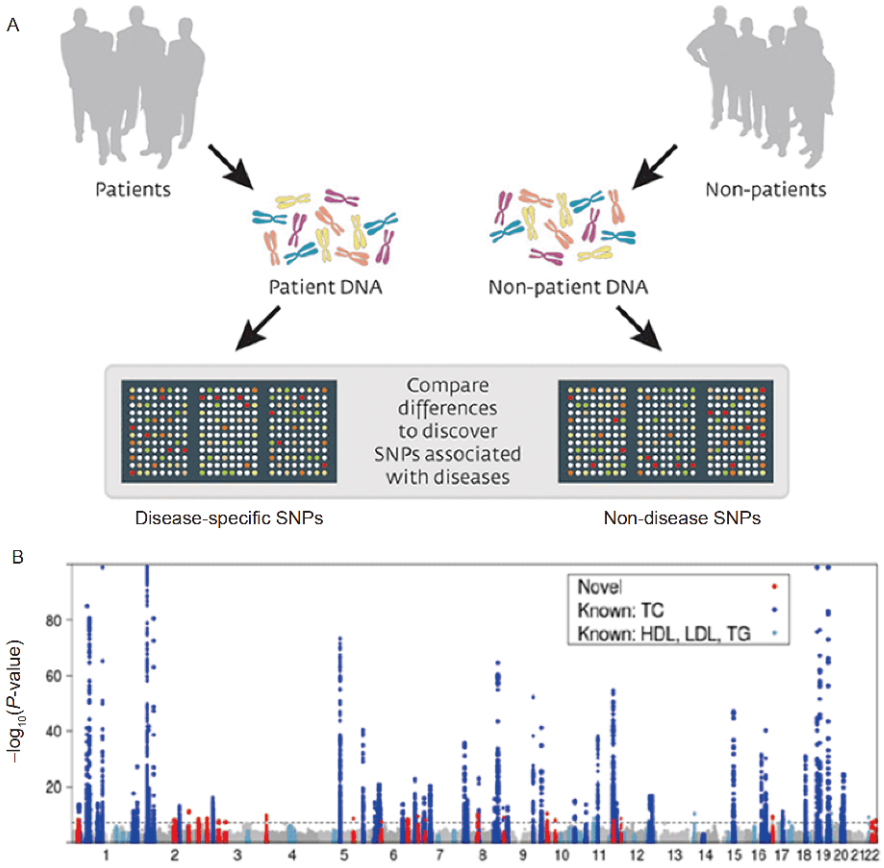

Figure 9 Identification of disease-associated SNP from GWAS. A, General overview of the Genome-Wide Association Study (image courtesy of Science Photo Library). B, Whole genome SNPs associated with TC. Adapted from Willer et al., 2013 with permission.

expression of the LDL receptor and ABCA1 (Wagschal et al., 2015).

Family-based linkage analysis and candidate gene approaches identified a number of key genes in the biology of lipoprotein for the past half century, such as *APOB*, *ABCG5*, *ABCG8*, *HMGCR*, *LDLR*, *PCSK9*, and *LPR1*. Almost all of these known genes localize in over 200 loci associated with blood lipid traits found through GWAS, which serves as a positive control for GWAS. However, among all the over 200 loci associated with blood lipids, about two-thirds of them harbor no documented gene known to play a role in lipid metabolism, which will be a gold mine to find the "novel" gene(s) or pathway(s) for lipoprotein metabolism.

Compared with the causal variants in Mendelian diseases, which typically confer large effects, the variants from GWAS are common and with modest effects for each of them. This is especially true for the large amounts of non-coding SNPs associated with blood lipids levels. Nevertheless, variants that explain a small proportion of blood lipid variation may provide substantial biological or therapeutic insights. Two of the examples are *HMGCR* and *NPC1L1*. The *HMGCR* gene encodes the rating limit enzyme for cholesterol synthesis and is the drug target of statin. The *NPC1L1* gene encodes a cholesterol transporter for cholesterol absorption in small intestine and is the drug target of ezetimibe. The common SNPs in the introns of *HMGCR* and *NPC1L1* confer a small effect on plasma LDL-C at 2.8 and 2.0 mg dL^{-1}, respectively (Teslovich et al., 2010). However, experimental evidence clearly demonstrated the critical roles of HMGCR and NPC1L1 in cholesterol homeostasis, and targeting these genes with statins or ezetimibe, respectively, has a much more profound effect on LDL-C. Until now, there is no rare, large-effect Mendelian mutation described in *HMGCR*, probably because *HMGCR* is so important that the deleterious mutations are not tolerated. Thus, some of the blood lipids associated genes may only be discovered through common, small-effect variants.

Current evidence suggests that the heritability for blood lipids is high, ranging from 35% to 60% (Kathiresan et al., 2007; Weiss et al., 2006). However, all the variants identified so far can only explain less than 50% of the heritability (Musunuru and Kathiresan, 2019; Teslovich et al., 2010). The provenance of the unaccounted heritability remains unknown. One reason is the existence of very rare variant (<1:1,000 frequency) (Bansal et al., 2010). Although its frequency is low, the effect could be high because of its

deleterious impact on phenotype. Those very rare variants could be identified with larger sample size. A second reason is the missing variant on the genotyping array used for GWAS or the biased reference genome, which is most derived from European descent. For example, there are about 6 million common variants in European populations, the commonly used Affymetrix 6.0 array only covers 900 k SNPs, leaving a large amount of SNPs un-genotyped (Raychaudhuri, 2011). With the progress of 1,000 genome project and cumulative genome sequence data from more diverse population, a new generation of genotyping array or whole genome sequence could be used for GWAS with blood lipid traits.

Challenges in post-GWAS era

Despite the success in identifying a large number of disease-associated loci, the current understanding of how SNPs predispose individuals to disease is very limited. First, there are a set of variants in linkage disequilibrium with the lead SNPs at each locus. Theoretically, any of those SNPs could be the causal variant, or one of multiple causal variants that are responsible for the association with disease. Second, at the whole genome level, more than 95% of disease-associated SNPs published in the literature sit in the non-coding region, with 41% localized in the introns and the left are in other regions that could be as far as 1 M base pairs away from the nearest gene body (Maurano et al., 2012). Third, the causal variant(s) must act upon one or more genes, microRNA or non-coding RNA, either through a local action within the locus or through long-range action on a more remote genome region. Fourth, the GWAS results tell the association, not the causality, with certain phenotypes. The identification of the causal gene(s) and underlying mechanism(s) need tremendous efforts with experimental evidence, which is far behind the progress of GWAS.

For common variants, fine-mapping through dense genotyping of markers in the region can identify the casual allele, or reduce the number of potential candidates. However, this method faces some challenges. First, in some instances, the association is being driven by a marker that has not been genotyped. Thus in order to fine-map effectively, dense genotyping all known markers in the region is critical. Second, in many instances there might be multiple causal alleles in the same region, and a large number of samples are required in order to have enough power to detect multiple effects (Raychaudhuri, 2011).

Systematic analysis found that most of the disease-associated non-coding variants are concentrated in the genome-active regions (Maurano et al., 2012). Those genome-active regions confer the promoter, enhancer and other regulatory elements. Identifying those regulatory elements can offer important complementary information to fine-mapping. Specific functional regulatory elements can be identified with DNase I sensitive sites which normally harbor promoters and enhancers. Histone modifications such as H3K4me1 mark enhancers, H3K4me3 marks active promoters, H3K4me2 and most histone acetylation mark both enhancers and promoters. With the development of ChIP-seq and DNase-seq, there are mounting public data on genome-wide chromatin profiles. The ChIP-seq and DNase-seq data from over 100 cell lines and tissues have now been generated through the ENCODE and Roadmap Epigenetics projects (Raychaudhuri, 2011).

Massively Parallel Reporter Assay (MPRA) is a recently developed method to simultaneously measure the reporter activity of thousands of oligonucleotides by deep sequencing. Normally, each plasmid contains one of the regulatory elements, an arbitrary open reading frame and a short identifying sequence tag. By constructing a library of such plasmids containing all the variations in a particular region, people will be able to measure the reporter activity by deep sequencing (Melnikov et al., 2012). This has been used to systematically dissect the function of common variants in red blood cell traits (Ulirsch et al., 2016) and offers important functional complementation to bioinformatics analysis.

Once the causal variant(s) was identified, the next challenge is to uncover the causal genes and mechanism. Expression quantitative loci (eQTL) are genome variants that correlate with the expression level of certain gene(s). It has been reported that the disease-associated common variants are significantly overlapped with eQTL, which suggests that many common disease variants act by changing the gene expression levels (Raychaudhuri, 2011). If a SNP in the loci is an eQTL, it suggests that it may be a specific disease-associated variant and the gene whose expression level is influenced by the risk allele might be the causal gene. Currently, the eQTL database containing over 60 separate tissues from over 600 deceased adult donors has been established (https://gtexportal.org/home/). One major challenge for eQTL analysis is that most of the eQTL are tissue-specific and may not be covered in the current database. In fact some of the eQTL may only be detectable with certain stimulus or stress. Nevertheless, the eQTL is a valuable complementary assay to MPRA and chromatin structure analysis. Patients derived induced pluripotent stem cells (iPSC) provide another opportunity to study the causal genes and underlying mechanism. Theoretically, iPSC could be differentiated into any cell types of interest for functional and mechanistic study. Gene editing, especially base editing on those cells provides another great promise to directly compare the functions of minor and major alleles (Warren et al., 2017).

Conclusions and perspectives

Human genetics is a powerful tool to identify gene(s) and

pathway(s) that predispose people to certain disease such as dyslipidemia. Both family-based linkage analysis and candidate gene approaches have uncovered a number of rare variants and key genes in lipid metabolism, which has greatly advanced our understanding of the genetic risk factors for dyslipidemia and lay the foundation for drug development. The GWAS has been shown successfully in identifying the genome loci associated with dyslipidemia. However, for the majority of the associated loci, the identities of causal variants and genes, as well as the function of these variants, remain unknown. With the newly developed methods, such as iPSC, gene editing, and MPRA, it holds great promise to solve those puzzles and identify new gene(s) and pathway(s) that function in the biology of lipid metabolism.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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