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Intracellular Cholesterol Transport Proteins: Roles in Health and Disease

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Abstract

Effective cholesterol homeostasis is essential in maintaining cellular function, and this is achieved by a network of lipid-responsive nuclear transcription factors, and enzymes, receptors and transporters subject to post-transcriptional and post-translational regulation, while loss of these elegant, tightly regulated homeostatic responses is integral to disease pathologies. Recent data suggest that sterol-binding sensors, exchangers and transporters contribute to regulation of cellular cholesterol homeostasis and that genetic overexpression or deletion, or mutations, in a number of these proteins are linked with diseases, including atherosclerosis, dyslipidaemia, diabetes, congenital lipoid adrenal hyperplasia, cancer, autosomal dominant hearing loss and male infertility. This review focuses on current evidence exploring the function of members of the 'START' (steroidogenic acute regulatory protein-related lipid transfer) and 'ORP' (oxysterol-binding protein-related proteins) families of sterol-binding proteins in sterol homeostasis in eukaryotic cells, and the evidence that they represent valid therapeutic targets to alleviate human disease. [144 words]

Short title Intracellular cholesterol transporters in health and disease

Keywords Cholesterol homeostasis; non-vesicular lipid trafficking; sterol-responsive transcription factors; Steroidogenic acute regulatory protein (StAR)-related lipid transfer domain; Oxysterol binding protein-related proteins

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Abbreviations

ABC	ATP binding cassette transporter
ACAT-1/2	Acyl CoA: Cholesterol Acyl Transferase (1/2)
AMPK	5'-AMP activated protein kinase
Apo	Apolipoprotein
ApoER2	Apolipoprotein E receptor-2
ATF6	Activating transcription factor 6
CE	Cholesteryl esters
CYP7A1	Cytochrome P540 family 7 subfamily A member 1
CYP27A1	Cytochrome P450 family 27 subfamily A member 1
DGAT	Diacylglycerol acyltransferase
ERBB2	ERBB2 receptor tyrosine kinase 2 (CD340)
ERC	Endocytic recycling compartment
ERSE	ER stress response element
FFAT	Two phenylalanines in an acid tract (domain)
GAP	GTP activating protein
GATA	'GATA' binding transcription factor
HDAC5	Histone deacetylase 5
HDL	High density lipoprotein
HMCGR	3-Hydroxy methyl glutaryl CoA reductase
HOPS	HOPS complex: 6 subunits of VPS (vacuole protein sorting) proteins [VPS11, VPS16, VPS18, VPS33, VPS39 and VPS41]
IDOL	Inducible Degrader of the Low Density Lipoprotein Receptor
IMM	Inner mitochondrial membrane
INSIG-1/2	Insulin Induced Gene (1/2)
Lam	Lipid transfer protein anchored at membrane contact sites
LCAT	Lecithin Cholesterol Acyl Transferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LRP	LDL receptor related protein
LXR	Liver X receptor
MENTAL	MLN64 N-terminal domain
MENTHO	STARD3 N-terminal like protein
MLN64	STARD3
NASH	Non-alcoholic steatohepatitis
NLRP4	NLR family, pyrin domain-containing 4
NPC-1/2	Niemann Pick disease type C1/2
Nup62	Nucleoporin 62kDa
OMM	Outer mitochondrial membrane
ORD	Oxysterol binding protein-related ligand-binding domain
ORP	Oxysterol binding protein related protein
OSBP	Oxysterol binding protein
PCSK9	Proprotein convertase subtilisin kexin type 9
PH	Pleckstrin homology (domain)
PI-3P	Phosphatidylinositol 3-phosphate
PI-4P	Phosphatidylinositol 4-phosphate
PM	Plasma membrane
PPAR	Peroxisome proliferator activated receptor
RAB7	Ras related protein Rab7a
RCT	Reverse cholesterol transport
RILP	Rab-interacting lysosomal protein

RXR	Retinoid X receptor
SCAP	Sterol regulatory element binding protein (SREBP) cleavage activating protein
SCP-2	Sterol Carrier Protein-2
SIRT1	Sirtuin 1
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
SQS/FDFT	Squalene synthase/farnesyl-diphosphate farnesyltransferase 1
SPAG5	Homo sapiens sperm associated antigen 5
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
StAR	Steroidogenic acute regulatory protein
STARD	StAR-related domain
START	StAR-related lipid transfer protein
SUMO-1	Small ubiquitin-like modifier 1
VAMP	Vesicle-associated membrane proteins
VAP	VAMP associated proteins
VLDLR	VLDL receptor
XBP1	X-box binding protein 1

INTRODUCTION

Cholesterol is the precursor for bile acids, steroid hormones and vitamin D, which regulate the absorption of dietary lipid, glucose metabolism, kidney and reproductive function and bone homeostasis, while imbalanced cholesterol metabolism results in embryonic and adult disease (recently reviewed by Cortes *et al* (2014) [1]. Aberrant serum and tissue cholesterol levels are caused by rare genetic disorders, and form part of the pathogenesis of more prevalent sporadic and complex disorders, including coronary heart disease, stroke, diabetes mellitus and Alzheimer's disease. Effective cellular cholesterol homeostasis is achieved by lipid-responsive transcription factors, and an array of post-transcriptional and post-translational mechanisms, controlling the biosynthesis, efflux (egress), oxidative metabolism, storage and uptake (ingress) of this sterol.

Biosynthesis of cholesterol occurs in the endoplasmic reticulum, from whence this sterol is directed to the Golgi apparatus and plasma membrane (PM), or esterified via Acyl CoA Cholesterol: Acyl Transferase (ACAT-1/2) and stored as cytosolic lipid droplets of cholesteryl ester (CE), recently reviewed by Rogers *et al* (2015) [2] and Khor *et al* (2013) [3]. Transcriptional regulation of cholesterol synthesis and uptake involves Sterol Regulatory Element Binding Proteins (SREBPs)[4], a class of transcription factors belonging to the basic helix-loop-helix leucine zipper (bHLH-Zip) family. Two genes, *SREBF-1* and *-2*, code for three distinct SREBP isoforms: -1a, -1c and -2. SREBP-1a is constitutively expressed in the cell as a 'low-specificity' regulator, targeting all genes that contain sterol response elements (SRE)[5]. By contrast, SREBP-1c and SREBP-2 are inducible and promote the expression of genes encoding proteins involved in fatty-acid and cholesterol metabolism, respectively [5] (**Figure 1A**).

The gene expression of *Srebf-2* is regulated in a 'feed-forward' manner, with SREBP-2 binding to the SREs present within its own promoter [6], and SREBPs are subjected to multiple levels of post-translational regulation [7-12]. Following translation, and in the presence of adequately high levels of intracellular sterols, SREBPs are inactive and retained within the endoplasmic reticulum by SREBP cleavage activating protein (SCAP), a chaperone protein characterised by a five transmembrane sterol-sensing domain, which interacts with the endoplasmic reticulum anchor, Insulin Induced Gene (INSIG-1/2). Conversely, when sterol levels within the cell fall, binding of INSIG to SCAP is disrupted, leading to the inclusion of the SCAP-SREBP complex in COPII-vesicles, and subsequent transfer to the Golgi apparatus. Golgi-resident site-1 and site-2 proteases (SP-1 and SP-2) cleave and release the amino terminal of SREBP [12, 13], the transcriptionally active portion that travels to the nucleus and targets sterol-responsive genes; this fragment is subsequently rapidly degraded to terminate this signalling pathway. Further, the stability of SREBP proteins is modulated by acetylation, increased by p300 [7], and reduced by SIRT1, which favours the rapid degradation of SREBPs [8]. Deacetylation of SREBPs facilitates the action of ubiquitin ligases, thus resulting in proteosomal degradation [9, 10], while SUMO-1 mediates degradation of SREBPs independently of the 26S proteasome [11].

Collectively, SREBP-2 regulates most the enzymes of the mevalonate pathway, which produces cholesterol as well as essential isoprenoids used for the synthesis of hormones and signalling molecules [14]. The rate limiting step in this pathway is mediated by 3-Hydroxy Methyl Glutaryl CoA Reductase (HMGCR) which is transcriptionally regulated by SREBP-2, but also by negative feedback [recently reviewed by Ness, 2015 [15]). The HMGCR protein undergoes ubiquitination upon accumulation of sterol intermediates (mainly lanosterol and 24,25-dehydrolanosterol) and cholesterol metabolites such as oxysterols[16-18], ultimately leading to its proteasomal degradation; squalene synthase (SQS) is also negatively regulated by accumulation of cholesterol in a similar manner. The activity of HMGCR is also controlled by 5' AMP-activated protein kinase (AMPK), that senses the energy status of the cell, which suppresses sterol synthesis following increases in the AMP/ATP ratio [19].

The transcription of SREBPs also stimulates the expression of intronic micro-RNAs -33a and -33b, which lie within the genomic sequence of *Srebf-2* and *Srebf-1*, respectively[20], which suppress the translation of ATP binding cassette transporter A1 (ABCA1) (*below*), reducing the efflux (egress) of cholesterol from the cell. This response ensures that newly synthesized cholesterol remains within the cell, rather than effluxed to extracellular acceptors. Indeed, a number of microRNA sequences are involved in regulation of the cholesterol homeostasis, either directly, by repressing the expression of genes and proteins involved in cholesterol synthesis and transport, or indirectly, by modulating the lipid metabolism and energy status of the cell [20, 21] and recently reviewed by Jeon and Osborne, 2016 [22] and DiMarco and Fernandez (2015) [23].

Cellular cholesterol can also be derived from the extracellular medium or the bloodstream by endocytosis of lipoproteins, such as low density lipoprotein (LDL) and other apoE-containing lipoproteins, via an array of receptors, including the LDL receptor (LDLR), LDL receptor related protein (LRP1 (CD91; α-2-macroglobulin receptor), LRP2 (GP300; megalin), LRP5 (LRP7), LRP6, LRP8 (apoE receptor 2) and the VLDL receptor (VLDLR) [24, 25]. Receptor-lipoprotein complexes are internalised via clathrin-coated vesicles, delivering LDL and other lipoproteins to the endocytic pathway, resulting in degradation of these complexes by hydrolytic enzymes in acidic (endo)lysosomal vesicles. Cholesterol leaves the endocytic pathway by the action of proteins that mediate its stepwise removal, as cargo associated with budding vesicles, or by intramembrane exchange with acceptor organelles via membrane contact sites. The first steps in protein-mediated egress of cholesterol are mediated by Niemann-Pick disease type-C1 and type-C2 proteins (NPC-1/2)[26,27], localized in the membrane and the lumen of late endosomes, respectively. NPC1 is thought to deliver cholesterol molecules to cytoplasmic transporters, which in turn supply this sterol to mitochondria and endoplasmic reticulum [28-36]. Small GTPases belonging to the Rab family regulate vesicle transport, by facilitating interactions with the actin filaments of the cytoskeleton [37-40], directing lipoprotein-derived cholesterol to intracellular organelles as well as recycling back to the plasma membrane [28].

The distribution of lipoprotein-derived cholesterol to organelles triggers the activation of counter-regulatory pathways. Delivery of cholesterol to the endoplasmic reticulum sequesters SREBPs at this site, by interaction with SCAP and INSIG, curtailing the generation of active SREBPs, while increased trafficking of cholesterol to mitochondria sterol 27-hydroxylase facilitates production of oxysterols, which act as ligands for nuclear transcription factors, Liver X Receptors (LXRs) [reviewed in 41-43]. These nuclear receptors heterodimerize with Retinoid X Receptors (RXRs), operating in functional opposition to SREBPs, reducing the expression levels of *Ldlr* and the enzymes in the mevalonate pathway (*above*)[14, 44]. Liver X Receptors also promote the protein degradation of LDLR, by increasing the expression of Proprotein convertase subtilisin/kexin type 9(PCSK9)[45] and the action of E3 ubiquitin ligase, inducible degrader of the low-density lipoprotein receptor (IDOL)[46].

Activation of LXRs also stimulates the reverse cholesterol transport (RCT) pathway [41-43], which removes excess cholesterol from cells and tissues, returning this sterol via high density lipoproteins (HDL) to the liver, reviewed by Rye and Barter (2014) [47] and Wang and Smith (2014) [48]. Oxysterol ligation of LXR/RXR heterodimers increases the transcription of ABCA1 and ABCG1/ABCG4, which efflux cholesterol to extracellular acceptors, apolipoprotein A-I (apoA-I) and HDL, respectively. The transcription of apolipoprotein E (ApoE) is also increased by ligation of LXRs, which can act as an extracellular acceptor of cholesterol, and enhance the efficiency of the RCT by stimulating the action of lecithin-cholesterol acyltransferase (LCAT), an HDL-bound enzyme that promotes the extracellular conversion of cholesterol to CE in the circulation[49], creating a concentration gradient favouring the continued removal of free cholesterol from tissues in the periphery. Removal of cholesterol from the body is achieved by hepatic conversion to bile acids: activated LXR/RXR heterodimers stimulate the expression of rate-limiting enzyme CYP7A1, resident in the ER [50],of mitochondrial Cyp27A1 responsible for the 'acidic' or alternative pathway,

and the half-transporters ABCG5 and ABCG8 which efflux cholesterol into bile [51] and recently reviewed by Li *et al.*, 2013 [52]. Mutations in ABCG5/8 cause β-sitosterolaemia: the accumulation of cholesterol and plant sterols which can eventually lead to premature coronary disease [51, 52].

The loss of these elegant, tightly regulated homeostatic responses plays an integral role in a number of disease pathologies, including metabolic diseases, tumourigenesis and disorders of the central nervous system. Within the arterial intima, for example, oxidation or enzymatic modification of LDL leads to recognition of modified LDL by scavenger receptors, including scavenger receptor (SR-)AI/AII, Cluster of Differentiation (CD)36, CD68 and lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1), leading to CE accumulation in LD and activation of inflammasomes [53-56]. Cholesterol and CE-laden macrophage 'foam' cells characterise early 'fatty streak' atheroma, while their apoptosis or necrosis contribute to the extracellular lipid core and cholesterol crystals found within more advanced lesions [53-56].

Cholesterol crystals are also found within hepatocyte lipid droplets in non-alcoholic steatohepatitis (NASH). These dead cells are removed by Kupffer cells, loading these cells with lipid and triggering the inflammatory responses linked with progression of steatosis to NASH [57-59]. Osteoporosis and deficient bone repair has been linked to elevated serum cholesterol [60-62], while cholesterol-laden histiocytes characterise the rare multi-system granulomatosis noted in Erdheim-Chester disease [63]. Lipid accumulation within alveolar macrophages is associated with pulmonary inflammation [64], while patients with pulmonary alveolar proteinosis display alveolar 'foam' cells, due to functional loss of granulocyte macrophage colony stimulating factor [65].

The supply of excess cholesterol and the provision of intermediates from the mevalonate pathway also appear to be central to the high rate of proliferation of some cancer cells, reviewed in [66] and more recently by Kuzu *et al.*, 2016 [67]. For example, cholesterol biosynthesis via HMGCR is enhanced in gastric cancer tissues: overexpression of this enzyme promoted growth and migration of gastric cancer cells, while knockout of HMGCR achieved the reverse [68]. Dietary cholesterol has also been linked with promotion of colorectal cancer, via activation of the NLR family, pyrin domain-containing 4 (NLRP4) inflammasome [69], and statin inhibitors of HMGCR have been proposed as anti-tumourigenic agents [reviewed in 70]. By contrast, activation of LXRα blocks the growth of human colorectal cells and of xenograft tumours in mice; protection is also provided against intestinal tumour formation after treatment with chemical carcinogens [71]. Further, while hypermethylation of *ABCA1* is associated with accelerated cell growth and poor prognosis in ovarian cancer patients [72], induction of ABCA1 expression and cholesterol efflux follows treatment with DNA topoisomerase II inhibitors, chemotherapeutics used in the treatment of various cancers [73].

Finally, loss of effective cholesterol homeostasis is a feature of ageing and of diseases of the central nervous system (CNS) [74-78] and a recent review by Courtney and Landreth (2016) [79]. ApoE is lipidated by astrocytes and glia within the CNS, generating lipoproteins that provide cholesterol to support the function of neuronal cells, and aid the transport and clearance of amyloid β peptide [reviewed in 74], and dysregulated cholesterol metabolism is an established feature of Alzheimer's disease, Parkinson's disease and traumatic central nervous system injury [74-79]. The neurodegenerative disorder, Huntington's disease, caused by the mutant huntingtin protein (mHtt) [77] is associated with loss of nuclear SREBPs and defective activation of LXR, resulting in lower cholesterol biosynthesis and reduced apoE-dependent transport of cholesterol from astrocytes to neurons [77]. Genetic defects in endosomal Niemann-Pick type C1 (NPC1) protein cause neurological dysfunction [78], and loss-of-function mutations in ABCA1, which cause low or absent levels of HDL, are thought to contribute to peripheral neuropathies in patients with Tangier disease [80].

INTRACELLULAR TRANSPORT OF CHOLESTEROL

Inter-organelle lipid transport occurs via the movement of transport vesicles, by transport by lipid binding/transfer proteins, and by diffusion across closely apposed membrane leaflets, although the major portion of inter-organelle transport seems to occur via non-vesicular mechanisms [28-32; 81,82]. In particular, it seems that transfer of lipid molecules over the narrow gap between lipid bilayers at membrane contact sites is a specific and efficient means of achieving lipid transport (**Figure 1B**).

A number of proteins, including Sterol Carrier Protein-2 (SCP-2) and the families of steroidogenic acute regulatory protein (StAR)-related domain lipid transfer (START) proteins, and oxysterol binding protein-related proteins (ORPs), are thought to contribute to the non-vesicular transport of cholesterol within the aqueous environment of the cytosol, and between organelles and the plasma membrane, sustaining the sterol gradient that exists between the endoplasmic reticulum and the plasma membrane [81-83]. Until around a decade or so ago, the role of most of these proteins remained relatively uncharacterised. However, it is becoming increasingly clear that sterol-binding, 'sensing' and trafficking proteins influence the expression and activity of sterol-responsive transcription factors, regulating the expression of genes encoding proteins which synthesize, metabolise and efflux cholesterol, and direct the trafficking of cholesterol between the plasma membrane, mitochondria, Golgi and endosomal and lysosomal compartments, defining the fate of this lipid [81-83] (**Figure 1B**). This review will focus on recent data exploring the function of the START and ORP proteins in cholesterol homeostasis in eukaryotic cells, and the evidence suggesting that they represent valid therapeutic targets to alleviate disease pathologies.

Human START proteins are a family of fifteen members, sharing a conserved 210-residue hydrophobic binding pocket, further classified into six sub-groups according to the degree of sequence homology of this StAR-related lipid transfer domain [84]. The structure of the START domain has been resolved for eight START proteins (STARD1 to D5, -D11, D13 and D14), showing that a single lipid molecule is accommodated in an hydrophobic cleft which arises from the spatial organization of 9 anti-parallel β-sheets arranged to form a hollow concave profile, closed at its extremities by two α-helices, while a third α-helix operates as 'lid', allowing the ingress and egress of the lipid molecule [85-89]. Additional domains are found in seven STARTs, specifically involved in determining their subcellular localization as well as conferring additional physiological functions [84].

The STARD1 subgroup is composed of StAR/STARD1 and STARD3. STARD1, the prototypic member of this family, possesses a mitochondrial targeting sequence and supplies cholesterol substrate for conversion to pregnenolone [90-92], while STARD3 localizes to the late endosomes, where it participates in the extraction of cholesterol as well as regulating their spatial organization [36, 39, 93]. The other subgroups of cholesterol-binding proteins (STARD4) contains STARD4, STARD5 and STARD6, which are devoid of targeting sequences and may operate as cytosolic cholesterol exchangers between membranes, their directionality being regulated by the intracellular gradient of sterols [35, 94, 95]. The STARD2 subgroup (STARD2, STARD10, STARD11/CERT) binds phospholipids and ceramides [96-99] while STARD9 possesses an N-terminal kinesin motor capable of interacting with and moving along microtubules [100, 101]. The other subgroups include Rho-GAP STARTs (STARD12, STARD13), which possess a Rho-GTP activating protein (GAP) domain involved in regulating the activity of Rho-GTPases and the cytoskeleton [102], and the thioesterase STARTs, STARD14 and STARD15, which hydrolyse long chain fatty acyl CoA and acetyl CoA, respectively [102-105].

By contrast, the family of human oxysterol binding protein (OSBP)-Related Proteins (ORPs) comprehends twelve distinct members (ORP1-12) divided into six subfamilies, with some ORPs occurring as short (S) and long (L) variants [reviewed in 106]. OSBP, the first ORP to be identified and characterized, was originally described as a 25-hydroxycholesterol transporter, and later shown to be able to bind other oxysterols, as well as cholesterol and ergosterol [106]. Ligands, which can include glycerophospholipids, are accommodated within an OSBP-related ligand-binding domain

(ORD) that is arranged as a hydrophobic β-barrel where the sterol is accommodated with the 3β-hydroxyl group oriented inwards and enclosed on the distal side by an N-terminal lid [106]. Most ORPs are found in association with the membranes of adjacent organelles and are constituents of membrane contact sites. The ORPs possess a pleckstrin homology (PH) domain, which can interact with phosphoinositide containing membranes, and a "two phenylalanines in an acid tract" (FFAT) domain, which binds to proteins (VAPs) associated with vesicle-associated membrane proteins (VAMPs)[106, 107]. Further, ORPs can interact with membrane-associated regulatory proteins, including small GTPases Arf1 and Rab7 [106]. The concept of 'dual targeting' describes the ability of ORPs to target two distinct membranes via these distinct binding sites, and in recent years it has become clear that this property may be critical in facilitating bi-directional lipid transfer at protein complexes found at membrane contact sites between differing organelles. Indeed, ORPs are thought to utilise the energy stored within the intracellular gradient of phosphatidylinositol 4-phosphate (PI-4P) to power the transport of sterols or other lipids against their concentration gradient at inter-organelle bridges [106].

Sterol-binding proteins: mitochondria

Steroidogenic acute regulatory protein (StAR; STARD1) plays a well-established, obligate role in the acute steroidogenic response regulated by StAR-dependent delivery of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), facilitating generation of pregnenolone from cholesterol by cytochrome P450scc (CYP11A1) [90-92] and reviewed extensively in 108-112]. StAR is synthesized as a 37kDa protein, with a mitochondrial leader sequence that is subsequently cleaved following mitochondrial entry to yield a 30kDa intra-mitochondrial protein. Overexpression of StAR increases steroidogenesis, primarily via its activity at the OMM, where a physical interaction between the C-terminal helical 'lid' and the membrane is required to allow release of cholesterol to the membrane [108]. Notably, loss-of-function mutations in StAR cause the autosomal recessive disorder, congenital lipoid adrenal hyperplasia, characterised by minimal production of all steroids and grossly enlarged adrenals filled with cholesterol and CE [110, 113], a phenotype replicated in StAR knockout mice [114].

At least 48 different mutations in *StAR* have been identified in various ethic groups, that typically cause severe adrenal failure in early infancy, although delayed onset of adrenal insufficiency has also been reported in some individuals [115]. Genetic clusters consistently occur in Japanese and Korean populations (p.Q258X), Palestinian Arab (p.R182L), eastern Saudi Arabian (p.R182H) and Swiss (p.L260P) populations [115]. Affected infants have low, but measurable, levels of steroid hormones, but unless hormone replacement is initiated soon after birth, the condition proves lethal during the first year of life due to loss of glucocorticoid and mineralocorticoid production and salt loss [116, 117]. Loss of StAR causes defective foetal testicular biosynthesis: affected 46,XY males are born with feminised genitalia reflecting the loss of androgen synthesis between 6 and 12 weeks of gestation [115]. Female 46,XX subjects with congenital lipoid hyperplasia are also affected at puberty, when ovarian steroidogenesis is programmed to initiate; loss of steroidogenesis and accumulation of cholesterol and cholesteryl esters leads to cellular damage, anovulatory cycles, polycystic ovaries and and ovarian failure [115, 117].

More recently, it has become evident that StAR is expressed and functional in non-steroidogenic cells, albeit at substantially lower levels than in adrenal or gonadal tissues [119]. For example, the expression of StAR is noted in endothelial cells, monocytes, macrophages and human aorta, and subject to regulation by cytokines, LXR, peroxisome proliferator activated receptor γ (PPARγ) and RXR agonists, and by modified LDL [120-122]. Notably, overexpression of StAR promotes cholesterol efflux to apoA-I and/or HDL, by enhancing the formation of oxysterols by mitochondrial cytochrome P450 enzyme, CYP27A1, activation of LXRα, and induction of ABCA1/ABCG1 mRNA and protein [34,123-125]. However, under pathological conditions, macrophage StAR can transfer

damaging cholesterol hydroperoxides to mitochondria, causing lipid peroxidation and loss of mitochondrial membrane polarisation, and reduced expression of ABCA1 [125]. The systemic effects of StAR are evident in apoE$^{-/-}$ mice fed a high fat/cholesterol diet, which display reduced levels of hepatic and hepatic and aortic lipids and decreased atheroma following tail vein administration of an adenoviral vector expressing StAR [126]. Furthermore, in primary hepatocytes overexpression of StAR enhances bile acid synthesis via CYP27A1 [127], while in rat hepatoma cells StAR overexpression increased utilisation of exogenous (pre-formed) fatty acids for the synthesis and secretion of triacylglycerol; these changes were associated with increased gene expression of diacylglycerol acyl transferase-1 (*Dgat1*) and *Dgat2* [128].

Until very recently, no ORPs had been observed at endoplasmic reticulum-mitochondrial contact sites, and no function ascribed for ORPs in endoplasmic reticulum-mitochondrial transport, biogenesis, division or fusion. However, Galmes *et al* (2016) have now described the localisation of ORP5 and ORP8 at endoplasmic reticulum-mitochondria contact sites, transporting phosphatidylserine to the mitochondria for subsequent synthesis of phosphatidylethanolamine [129]. This localisation requires a functional ORD, and involves interaction with the OMM protein, PTPIP51. Notably, depletion of ORP5 and ORP8 (discussed further below) led to defects in mitochondrial morphology and mitochondrial respiration [129].

Sterol-binding proteins: cytosolic

The STARD4 subgroup of START proteins, STARD4, STARD5 and STARD6, consist predominantly of a 205-233 amino acid START domain [31, 35, 94, 95]. StarD4 was discovered as a gene whose expression decreased >2-fold in the livers of mice fed a high cholesterol diet [130], and together with StarD5 and StarD6 constitute a subfamily sharing approximately 30% amino acid identity with one another [83, 84; 116]. Recently, it has become clear that the conserved residues of STARD1 (above), STARD3 (below), STARD4, STARD5 and STARD6 STARD create the global protein fold, those lining the ligand binding site diverge, conferring specificity in ligand binding [94, 95, 131].

Levels of StarD4 mRNA and protein are repressed by the accumulation of cholesterol in fibroblasts and liver tissues, StarD4 is an established target of SREBP-2, and genetic obesity in rodents is associated with reduced hepatic expression of StarD4 [31, 35, 94, 95, 131-133]. Endoplasmic reticulum stress has also been reported to induce STARD4 via Activating transcription factor 6 (ATF6) and an endoplasmic reticulum stress response (ERSE)-like element within its promoter [95]. The interaction of STARD4 with membranes, and its sterol binding and release, reveals a model requiring dynamic movement of the Omega-1 loop, covering the sterol binding pocket, and insertion of the C-terminal α-helix into the membrane bilayer [131].

Overexpression of StarD4 increases sterol delivery to the endocytic recycling compartment and the endoplasmic reticulum, and enhances cholesterol esterification via ACAT in macrophages, fibroblasts and hepatocytes; silencing of STARD4 diminishes cholesterol-mediated regulation of SREBP-2 activation, while its overexpression achieves the reverse [35, 131-139]. In rodent hepatoma cells, overexpression of StarD4 increased the lipidation of exogenous apoA-I, without impacting on triacylglycerol synthesis or secretion [133] while knockdown of STARD4 led to the accumulation of cholesterol at the plasma membrane [138]. Overexpression of STARD4 can also increase the formation of bile acids, so that cholesterol catabolism is enhanced [135]. In keratinocytes (HaCaT), transient overexpression of STARD4 decreased the biosynthesis of cholesterol and CE, and triggers increased expression of *SREBF2*, *ABCG4* and loricrin, but represses expression of *ABCA1*, suggesting modulation of both keratinocyte cholesterol metabolism and differentiation status [138]. Bazuine *et al* (2009) have suggested that both StarD4 and OSBP are targets of the p53 tumour suppressor protein, modifying cellular responses to stress caused by reactive oxygen species and lipotoxicity [139]. However, like StAR, StarD4 can facilitate the translocation of 7-hydroperoxycholesterol to isolated mitochondria resulting in greater vulnerability to peroxidation by free radicals and mitochondrial membrane depolarisation [140]. Global

knockout of *StarD4* leads to modest weight loss, and decreased bile cholesterol and phospholipid concentrations in female mice, which also evinced modest decreases in total cholesterol, LDL and CE concentrations when challenged with a diet enriched with cholesterol [141].

The cholesterol-binding properties of StarD5 remain controversial: this protein has been reported to bind cholesterol and 25-hydroxycholesterol [142, 143], while other studies indicate primary and secondary bile acids as its preferred ligands [131,145-1457]. Unlike StarD4, the promoter region of StarD5 does not contain a sterol response element. Instead, StarD5 expression appears to be increased in macrophages enriched in free cholesterol [130], due to activation of the endoplasmic reticulum stress response mediated by the transcriptional stress factor, XBP1; induction of endoplasmic reticulum stress is also associated with stabilisation of StarD5 mRNA, and with movement of StarD5 from the nucleus to cytosol and membranes [147]. Levels of StarD5 mRNA and protein are decreased in hepatic tissues from genetically obese rodents [133], and are thought to be concentrated in Kupffer cells within human liver [143-144; 147]. StarD5 is targeted by c-Jun N-terminal kinase and cyclooxygenase-2, possibly as part of a protective stress response, within specialised epithelial Sertoli cells [148]. Overexpression of STARD5 in hepatocytes increased the free cholesterol content of intracellular membranes [143, 144]; in rodent hepatoma cells, STARD5 overexpression did not alter cholesterol homeostasis, but increased the basal synthesis of triacylglycerol, promoted the incorporation of pre-formed fatty acids into this neutral lipid and stimulated the expression of *Dgat1* [133].

The cholesterol binding affinity of STARD6 is similar to that of STARD1/StAR in that it induces adrenal and gonadal steroidogenesis, although the protease-sensitive C-terminal is smaller than that of StAR [149]. Both STARD1 and STARD6 unfold and refold with similar (slow) kinetic patterns, and interact equally well with mitochondrial membranes [149]. Initially STARD6 was reported to be limited to male germ-line cells [84, 150, 151], but recent studies have shown that this protein is expressed in steroidogenic cells of the ovary, where it lies under the control of GATA4 and GATA6 transcription factors, and facilitates pregnenolone production [150]. Further, Chang and coworkers have reported STARD6 to be present in the brain, spinal cord and dorsal root ganglia, where it may play a role in the formation of neuroprotective steroids after excitotoxic brain injury [151-153].

Since the majority of ORPs are found at membrane contact sites, there are few reports of cytosolic versions of this family of proteins. However, two variants of ORP1 exist, which display different tissue expression patterns, intracellular localisation and functions [154]. The 'short' form of ORP1 (ORP1S) is known to translocate from the cytosol to the nucleus after binding sterol, where it binds to LXR and enhances LXR-dependent transcription of apoE via the ME.1 and ME.2 enhancer elements of this gene [155]. Overexpression of ORP1s (together with ORP2) has also been shown to stimulate transport of sterol from the plasma membrane to lipid droplets, by enhancing plasma membrane to endoplasmic reticulum transport [156], and it is clear that even the 'short' variants of ORPs can cluster at membrane contact sites [157].

Sterol-binding proteins: endoplasmic reticulum and the trans-Golgi network

The prototypic member of the ORP family, the 57kDa oxysterol binding protein (OSBP) regulates the movement of cholesterol from the endoplasmic reticulum to the trans-Golgi network at membrane contact sites between these organelles, driven by the transport of PI-4P in the opposite direction [106,107, 156-160]. This exchange of lipid is inhibited by 25-hydroxycholesterol, and also triggers recruitment of STARD11 (CERT) for sphingomyelin synthesis, demonstrating cross-talk between the ORP and START families of lipid trafficking proteins [156-160]. Depletion of VAMP-associated proteins, VAPs (VAP-A, VAP-B) inhibits Golgi-mediated transport events, which are co-ordinately regulated by the phosphatidylinositol/ phosphatidylcholine transfer protein, Nir2, together with OSBP and CERT, all of which interact with VAPs via the FFAT motif. Notably, Mesmin *et al* (2013) recently described a four-step cycle, driven by PI-4P hydrolysis: the PH domain

tethers OSBP at the membrane, the FFAT motif enables sterol transfer via ORD, followed by retrograde transfer of PI-4P, and hydrolysis of PI-4P by the endoplasmic reticulum protein Sac1 [161]. The OSBP controls the Golgi cholesterol level, which is essential for the correct localisation of GS28 and GS15 and effective coat protein complex (COP)-I vesicle transport: knockdown of OSBP results in cytosolic mis-localisation of intra-Golgi vSNAREs, GS28 and GS15, and reduces the amount of the Golgi enzyme, mannosidase II, a phenotype which can also be induced by depletion of cellular cholesterol [160]. Some of these properties of OSBP have apparently been exploited by RNA viruses, such as poliovirus and hepatitis C virus, which require PI 4-kinases and their effectors for replication [162].

Overexpression of OSBP in hepatocytes, or injection of adenovirus expressing OSBP in mice, results in a marked increase in hepatic triglycerides, and increased output of VLDL (+70%), mediated via upregulation of SREBP-1 and increased expression of enzymes involved in fatty acid biosynthesis; by contrast, silencing of OSBP blocked insulin-induction of SREBP-1c, reducing triglyceride synthesis [163]. Suppression of OSBP in Chinese hamster ovary cells or macrophages increases the expression of ABCA1, via stabilisation of the ABCA1 protein, an effect that was abrogated by a mutation in the ORD [164]. Mutants of OSBP in Drosophila exhibit male-sterility and defects in spermatid packaging into its own membrane, suggesting sterol trafficking plays a key role in this process [165] and discussed further below.

Another ORP known to influence the intra-Golgi vSNARE protein GS28 is ORP7, which is enriched in the gastrointestinal tract, and interacts with GATE-16, a regulator of GS28 function and stability. Knockdown of ORP7 increased the levels of GS28 protein (+40%), while ORP7 overexpression achieved the reverse (-25%) by proteasomal degradation; equally, cells treated with the ORP7 ligand, 25-hydroxycholesterol, displayed destabilised GS28 [166]. Overexpression of ORP7 also led to the formation of vacuolar structures, autophagosomes, which stained positive for GATE-16 [166].

Lastly, ORP9L is known to interact with the Golgi and trans-Golgi network via its PH domain and the endoplasmic reticulum via interactions with VAP-A, and is thought to aid in the maintenance of the early secretory pathway [167, 168]. The function of ORP9L seems similar to that of OSBP, in that both can facilitate PI-4P dependent cholesterol transport between liposomes; ORP9 can also interact with ORP11 as a dimer. Silencing of ORP9L causes fragmentation of Golgi and build-up of cholesterol within endosomes and lysosomes [167, 168]. By contrast, ORP9S, which lacks the PH domain, acts as a dominant negative variant, and overexpression of this protein inhibits protein transport and cell growth [167, 168]. More recently, Liu and Ridgway (2014) have demonstrated that both ORP9L and ORP9S bind sterol and PI-4P, and that sequestration of Golgi PI-4P by ORP9S may explain the growth inhibitory effects of this variant [169].

Sterol-binding proteins: endoplasmic reticulum and the plasma membrane

As indicated above, ORP5 and ORP8, both tail-anchored ORPs integral to the endoplasmic reticulum, can transport phosphatidylserine from the endoplasmic reticulum to mitochondria at membrane contact sites [129]. The same combination of ORPs can also tether the endoplasmic reticulum to the plasma membrane, via the interaction of the PH domain with PI-4P in the plasma membrane [170]. Again, glycerophospholipids are exchanged between these bilayers, rather than sterol, such that PI-4P is delivered to the endoplasmic reticulum PI-4P phosphatase Sac1 for degradation, and the plasma membrane is enriched in phosphatidylserine [170]. ORP5 is also involved in endosomal cholesterol trafficking, via interaction with NPC-1 protein: knockdown of ORP5 triggers the build-up of cholesterol in the limiting membranes of late endosomes and lysosomes, and causes the mislocalisation of trans-Golgi resident proteins to endosomal compartments [171].

Notably, the expression of ORP5 is associated with invasion and poor prognosis in patients with pancreatic cancer [172, 173]. In some pancreatic cancers, strong expression of ORP5 augments the cholesterol biosynthesis pathway, via induction of SREBP-2 and its downstream target histone

deacetylase 5 (HDAC5), rendering invasion rate and growth susceptible to inhibition by statin inhibitors of HMG CoA reductase [172, 173]. This finding may prove clinically significant, given the lack of current therapies with positive impact on this highly lethal disease [174]. High expression levels of ORP5 have also been linked to the metastatic potential of lung cancer cells, as overexpression increased lung cancer cell invasiveness, while knockdown achieved the reverse [175]. Notably, survival rates were significantly lower in patients with ORP-5 positive cancer: the median survival time of the ORP-5 negative group was longer than that of the ORP-5 positive group by 8.9 months [175].

The C-terminal ORD of ORP8 anchors this protein to the endoplasmic reticulum, binds 25-hydroxycholesterol, and ORP8 known to interact with the nuclear porin Nup62 thereby reducing the levels of nuclear SREBP-1, SREBP-2 and their downstream target genes [176]. Thus, hepatic overexpression of ORP8 in Huh7 cells reduced cholesterol biosynthesis from radiolabelled acetate, and adenoviral administration of ORP8 decreased serum and hepatic levels of cholesterol, phospholipids and triglycerides in serum and liver tissues in mice [176]. Treatment of HepG2 cells with 25-hydroxycholesterol, or overexpression of ORP8, also slows the cell cycle, causing cells to remain longer in the G2/M phase, by recruitment of SPAG5 (Homo sapiens sperm associated antigen 5)/Astrin to the endoplasmic reticulum during interphase [177].

In macrophages, the interaction of ORP8 with Nup62 is thought to suppress migration and suppress microtubule formation [178], and overexpression of ORP8 is associated with decreased expression of ABCA1 and attenuated cholesterol efflux to apoA-I in murine macrophages [179]. Indeed, silencing of ORP8 in human macrophages enhances *ABCA1* transcription, by negating the suppressive effects of ORP8 at the LXRE and E-box sites within the promoter region of *ABCA1*. In good agreement, ORP8 knockout in macrophages resulted in lower susceptibility to foam cell formation, decreased output of inflammatory cytokines, and reduced levels of differentiation markers [178, 179].

Mice lacking ORP8 showed increased levels of HDL cholesterol in the bloodstream [180], suggesting increased hepatic expression of ABCA1, while LDL receptor$^{-/-}$ mice transplanted with ORP8$^{-/-}$ bone marrow displayed higher levels of VLDL-cholesterol (+50%) but no increase in HDL-C when fed a high fat/high cholesterol diet [181]. However, LDL receptor$^{-/-}$ mice transplanted with ORP8$^{-/-}$ bone marrow developed smaller atherosclerotic lesions (-20%), despite a modest increase in macrophage content, compared with wild type transplanted controls, on the same atherogenic diet [180]. In human coronary atherosclerotic lesions, ORP8 co-localises with CD68(+) macrophages in the shoulder regions of the plaque, and is significantly increased (2.7-fold) compared with healthy arterial tissue [180], suggesting that ORP8 may be a good therapeutic target for prevention of disease. In support of this miR-143b, which down-regulates ORP8, is upregulated in hepatic tissues in genetic and dietary murine models of obesity, characterised by impaired insulin-signalling to AKT and glucose metabolism [182]. Importantly, repression of ORP8 also limits insulin-stimulation of AKT, highlighting the miR-143b-ORP8 pathway as an important target in treatment of diabetes and cardiovascular disease [182].

Sterol-binding proteins: endosomes, late endosomes and the ER

Members of the ORP and START families are involved in late endosome-endoplasmic reticulum membrane contact sites, together with a newly discovered third family of sterol-binding proteins, conserved between yeast and man, called the lipid transfer protein anchored at membrane contact sites (Lam) proteins [183, 184].

The sterol sensor ORP1L has been shown to be involved in the NPC2-dependent transport of endosomal LDL cholesterol to a specific pool within the endoplasmic reticulum, destined for esterification into lipid droplets, and that mutations in *OSBP1LA* contribute to extremely low levels of serum HDL [106, 185, 186]. It is known that endosomes fated for eventual fusion have to be transported intracellularly before tethering to initiate contact, and ORP1L is part of a multi-protein

complex (RAB7-RILP-ORP1L) involved in late endosomal transport and tethering [186]. Here, ORL1L acts as a cholesterol-sensing switch controlling the interactions between RILP-HOPS-p150(Glued). The RAB7-RILP-ORP1L complex recruits the dynein motor and HOPS protein complex, and regulates microtubule minus-end transport and fusion, both key events in endosomal fusion [185, 186]. Further, it seems ORP1L and the other member of the STARD1 subfamily, STARD3, mark distinct late endosomes [187]. ORP1L is localised to late endosomes containing NPC1, which deliver cholesterol to the endoplasmic reticulum, while STARD3 is present in a distinct population containing the cholesterol transporter ABCA3 which can recycle cholesterol to the plasma membrane [187].

STARD3, also called Metastatic Lymph Node 64 (MLN64) is co-amplified with the oncogene ERBB2 in human breast carcinoma, and has two distinct conserved cholesterol binding regions: the classical C-terminal START domain and a region of four transmembrane helices with three short intervening loops, termed the N-terminal (MENTAL) domain [36, 39, 93, 188]. STARD3 also has a close paralogue, the STARD3 N-terminal like (MENTHO) protein, and recent data suggest that both are anchored on the limiting membrane of late endosomes where they interact with endoplasmic reticulum-anchored VAP-A and VAP-B proteins to form membrane contact sites which may influence endosome dynamics [189, 190]. STARD3 undergoes endocytosis from the plasma membrane to late endocytic compartments, although in cholesterol-laden cells, this movement is inhibited, and STARD3 becomes trapped at the edges of cholesterol-enriched lysosomes [39]. Knockdown of STARD3 disperses late endosomes to the cell periphery, decreases the association of these organelles with actin and impairs the fusion of late endocytic vesicles. Equally, overexpression of STARD3 increased the association of actin with late endosomes, and stimulated the accumulation of sterol in these organelles [39]. STARD3 can also enhance the movement of lysosomal cholesterol to mitochondria and stimulate steroidogenesis, an effect which is enhanced by removal of N-terminal sequences [36, 191].

The expression of STARD3 mRNA and protein are repressed during macrophage differentiation, and by sterol accumulation, but stimulated by the hypocholesterolaemic agent LY295427 in macrophages, suggesting that STARD3 is a SREBP target [122]. Overexpression of STARD3 in human macrophages induces the expression of ABCA1, enhances efflux of cholesterol to apoA-I and inhibits lipogenesis, and blocks cholesterol ester deposition following exposure to modified LDL, suggesting an anti-atherogenic phenotype [192]. Hepatic levels of StarD3 protein decreased in genetic obesity, suggesting a link with lipid storage or lipoprotein export from the liver [133]. Genetic deletion of the START domain of STARD3 in mice induced only modest changes in lipid metabolism in mice [193]. However, overexpression of StarD3 increases bile acid synthesis via CYP27A1 in primary rat hepatocytes [127] and in rat hepatoma cells overexpression of StarD3 increased lipidation of exogenous apoA-I, and facilitated *de novo* biosynthesis of neutral lipids [133].

Notably, the functional role of STARD3 in promoting steroidogenesis, and the overexpression of STARD3 in human breast carcinomas, led to the suggestion that this protein could promote intraneoplastic autonomous steroidogenesis and contribute to tumour growth. High levels of STARD3 are also found in neoplastic prostatic tumours, correlating with stage of disease and short relapse time [194]. Cai *et al* (2010) have also described an association between elevated STARD3 and poor prognosis and overall survival in breast cancer patients, possibly via regulation of cell proliferation and adhesion [195].

Finally, ORP6, which is a target for LXRs and is regulated by cholesterol loading in cells, and by a Western diet in animal models, also appears to associate with the endolysosomal network [196]. Overexpression of ORP6 enhances endosomal trafficking and cholesterol efflux in macrophages and hepatocytes, while knockdown of ORP6 triggers aberrant endosome clusters, enriched in free cholesterol, and reduced cholesterol esterification at the endoplasmic reticulum [196]. Levels of ORP6 correlate direct with plasma HDL concentrations, are repressed by miR-33 and miR-27b, and are reduced in human atherosclerotic plaques [196].

Sterol-binding proteins: ER and lipid droplets

Recent data suggests that cholesterol metabolism is also regulated by ORP2 [190], which possesses ORD and FFAT motifs, but not a PH domain. This protein targets lipid droplets and, when complexed with VAP, decorates endoplasmic reticulum structures with lipid droplets attached to them, facilitating the synthesis (and inhibiting the lipolysis) of triglycerides in hepatic cells [197]. Whether ORP2 plays a similar role in promoting the formation of lipid droplets of CE is not known, but knockdown of ORP2 in adrenocortical cells reduces the production of some steroids, including progesterone, 11-deoxycortisol and cortisol, but enhances the production of androgens and estrogens [198]. Loss of ORP2 is also associated with reduced levels of 22-hydroxycholesterol and 7-ketocholesterol, both of which bind to ORP2; ORP2 also binds to LXR, facilitating recruitment to the *CYP11B1* promoter in response to cAMP, and is required for the expression of a number of LXR targets, including ABCA1 [198].

Frameshift mutations in *OSBPL2*, which encodes ORP2, cause a form of autosomal dominant hearing loss (DFNA67) [199, 200], although whether this effect is related to its sterol-binding/transfer properties remains unknown. The genetics of nonsyndromic congenital hearing loss are complex: to date more than 125 deafness loci have been identified, 58 of which cause autosomal dominant hearing loss (DFNA), 63 cause autosomal recessive inherited hearing loss (DFNB) and 4 are X-linked [201]. This reflects the large number of genes involved in inner-ear function, with mutations affecting adhesion of hair cells, intracellular transport and cytoskeletal function, and neurotransmitter release all known to contribute to malfunction of the cochlea and inner ear [201]. Mutated *OSBPL2* was discovered as a novel locus causing onset of hearing loss in childhood, progressing to profound deafness in adulthood [199, 200]. Wild type ORP2 encodes a protein located in inner and outer hair cell stereocilia, where it interacts with diaphanous homologue 1 (DIAPH1), a Rho effector protein that regulates cytoskeletal dynamics. A mutated (truncated) form of ORP2 may interfere with cytoskeletal function, which is critical for the structure of stereocilia; since ORP2 also binds PI-3P, ORP2 may also be involved in creating or sustaining the polarity of hair cells [199, 200]. It remains an intriguing possibility that cholesterol metabolism may play a role in hair cell function and hearing loss: Malgrange *et al* (2015) recently speculated that cholesterol homeostasis may contribute to sensorineural hearing loss [202].

ORP4, which displays the highest degree of similarity with OSBP, has two variants: the long form ORP4L) has both a PH and an oxysterol binding domain, while the short form (ORP4S) lacks the the PH domain and part of the ORD. ORP4S colocalises with vimentin, causing the intermediate filament network to aggregate, and when overexpressed limits (-40%) esterification of cholesterol derived from endocytosis of LDL [203]. The short form of ORP4 inhibits the replication of hepatitis C virus in liver cells, but promotes the formation of lipid droplets in this context [204]. Notably, genetic deletion of ORP4 causes male infertility, due to severe oligo-astheno-teratozoospermia (OAT) (low sperm number, motility and abnormal morphology) [205]. Approximately 60% of OAT is diagnosed as idiopathic, implying the contribution of genetic abnormalities, although these have proved elusive to date. Loss of ORP4 in mice causes a marked reduction in sperm count, due to apoptosis of post-meiotic spermatids; abnormal sperm morphology is also observed, due to defects in the post-meiotic differentiation of germ cells, causing the build-up of deformed spermatids in the seminiferous tubules of $ORP4^{-/-}$ mice. The abnormal distribution of mitochondria in $OPR4^{-/-}$ sperm may contribute to low sperm motility [205], and although total cholesterol mass was not altered in the testis of wild type and knockout mouse, it remains possible that subcellular cholesterol distribution could be affected. This concept is supported by studies in *Drosophila melanogaster*, where mutations in the single homologue of OSBP and ORP4, CG6708, cause abnormal sterol distribution during 'germ cell individualisation' and sterility can be rescued by treatment with cholesterol or 7-dehydrocholesterol [207].

Sterol-binding proteins: microtubules and Golgi

The cholesterol-binding protein ORP10 possesses both ORD and PH domains, and is found associated with microtubules and with Golgi membranes [207]. Knockdown of ORP10 is associated with increased output of apoB100 by human (Huh7) hepatocytes, and reduced levels of intracellular apoB100, indicating a repressive role for ORP10 in the output of VLDL [207]. Polymorphisms in the OSBP10 gene are associated with elevated LDL cholesterol, and with peripheral arterial disease [208].

TARGETING STEROL-BINDING PROTEINS

The increasing numbers of roles assigned to sterol-binding proteins has led to interest in identifying or developing small molecules which can bind and modify the function of these proteins. Chitrala and Yeguvapalli (2014) have recently described a ligand-based virtual screening approach to identify molecules which inhibit the steroidogenic function of STARD3 [209] and the dietary carotenoid lutein has recently been described as a high affinity ligand for STARD3 (Kd=0.45μM) [210]; no impact on cholesterol metabolism has, as yet, been reported.

By contrast, a number of natural compounds which bind ORPs, termed 'ORPphilins' have been identified, and it is likely that these molecules will be useful in deciphering the cellular functions and roles of ORPs in human disease. Some of these compounds, such as OSW-1 cephalostatin, ritterazine B and schweinfurthin A, are currently under development as anti-cancer drugs, but have also proved useful as inhibitors of enterovirus replication [211-214]. For example, schweinfurthins inhibit trans-Golgi network trafficking by binding to OSBP and inhibit proliferation of PTEN deficient cancer cells by blocking phosphorylation of AKT [212]. Itraconazole is an anti-fungal agent with anti-cancer activity, and is known to target OSBP and ORP4L, inhibiting enteroviral replication by disrupting the membrane changes needed for viral replication organelle formation [213].

FUTURE DIRECTIONS

It is becoming increasingly clear that sterol-binding sensors, exchangers and transporters contribute to regulation of cellular cholesterol homeostasis, and that mutations in a number of these proteins cause disease (summarised in **Figure 1C**). Congenital lipoid hyperplasia, male sterility and autosomal dominant hearing loss are caused by loss-of-function mutations in StAR, OSBP and ORP4, and ORP2, respectively, while studies in mice suggest that knockdown of ORP8 may help to prevent the development of atherosclerotic lesions and diabetes. Loss of StarD4 may aid weight loss and reduce serum lipid concentrations, while knockdown of ORP10 could limit the output of triglyceride-rich lipoproteins and modulate peripheral arterial disease. Enhanced expression of ORP5 is linked to poor outcome in pancreatic cancer, and co-amplification of STARD3 with ERBB2 is linked with breast carcinoma. By contrast, cellular studies indicate that overexpression of StAR, STARD3, STARD4 ORP1L, ORP2 or ORP6 could help to reduce cholesterol accumulation and/or inflammation within vascular or hepatic tissues, and enhanced expression of ORP4S can limit replication of hepatitis C. However, much remains to be understood about their differing modes of action and the polymorphisms which affect their function; the extent to which differing family members can compensate for genetic deletion of another also demands attention, as does the crosstalk between family members, and between the distinct ORP and START families of proteins.

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Legend to Figure 1 Intracellular sterol transport via membrane contact sites (MCS) and non-vesicular trafficking

A) Lipoprotein-derived cholesterol is delivered to the endocytic pathway, via lipoprotein receptors internalised by clathrin-coated pits, to early endosomes (EE) where the lower pH separates the lipoprotein from its receptor, which is recycled back to the plasma membrane via the endocytic recycling compartment (ERC). The lipoprotein remains within the EE as they mature to late endosomes (LE)/lysosomes wherein it is hydrolysed to free cholesterol, fatty acids, and amino acids. Niemann Pick C-1/2 proteins aid transport of cholesterol out of the LE/lysosomes for transport to the plasma membrane (PM) and endoplasmic reticulum (ER). Cholesterol transported to the endoplasmic reticulum (ER) sequesters Sterol Regulatory Element Binding Protein (SREBP)/SREBP-cleavage activating protein complex at this site; oxysterols (*below*) enhance this process by binding to Insig-1/2 (insulin-induced gene-1 or -2), thereby achieving repression of endogenous cholesterol biosynthesis. Excess cholesterol is esterified via ACAT-1/2, storing cholesteryl ester as lipid droplets within the cytoplasm as 'foamy' deposits. Delivery of cholesterol to mitochondria can increase the endogenous production of oxysterol ligands by sterol 27-hydroxylaase (CYP27A1) for Liver X Receptors (LXR α/β). Ligation of the LXR/Retinoid X Receptor (RXR) heterodimer allows the recruitment of coactivator proteins, increasing the transcription of ATP binding cassette (ABC) transporters, ABCA1, ABCG1 and ABCG4, which efflux cholesterol to apolipoprotein (apo) A-I, apoE or nascent high density lipoprotein (HDL). EE= early endosome; ER= endoplasmic reticulum; ERC= endocytic recycling compartment; LD= lipid droplets; LE= late endosome; PM= plasma membrane; CG= cis-Golgi; TG= trans-Golgi.

B) Intracellular trafficking of cholesterol can be achieved by vesicular (blue arrows) and non-vesicular (red dotted arrows) transport, and by exchange over membrane contact sites (green arrows). The distribution of cholesterol differs significantly between organelles, with the PM, ERC and EE being highly enriched with cholesterol compared with the ER, mitochondria and lysosomes, which are relatively cholesterol-poor; the Golgi

cholesterol:phospholipid ratio is intermediate between that of PM and the ER, with the cholesterol content increasing from the *cis* (CG) to the *trans*-Golgi (TG) network [207]. Cholesterol transfer between organelles does occur by vesicular trafficking, particularly within the endocytic and secretory pathways, but the majority appears to occur via non-vesicular, carrier-mediated transport [215].

The key members of the ORP and START families of lipid-trafficking/exchange proteins implicated in exchange of lipids across MCS, and in trafficking sterol between organelles. Mitochondrial cholesterol transport from the outer to the inner mitochondrial membrane can be facilitated by StAR, STARD3 and STARD6, while the presence of ORP5 and ORP8 at ER-mitochondrial membrane contact sites influences mitochondrial morphology and respiration; ORP5 and ORP8 can also tether the ER to the PM, aid endosomal trafficking and modulate cholesterol and lipoprotein metabolism. Cytosolic STARD4 modulates cholesterol trafficking between the ERC, ER, mitochondria and the PM, while ORP1S and ORP2 can stimulate sterol transport from PM to LD. STARD5 is involved in the ER stress response, can modulate the free cholesterol content of intracellular membranes and increase the basal synthesis of triacylglycerol. At ER-Golgi membrane contact sites OSBP, ORP9 and ORP11 are involved with sterol/PI-4P exchange, influencing the localisation of vSNAREs, cell growth, cholesterol and triacylglycerol metabolism. Within the endocytic pathway, ORP1L interacts with NPC-2 to deliver cholesterol to LD, and sustain serum levels of HDL; STARD3 and ORP6 facilitate endosomal cholesterol trafficking to mitochondria, ER and/or the PM, regulating cholesterol efflux and HDL levels. Finally, ORP2 and ORP4 are found at ER-LD membrane contact sites, influencing cholesterol metabolism and cytoskeletal function, ORP10 influences the packaging and secretion of VLDL, and evidence exists for nuclear localisation of ORP1S, ORP8 and ORP2.

Figure 1C The links between dysregulated expression (↑ or ↓) or mutations in members of the ORP and START lipid trafficking families of proteins, and disease conditions, including atherosclerosis, dyslipidaemia, diabetes, congenital lipoid hyperplasia, cancer, autosomal dominant hearing loss and male infertility. The images used here were adapted from Iaea and Maxfield (2015) [215].

Figure 1A

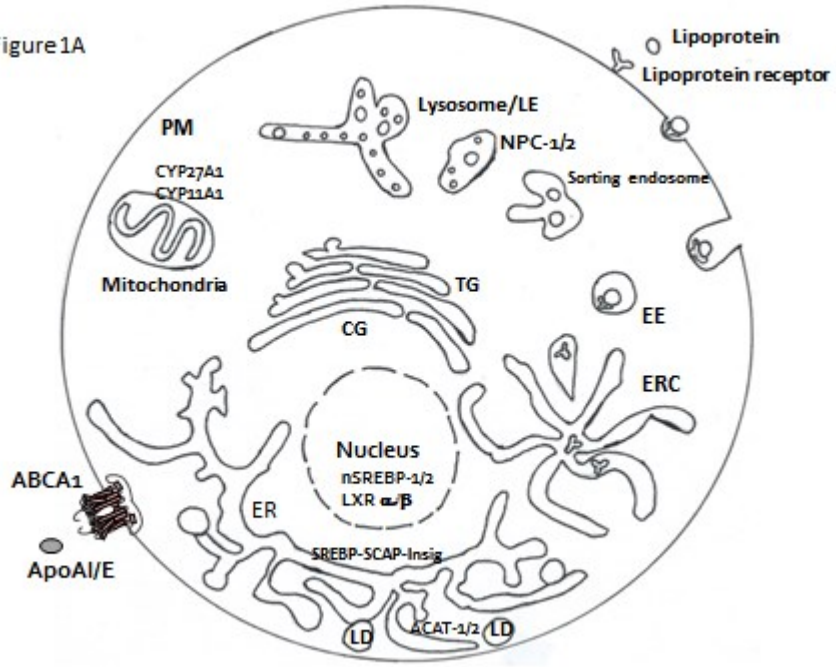
Lipoprotein
Lipoprotein receptor
PM
Lysosome/LE
NPC-1/2
Sorting endosome
CYP27A1
CYP11A1
Mitochondria
TG
CG
EE
ERC
Nucleus
nSREBP-1/2
LXR α/β
ABCA1
ER
ApoAI/E
SREBP-SCAP-Insig
LD
ACAT-1/2
LD

Figure 1B

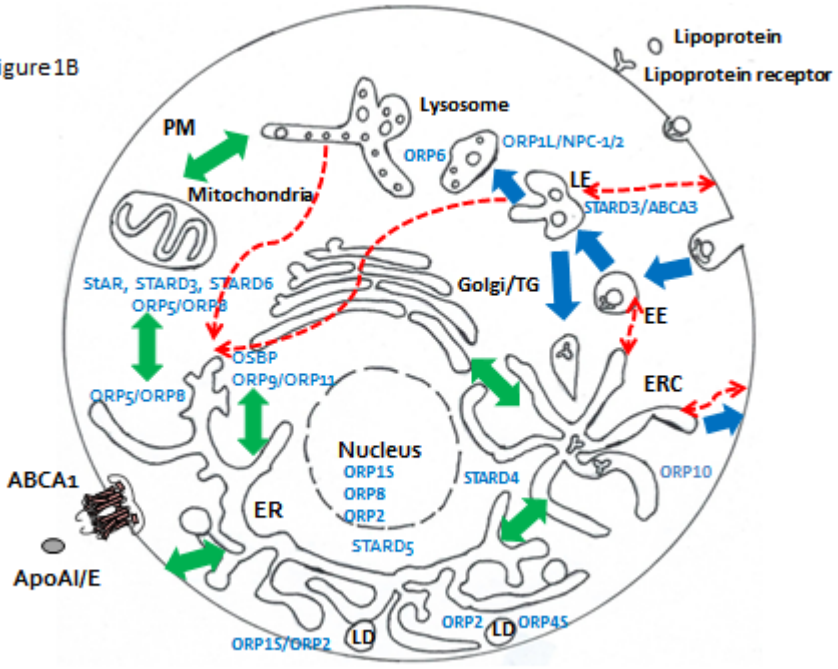

Figure 1C