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Structure, function and small molecule modulation of intracellular sterol transport proteins

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ABSTRACT

Intracellular sterol transport proteins (STPs) are crucial for maintaining cellular lipid homeostasis by regulating local sterol pools. Despite structural similarities in their sterol binding domains, STPs have different substrate specificities, intracellular localisation and biological functions. In this review, we highlight recent advances in the determination of STP structures and how this regulates their lipid specificities. Furthermore, we cover the important discoveries relating to the intracellular localisation of STPs, and the organelles between which lipid transport is carried out, giving rise to specific functions in health and disease. Finally, serendipitous and targeted efforts to identify small molecule modulators of STPs, as well as their ability to act as tool compounds and potential therapeutics, will be discussed.

1. Introduction to sterol homeostasis

Maintaining intracellular cholesterol homeostasis is essential for healthy human physiology.[1] The functions of this important lipid are wide-ranging, from regulating membrane structure and fluidity to serving as a precursor to steroid hormones. Enzymatic and non-enzymatic oxidation of cholesterol can give rise to an array of different oxysterols, some of which can be further converted to bile acids.[2] These metabolites can themselves carry out a wide variety of signalling functions including the modulation of steroid receptors. Furthermore, both binding to, and post-translational modification of the hedgehog family of proteins with cholesterol also contributes to hedgehog signalling in development.[3,4] Given the plethora of cellular functions modulated by cholesterol, it is no surprise that species have evolved a range of processes to tightly regulate its levels and localisation, as well as those of its metabolites.

Cholesterol can either be taken up by external sources (diet) or biosynthesised in the body. There are many excellent reviews covering both subjects,[1,5] and as such these will only be summarised here. *De novo* biosynthesis of cholesterol is a highly complex process, involving the concerted action of numerous enzymes over multiple individual steps, and serves as the major source of cellular cholesterol.[5] Biosynthesis commences via the conversion of acetate in the form of acetyl-coenzyme A (acetyl-CoA) to mevalonate, including the rate-limiting reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonate by the endoplasmic reticulum (ER)-resident HMG-CoA reductase (HMGCoR).[6] Through conversion of mevalonate to activated isoprene building blocks and subsequent condensations, the 30-carbon terpenoid squalene is synthesised. Oxidation of squalene by squalene monooxygenase (SM) to 2,3-oxidosqualene represents the second major rate-limiting reaction of the sequence.[7] 2,3-Oxidosqualene is then cyclised to yield lanosterol, which through a series of reductions affords cholesterol and closes the biosynthetic pathway.[5]

Cellular uptake of dietary cholesterol occurs in one of two main processes, either by the Niemann-Pick type C1-like 1 (NPC1L1) protein in enterocytes or by the low-density lipoprotein (LDL) receptor (LDLR) from the blood, both of which occur in a clathrin-dependent manner.[1] Under low levels of cellular cholesterol, NPC1L1 translocates to the plasma membrane (PM) of intestinal cells and binds to unesterified cholesterol.[8] Endocytosis of NPC1L1-bound cholesterol delivers the receptor and cholesterol to the endocytic recycling compartment (ERC), from which cholesterol can be transported to the ER via unknown mechanisms.[9]

The LDLR is a plasma-membrane bound protein, which binds to LDL-particles containing cholesterol esters, through its extracellular ligand binding domain. Once bound, the receptor undergoes endocytosis – releasing its cargo of LDL upon undergoing a conformational change in the acidic endosomes.[10] The cholesterol esters found in the LDL are ultimately hydrolysed by lysosomal acid lipases, releasing free cholesterol into the lysosomal lumen. Cholesterol is then transported from lysosomes, ultimately to the ER, by the Niemann-Pick type C1 (NPC1), Niemann-Pick type *C*2 (NPC2), and lysosome-associated membrane protein-2 (LAMP2) proteins.[11]

In order to maintain correct cholesterol homeostasis, the interplay of

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cholesterol biosynthesis and uptake must be tightly regulated. A number of mechanisms are present to achieve this, of which the ER-bound sterol regulatory element-binding protein 2 (SREBP2) plays a significant role; regulating cholesterol biosynthesis genes as well as influencing LDLR-mediated uptake of cholesterol.[12] When cellular cholesterol levels are high, cholesterol binds to the SREBP-cleavage activating protein (SCAP) which in-turn results in binding to insulin-induced gene (INSIG) proteins 1 and 2, blocking its export from the ER and subsequent upregulation of the transcription of cholesterol biosynthesis genes.[13,14] Oxysterols that can form when ER cholesterol levels are high also promote the retention of SREBP2 by direct binding to INSIG, enhancing its binding to SCAP.[15]

When ER cholesterol levels are low, SCAP adopts an alternative conformation that allows binding to COPII vesicles and dissociation from INSIG, allowing translocation of the SCAP-SREBP2 complex to the Golgi apparatus.[15] At the Golgi, proteolytic cleavage of SREBP2 releases nuclear SREBP2 (nSREBP2), which is subsequently translocated to the nucleus.[16] In the nucleus, nSREBP2 binds to sterol-regulatory elements (SREs) of genes involved in cholesterol biosynthesis and uptake including HMGCoR, SM, LDLR, and NPC1L1 as well as the SREBP2 gene itself; resulting in an upregulation of their transcription.[17] As well as upregulating the transcription of the LDLR, nSREBP2 can also lead to its down-regulation via upregulating transcription of proprotein convertase subtilisin/kexin type-9 (PCSK9) which binds to and directs LDLR for degradation in the lysosomes.[18]

In addition to the SREBP2/SCAP regulatory axis, additional transcriptional regulation occurs to maintain cholesterol homeostasis. Uptake of cholesterol by NPC1L1 can, for example, be regulated positively and negatively by the proliferator-activated receptor α – retinoid X receptor α (PPARα-RXRα) complex and the cyclic adenosine monophosphate (cAMP)-responsive element-binding protein H (CREBH) protein respectively.[19,20] LDLR-mediated uptake of LDL is also further regulated by the E3 ubiquitin ligase inducible degrader of LDL-receptor (IDOL), both of which are in turn regulated by liver X receptors.[21] The mechanisms of these transcriptional regulations will not be introduced here, but have been discussed in detail in other reviews.[9,22]

When levels of cellular cholesterol are too high, cholesterol can either be removed from the cell via efflux or processed for storage.[23] A significant source of cholesterol efflux occurs via the ATP-binding cassette (ABC) family of transport proteins, particularly the protein ABCA1. ABCA1 can transport cholesterol to apolipoprotein A1 (ApoA-1) and subsequently form high-density lipoproteins (HDL).[24] In contrast to direct removal from cells as HDL, cholesterol can be esterified by the enzymes acyl-Coenzyme A: cholesterol acytransferase (ACAT) 1 and 2, and the cholesterol esters stored in lipid droplets – wherein the cholesterol can be released as necessary by either lipophagy or lipolysis.[25] In contrast to the transcriptional regulation of cholesterol homeostasis, intracellular transport of cholesterol between different organelles is achieved by vesicular and non-vesicular transport, mediated by sterol transport proteins (STPs). In the following sections we will discuss the structure and lipid binding profiles, (patho)physiological functions and small molecule modulators of STPs involved in non-vesicular sterol transport. Key information relating to each STP covered is also summarised in Table 1.

2. Structure and lipid binding selectivity of STPs

Non-vesicular sterol transport is mainly carried out by three protein families, the Oxysterol-binding Protein-Related Protein (ORP) Family Proteins, the Steroidogenic Acute Regulatory Protein-related Lipid Transfer Domain (STARD) Family Proteins and the Aster-domain containing protein family.[26] All Asters transport cholesterol, while selected members of the STARDs and ORPs transfer other lipids including phosphatidylcholine (PC), ceramide (CE) and phosphatidylinositol phosphates (PIPs). The precise lipid selectivity of individual STPs has not been systematically determined; however, several are also known to transfer other lipids either individually, or in exchange with cholesterol.[27] Structurally, STPs can be subdivided further into those that consist almost entirely of their sterol-binding domain (SBD) and those that contain additional domains. The presence and nature of these domains dictates their distinct intracellular localisation and cholesterol transport activity. A large proportion of cholesterol transport occurs from the endoplasmic reticulum (ER), where it is biosynthesised, and at the lysosome, where imported cholesterol is taken before being delivered to other compartments.[28] Within the context of this review we will focus on lipid transport proteins, which are known for sterol-binding.

2.1. Oxysterol-binding Protein-Related protein family proteins

The Oxysterol-binding Protein (OSBP) and OSBP-related proteins (ORPs) are evolutionary conserved and expressed in all eukaryotes. There are 12 ORP genes in mammals, spliced in 16 ORP variants (Fig. 1A). A main characteristic of this protein class, except for ORP3 (2),[29] is the presence of a conserved OSBP-related domain (ORD), serving as a binding pocket for one lipid molecule.[30–32] The ORD domain consists of an incomplete β-barrel forming a hydrophobic tunnel. Enclosed to the pocket entrance reside the EQVSHHPP signature and the *N*-terminal lid, containing the helix α1.[33] In 2019, Dong *et al.* solved the first crystal structure of a mammalian ORD (Fig. 2A, PDB: 5ZM5, ORD1) in complex with cholesterol. The rigid cholesterol backbone and the hydrocarbon tail firmly interact with multiple hydrophobic residues at the tunnel wall, allowing a head-down binding mode of cholesterol. A sequence comparison between sterol binding ORPs displayed similar spatial distribution of hydrophobic interacting residues in the binding tunnel, suggesting a conserved sterol binding mode among these proteins. Furthermore, the 3-OH group of cholesterol forms direct or water-mediated hydrogen bonds to Y583 and Q724 at the bottom of the binding tunnel (Fig. 2B).[34] Subtle sequence differences in this region between ORP proteins indicate different ligand affinities.

ORP1S and ORP4S only contain the ORD domain, whereas others comprise a pleckstrin homology (PH) domain and/or a diphenylalanine in an acidic tract (FFAT) motif, enabling them to target distinct membranes.[37–39] ORP5 and ORP8 include transmembrane domains at the C-terminus for anchoring the ER.[40,41] ORP1L has an additional *N*-terminal extension of three ankyrin repeats, targeting the protein to the late endosomes.[42,43] In mammals, OSBP, ORP1, ORP2, ORP4, and ORP9 have been confirmed to harbour and transport cholesterol.[33,44,45] OSBP, the prototypic member of this family, binds cholesterol, 25-hydroxycholesterol (25-HC) and PI4P competitively.[28] OSBP contains additional motifs in its *N*-terminus (PH domain and FFAT motif) and localises to membrane contact sites formed between the ER and other organelles, including the *trans*-Golgi network (TGN), endosomes, and lysosomes (Fig. 3).[46–48]

The ORP1 gene transcription results in two spliced isoforms: ORP1L and ORP1S, which contain the ORD only. ORP1L is predominantly found on late endosome/lysosomes (LELs), arising from the interaction between the ankyrin repeats and LEL-anchored Rab7 in addition to phosphoinositide binding by the PH domain.[42,43,49,50] Due to its lack of additional domains, ORP1S is located diffusely in the cytosol and nucleus.[43,51] The ORP1-ORD binds a variety of ligands including cholesterol, oxysterols and various phosphoinositides.[38,52,53] The ORD also transports cholesterol and orientates on membranes by PIP binding (i.e. PI(4,5)*P*2, PI(3,5)*P*2) via basic patches.[34]

ORP2 is closely related to ORP1 and like OSBP, their ORD can also transfer cholesterol.[54] ORP2 is the only short ORP produced from a full-

Table 1
Overview of STP family members and their respective domains, localisations, functions and known ligands; Chol = cholesterol; 25-HC = 25-hydroxy cholesterol; Preg. = pregnenolone; Testost. = Testosterone; ER = endoplasmic reticulum; TGN = trans-Golgi network; LY = lysosomes; LE = late endosomes; PM = plasma membrane; LD = lipid droplets; RE = recycling endosomes; OMM = outer mitochondrial membrane; n/a = none available.

Class	*Name*	*Lipid*	*Domains*	*Localisation*	*Organelle transport*	*(Patho)physiological relevance*	*Inhibitors*
ORP I	OSBP	Chol PI4P 25-HC	ORD, PH	ER, Golgi	ER → TGN[117] ER → LY[47]	p21$^{-/-}$ cancer,[99] inhibition induces autophagy[47]	Cephalostatin-1 OSW-1, Ritterazine [99] Schweinfürthin G [100]
	ORP4L	Chol PI4P 25-HC PI (4,5)*P*2	ORD, PH	ER (PM)[76]	ER – Golgi	Inhibition eradicates leukemic stem cells[76]	Cephalostatin-1 OSW-1, Ritterazine[99] LYZ-81 [76]
	ORP4M	Chol	ORD				–
	ORP4S	Chol	ORD	Vimentin filaments[77]			–
ORP II	ORP1L	Chol, PI4P Oxysterols	ORD, PH, FFAT	ER, LE/LY	ER → LE/LY[78]	Autophagy maturation[79]	n/a
	ORP1S	Chol	ORD	Cytosol, Nucleus	LE/LY → PM[80] PM → ER, LD[80]		–
	ORP2	Chol 22-HC PI4P	ORD, PH, FFAT	Cytosol	LE/ER → PM LE → RE[82]	Lipolysis of LDs[83] knockdown reduces angiogenic potential[84]	n/a
ORP III	ORP3(1)	PI4P	ORD, PH, FFAT	ER-PM			n/a
	ORP3(2)	PI4P	PH				–
	ORP6	PI4P Chol?	ORD, PH, FFAT	ER-PM[118]		Mediates cholesterol efflux[119]	n/a
	ORP7	PI4P Chol?	ORD, PH, FFAT	ER-PM[120]		Mediates cholesterol efflux[109]	Cpd G[109]
ORP IV	ORP5	PI4P PS Chol[41]	ORD, PH, TM	ER	ER → PM [121] ER – mito[122] ER – LD [123]	Induces proliferation by activating mTOR signaling[124]	n/a
	ORP8	PI4P PS PI(4,5)P2 Chol?	ORD, PH, TM	ER	ER → PM [121] ER – mito[122]		n/a
ORP V	ORP9L	PI4P Chol[85]	ORD, PH		ER – TGN[87]	Golgi integrity[87]	n/a
	ORP9S	PI4P Chol[85]	ORD				n/a
ORP VI	ORP10	PS[125] Chol[126]	ORD, PH	Microtubules, Golgi[126]	ER – LE[127]	β-lipoprotein secretion[126]	n/a
	ORP11	Chol? PI4P	ORD, PH	Golgi, LE[128]		Dimerisation with ORP9[128]	n/a
STAR I	STAR	Chol[62]	StART[63]	OMM[59]	OMM → IMM[35,63]	Steroidogenesis, mutations lead to lipoid CAH[56,88]	n/a
	STARD3	Chol[65]	StART, MENTAL FFAT[61,64,65]	ER[61,64,65]	ER endosome[61,64,65]	Overexpressed in Her-2 positive breast cancer[89]	VS1 [110]
STAR II	STARD4	Chol[70]	StART[66,67]	Cytosol[66,67]	Endosome → PM[35]		n/a
	STARD5	Chol[60] Cholic acid[35]	StART[56]	Cytosol[67]	ER-PM[35]		n/a
	STARD6	Preg.[35] Testost.[90]	StART[66,67]	Cytosol[67]		Primarily localised in the testis[90]	n/a
Aster	Aster-A	Chol	GRAM, ASTER TM	ER	ER-PM[57]	Autophagy initiation[72]	Autogramins-1/2[72] Pyrazolopyrimidine 2 [116]
	Aster-B	Chol	GRAM, ASTER TM	ER	ER-PM[57] ER → Mito	Involved in supplying sterol for steroidogenesis[57]	3d [115]
	Aster-C	Chol	GRAM, ASTER TM	ER	ER-PM[57]	Autophagy initiation[95]	(-)-Astercin1[116] AI-11 [115]

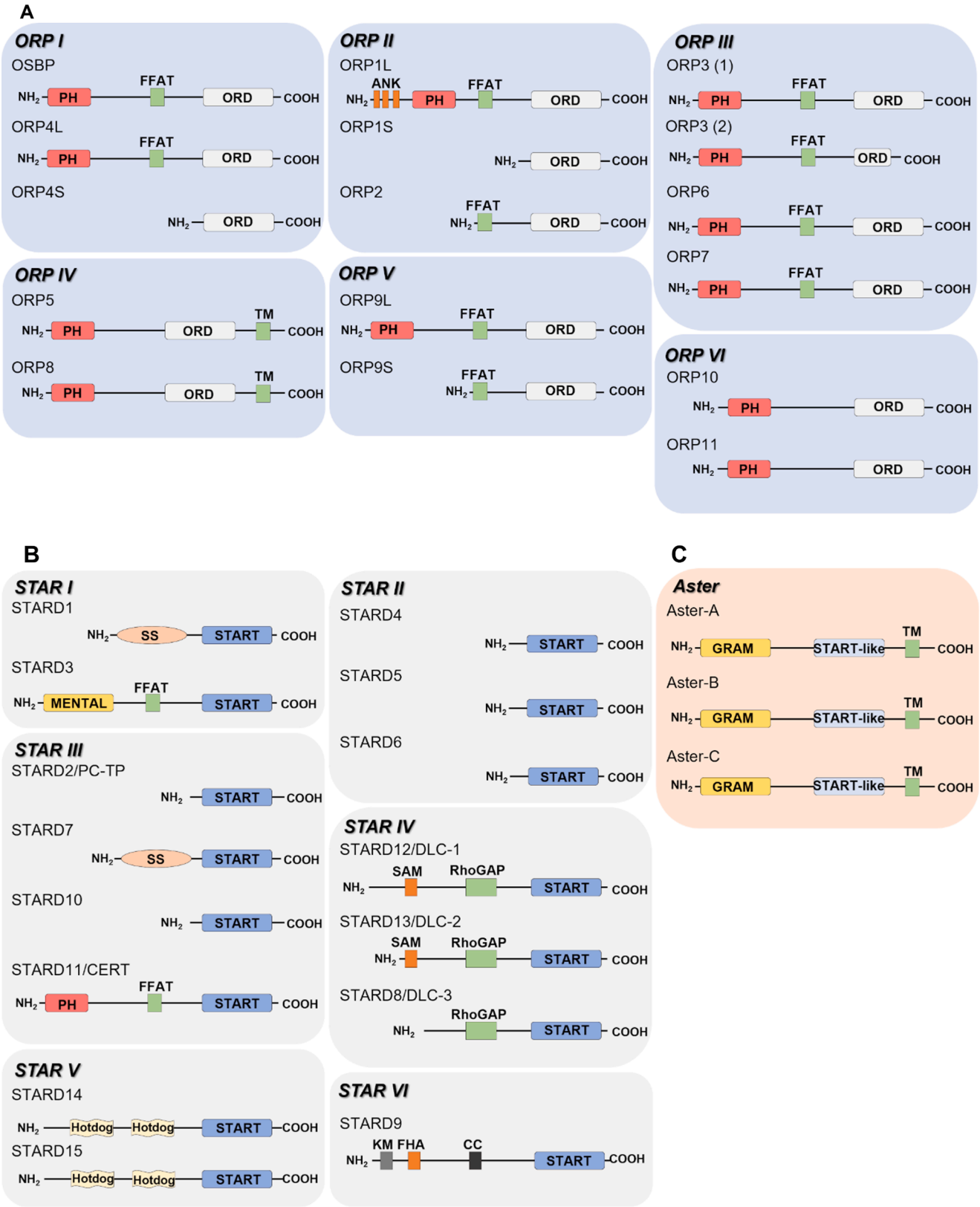

Figure 1. Domain organisation of STPs. (A) The Oxysterol-binding Protein (OSBP) and OSBP-related proteins (ORPs) are evolutionary conserved and present in all eukaryotes. In mammals there are 12 ORP genes, which generate up to 16 ORP variants.[33] **(B)** The mammalian Steroidogenic Acute Regulatory Protein (StAR)-related Lipid Transfer (START) Domain (STARD) family contains 15 members, which all have the StART lipid transfer domain. They are divided into 6 subfamilies according to their ligand binding properties and sequence similarities.[35] **(C)** The lipid transfer proteins anchored at a membrane contact site (Lam) family comprises three members in mammals (GRAMD1a/Aster-A, GRAMD1b/Aster-B, and GRAMD1c/Aster-C)). These proteins share a PH-like glucosyltransferases, Rab-like GTPase activators, and myotubularins (GRAM) domain, one or two START-like ASTER domains, and one or two transmembrane segments for ER localization.[36] Adapted from Luo *et al.*[27.]

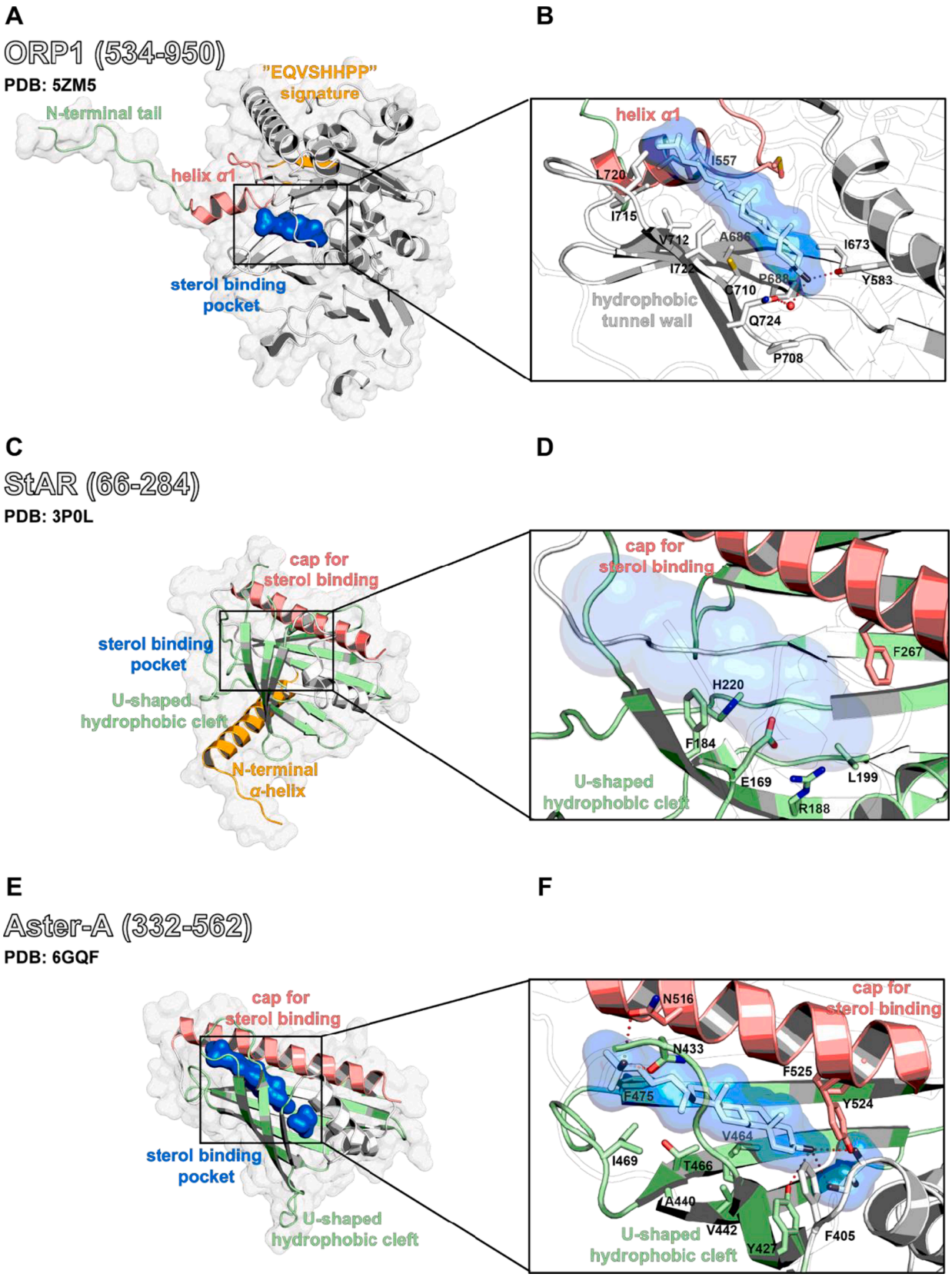

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Figure 2. Exemplary structural insights into the sterol binding domains of STPs. (A) Structure of the ORP1-ORD (PDB: 5ZM5 in complex with cholesterol (blue)), consisting of an incomplete β-barrel forming a hydrophobic tunnel, which serves as a binding pocket for one lipid molecule. Enclosed to the pocket entrance reside the EQVSHHPP signature (orange) and the *N*-terminal lid (green), containing the helix α1 (pink). **(B)** Representation of the cholesterol binding mode in ORP1. The rigid cholesterol backbone and the hydrocarbon tail interact with multiple hydrophobic residues at the tunnel wall. The 3-OH of cholesterol forms direct (Q724) or water-mediated hydrogen bonds (Y583) at the bottom of the binding tunnel.[46] **(C)** Structure of the STARD1-START domain (PDB: 3P0L), folding into an α-helix/β-grip fold globular structure, comprising of a curved β-sheet gripped (green) by two α-helices (pink and orange). The concave face of the β-sheet and the C-terminal α-helix enclose a hydrophobic cavity for sterol binding. **(D)** Representation of the sterol binding pocket in StAR (blue). Amino acids E169, F184, R188, L199, H220 and F267 seem to define the size of the pocket.[56] **(E)** Structure of the Aster domain of Aster-A in complex with 25-HC (PDB: 6GQF), consisting of a curved seven-stranded β-sheet that forms a hydrophobic groove for sterol binding, two short *N*-terminal α-helices and a long C-terminal α-helix that closes the hydrophobic cavity. **(F)** Representation of the 25-HC binding mode in Aster-A. F405, Y524 and F525 determine the ligand orientation, which are conserved in all three mammalian Aster proteins.[57]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

length transcript, consisting only of an FFAT motif and an ORD. In cells, ORP2 is mainly cytosolic with some aggregation on LD surfaces and at the PM. The ORP2-ORD can bind cholesterol, most phosphoinositides and various oxysterols. The FFAT motif associates with ER-localised VAPs.[28,53–55]

ORP4 is present in two splicing variants, ORP4L and OPR4S. Despite its similarity to OSBP, Studies revealed that the ORP4 ORD binds sterols and PI4P, while the PH domain targets PI4P in the Golgi apparatus. ORP9 can be found at the Golgi apparatus. ORP9L, as well as the ORP9S variant without the PH domain, are cholesterol and PI4P binding proteins that sequester and/or modify Golgi PI4P content when expressed in cells.[33]

2.2. Steroidogenic Acute regulatory Protein-related lipid transfer domain family proteins

The mammalian Steroidogenic Acute Regulatory Protein (StAR)-related Lipid Transfer (START) Domain (STARD) family contains 15 members. All members have the StART lipid transfer domain.[28] They are divided into 6 subfamilies according to their ligand binding properties and sequence similarities: the sterol-specific binding and membrane targeted STARD1/D3 subfamily, the sterol-binding and soluble STARD4 subfamily (STARD4/D5/D6), and the phospholipid/ceramide-binding STARD2 subfamily (STARD2/D7/D10/D11). Members of the remaining subfamilies are multi-domain proteins with currently no identified ligand: the RhoGap STARD8/12/13 subfamily, the acyl-CoA thioesterase STARD14/15 subfamily, and the kinesin motor STARD9 subfamily (Fig. 1B). [27,35]

The START domain is defined by a conserved sequence of approximately 210 amino acids, folding into an α-helix/β-grip fold globular structure, and comprising a curved β-sheet gripped by two α-helices (Fig. 2C).[58] The concave face of the β-sheet and the C-terminal α-helix enclose a hydrophobic cavity that can accommodate lipid molecules (Fig. 2D).[56] Lipid binding requires a conformational change involving movement of the C-terminal helix with specificity for lipid binding driven by residues that form the hydrophobic binding cavity. Membrane interactions via the C-terminal α-helix and movement of the Ω-1 loop promote access to the lipid binding pocket.[59]

Five STARD family members have been shown to harbour and transport sterols (STARD1, 3, 4, 5 and 6).[35,60–62] STARD1 is located at mitochondrial membranes, induced by an *N*-terminal membrane targeting sequence.[62,63] STARD3 harbours an *N*-terminal MLN64 NH_2-terminal (MENTAL) domain that targets the protein to the late endosomes and orients the C-terminal START domain towards the cytoplasm.

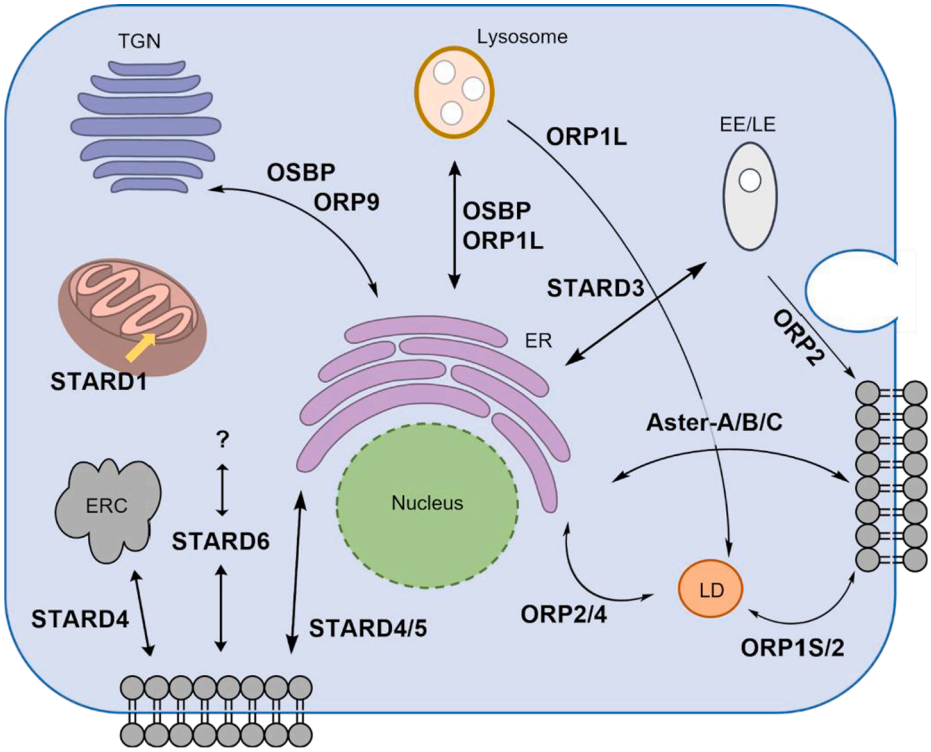

Figure 3. Intracellular cholesterol transport. STPs are written in black and black arrows represent interorganellar cholesterol transport. The yellow arrow represents intermitochondrial transport. EE = early endosome, ER = endoplasmic reticulum, ERC = endocytic recycling compartment, LD = lipid droplet, LE = late endosome, TGN = Trans Golgi Network. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

At the end of the MENTAL domain locates a FFAT-like motif that interacts with ER-anchored vesicle-associated membrane protein (VAMP)-associated proteins (VAPs).[64,65] STARD4/5/6 comprise almost entirely of a START domain, with 30% sequence similarity with one another, their soluble nature contributes to dynamic membrane associations of these proteins (Fig. 3).[66,67] Models of cholesterol complexes of STARD1,[68,69] STARD3,[59,68] and STARD5[56] have been proposed based on molecular docking and molecular dynamics simulations, suggesting a "head-first" binding mode. However, while structures of non-sterol binding START domains are available with their respective ligands, no structure of a START domain-sterol complex currently exists. Hence, the actual mode of binding and the determinants of the specificity of STARD1, 3, 4, 5 and 6 towards different sterols remain to be understood.

2.3. Aster/GRAMD1 family proteins

The lipid transfer proteins anchored at a membrane contact site (Lam) family comprises three members in mammals (GRAMD1a/Aster-A, GRAMD1b/Aster-B, and GRAMD1c/Aster-C)). They share an *N*-terminal GRAM and a StART-like domain followed by a C-terminal transmembrane domain.[36] Mediated through interactions between their transmembrane domains and their luminal amphipathic helices, Asters form homo- and heteromeric complexes. While localizing at the ER throughout steady state, they rapidly move to ER-membrane contact sites via the GRAM domain dependent detection of accessible cholesterol and anionic lipids within the PM (Fig. 3).[27,28]

The GRAM domain has a typical PH domain β-sandwich fold, consisting of seven antiparallel β-strands arranged in two β-sheets followed by a C-terminal α-helix. Modelling studies of the Aster-B-GRAM domain based on the Lam6-GRAM revealed the presence of a basic patch, important for sensing anionic lipids in membranes. In addition, the GRAM domain of Aster-B has a distinct site near the basic patch that is dedicated for recognizing accessible cholesterol.[36,70,71]

One of the key elements of the Aster protein family is the presence of a StART-like ASTER domain, consisting of a highly curved seven-stranded β-sheet that forms a hydrophobic groove for sterol binding, two short *N*-terminal α-helices between the β1 and β2 strands, and a long C-terminal α-helix that closes the hydrophobic cavity together with the two shorter helices. The Ω1 loop is located between the β3 and β4 strands and has been associated with regulating sterol entry and exit.[36,72,73]

So far, two crystal structures of murine Aster-A and Aster-C have been solved with sterol derivatives: Aster-A in complex with 25-HC (Fig. 2E, PDB: 6GQF)[57] and Aster-C with cholesterol derived ligand AI-1 l (PDB: 7AZN)[74]. In the binding pocket, an additional volume within the cholesterol-binding cavity, occupied by a glycerol molecule, presumably provides space for larger ligands. The structure of Aster-A has a high structural similarity to the START domain fold. However, sequence differences within the cholesterol-binding pocket result in a different binding mode for the ligand, such that in Aster-A the sterol is slightly rotated. In particular, F405, Y524 and F525 determine the ligand orientation, which are conserved in all three mammalian Aster proteins (Fig. 2E).[57]

3. (Patho)physiological functions of STPs

3.1. Functions of the oxysterol-binding protein related proteins (ORPs)

The prototypical ORP, OSBP, is responsible for regulating cholesterol transport from the ER.[28] It promotes the transport of cholesterol to the TGN, cycling PI4P in the opposite direction.[48] Through a feedback loop where PI4P is hydrolysed to PI by Sac1 in the ER, OSBP does not maintain contact to the TGN for extended periods, though this can be forced by employing OSBP inhibitors. Through its function at the TGN, OSBP maintains the correct lipid composition required for the function of TGN-associated processes. More recently, a role for OSBP in

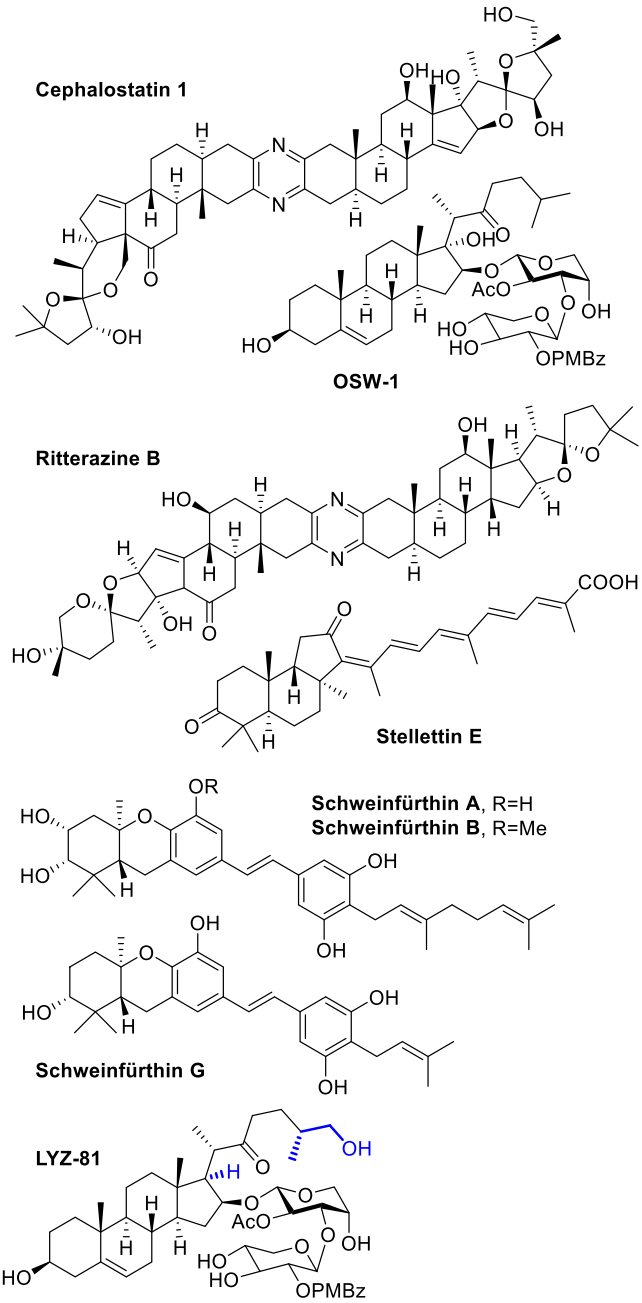

Figure 4. Structures of natural product inhibitors of OSBP and ORP4L. Structural differences between OSW-1 and LYZ-81, which inhibits ORP4L selectively, are highlighted in blue on LYZ-81. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mediating cholesterol transport from the ER to the lysosome has been reported.[47] This controls cholesterol levels at the lysosome, activating mammalian target of rapamycin (mTOR) complex 1 activity, leading to autophagy inhibition. As such, inhibiting OSBP with small molecules induces autophagy.

The closest structural relative to OSBP is ORP4. Its splicing isoforms display different localisation and functions, with ORP4L localising to ER-Golgi contact sites and contributing to the maintenance of cholesterol and PI4P homeostasis, in part through a heterodimerisation with OSBP.[75] More recently, ORP4L has also been shown to also bind and extract PI(4,5)*P*2 from the PM, leading to its subsequent hydrolysis by phospholipase Cβ3 (PLC-β3) to inositol 1,4,5-trisphosphate (IP3).[76] This activity has been associated with leukemic cancer cell survival, and

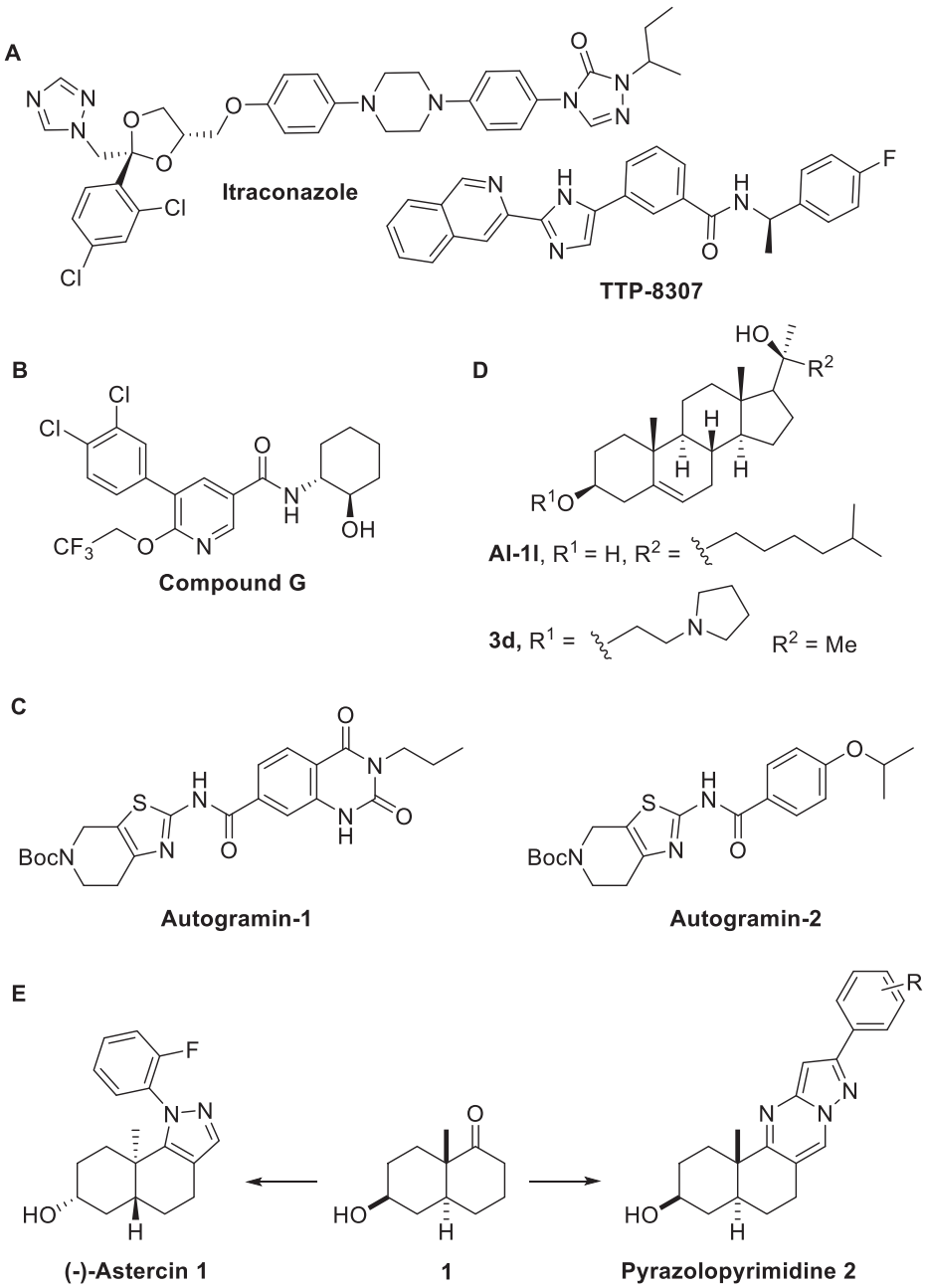

Figure 5. Small molecule STP inhibitors. (A) OSBP inhibitors identified through antiviral screens. (B) Recently identified inhibitors of ORP7. (C) Aster-A inhibitors identified through phenotypic screening for new autophagy inhibitors. (D) Structure of oxysterol derived Aster inhibitors. (E) Aster-A and –C inhibitors identified from the screening of a sterol-inspired compound library.

inhibition of ORP4L is proposed as a strategy to target this vulnerability. In contrast to ORP4L, ORP4S predominantly associates with vimentin filaments in a manner that appears independent of its sterol-binding capabilities.[77]

Additional sterol-binding ORPs include ORP1 and ORP2, which are also structurally related. The long isoform ORP1L is localised at the LEL. Its specific role as a sterol sensor or transporter has not been clearly delineated, though its role in transporting cholesterol from the ER to the endolysosome has been proposed.[78] Through its role in mediating ER/LY contacts, it has also been shown to play an important role in the late stage (maturation) of autophagy, promoting the autophagosome-lysosome fusion.[79] In contrast to ORP1L, ORP1S is localised to the cytoplasm and regulates PM cholesterol levels by shuttling cholesterol from the LEL to the PM and from the PM to the ER and lipid droplets.[80] Similarly, ORP2 also drives sterol transfer to the PM from the LE, but also from the ER,[81] with these processes occurring in exchange with PI(4,5)*P*2.[54,82] Intriguingly, ORP2 has been reported to play roles in regulating lipolysis from lipid droplets[83] as well as focal adhesion[82] and angiogenesis,[84] suggesting that it may be worth exploring its therapeutic targeting. The final ORP with confirmed cholesterol binding and transport capabilities is ORP9. Both long and short splice variants were shown to possess sterol transport activity in vitro and knockdown resulted in loss of Golgi integrity, suggesting a function in the transport of cholesterol between the ER and the TGN.[52,85]

Other ORPs have been suggested to regulate cholesterol homeostasis, however a direct binding interaction with cholesterol has not been described yet. For example, ORP6 and 7, which localise at ER-PM contact sites, have both independently been shown to regulate cholesterol efflux. In contrast, ORP5 and 8 have been shown to transport PS from the ER to the PM employing the ORD, with PI4P being transported in the

opposite direction.[41] This is also coupled with PI4P hydrolysis in the ER by Sac1. While other reports suggest that ORP5 can transfer sterol analogues in vitro, the majority of the literature reports their phosphoinositide binding and transport ability.[86] Finally, ORPs 10 and 11 are the least well studied. Despite this, a putative cholesterol binding function has been ascribed to ORP10, although its primary role appears to be the transfer of PS from between the ER and the LE.[85,87]

3.2. Functions of the steroidogenic Acute regulatory Protein-related lipid transfer domain family proteins

The biological function of STARD1 in regulating the delivery of cholesterol from the outer (OMM) to the inner mitochondrial membrane (IMM) in steroidogenesis is well established. At the IMM cholesterol is converted to pregnenolone catalysed by the mitochondrial cytochrome P450 cholesterol side chain cleavage enzyme and thereby requires StAR synthesis for cholesterol delivery. Consequently, StAR protein expression levels are predominantly high in tissues with de novo steroidogenic capacity, for example in the adrenal cortex, and corpora luteal cells.[35,63] Mutations in the human STAR gene are the basis for the genetic disorder congenital lipoid adrenal hyperplasia (lipoid CAH). Affected patients have decreased steroid hormone levels and accumulation of cholesterol in lipid droplets in the steroidogenic cells, indicating that *de novo* steroid hormone biosynthesis is blocked in the absence of STARD1 at the step of cholesterol trafficking from the OMM to the IMM.[88] STARD3 transfers cholesterol from the ER to the late endosome and mediates contact between the two organelles. While the cholesterol transfer function of STARD3 is well known, studying the biological function remains a challenge. Early work eliminated STARD3 as the acceptor protein for Niemann Pick type C protein (NPC1)-mediated cholesterol efflux from the late endosomes.[35,61] It is overexpressed in 25% of breast cancers in conjunction with human epidermal growth factor receptor 2 (HER-2).[89] However, the reason for this co-dependency remains unclear and has to be determined. STARD4 shuttles cholesterol between PM and ER as well as PM and ERC, while STARD5 was found to presumably transport cholesterol and primary bile acids between PM and ER. STARD6 is predominantly expressed in testis, with the highest expression levels in round spermatids. In this context, it has been proposed to act as a testosterone transfer protein.[35,90] The biological functions of STARD4/5/6 are not fully understood, however STARD4 knockout mice had decreased body weight and lower serum cholesterol concentrations while otherwise displaying normal physiology.[91]

3.3. Functions of the Aster/GRAMD1 proteins

Despite their recent description, specific physiological functions of the Aster family of STPs have started to be elucidated. The differential patterns of tissue expression observed for the Asters suggest that each should possess distinct functions from one another, with Aster-A having highest expression in the brain, Aster-B in adrenal tissues, and Aster-C in the testes and liver.[57] The role of Aster-A in autophagy was elucidated through identification of the small molecule inhibitor Autogramin-2, which inhibits Aster-A mediated cholesterol transfer between the ER and forming phagophore.[72,92] On the other hand, Aster-B has been identified as a key regulator of HDL-derived cholesterol transport to the ER in steroidogenic tissues, acting downstream of the known scavenger receptor class B type 1 (SR-BI) receptor.[57] Aster-B has additionally been identified as a regulator of ER – mitochondria cholesterol transport and the uptake of cholesterol ester-derived fatty acids, further supporting its role as a regulator of steroidogenesis.[93] Mutations in the GRAM domain of Aster-B have also been associated with intellectual disability, arising from defective cholesterol sensing.[94] Studies of Aster-C have identified it as a negative regulator of mTORC1 signalling under conditions of nutrient starvation. Similarly to Aster-A, a putative role of Aster-C as a negative regulator of starvation-induced autophagy has been suggested, as well as regulating mitochondrial bioenergetics by mediating mitochondria – ER cholesterol transfer.[95] Changes in expression levels of the Aster proteins have also been associated with differential prognoses in cancers including renal carcinoma and breast cancer, highlighting how small-molecule modulation of the Asters could prove to have therapeutic relevance.[96,97] Recently, Bandara *et al.* provided evidence that Aster proteins can bind carotenoids. Among these proteins, Aster-B protein is highly expressed in carotenoid-rich tissues of human and mice, suggesting its critical role in carotenoid transfer and retention.[98]

4. Small molecule modulators of STPs

To date, small molecule modulators of STPs have mostly been identified serendipitously, with only a limited number of STPs being targeted at all. In the seminal work by Burgett *et al.* a seemingly disparate group of natural products was identified as targeting OSBP and ORP4L.[99] These molecules, which were termed ORPphilins, include cephalostatin 1, OSW-1, ritterazine B and schweinfurthin A (Fig. 4). These were identified as selective growth inhibitors of *p21*$^{-/-}$ HCT116 colorectal carcinoma cells, and were shown to target both OSBP and ORP4L, although their selectivity across other families of STPs remains to be established. Of these compounds, Schweinfürthin A showed the most interesting selectivity for OSBP over ORP4L. This trend was also observed for the closely related Schweinfürthin G, whose additional intrinsic fluorescent has been employed to track OSBP levels and localisation in cells.[100,101] In separate work, analogues of OSW-1 were synthesised to explore their antiproliferative effects in the context different cancer cell lines.[102] Following the identification of OSBP/ORP4L as the targets of OSW-1, one of the identified analogues (termed LYZ-81) was identified as possessing improved selectivity for ORP4L over OSBP,[76] complementing Schweinfürthin G in the context of selective STP modulators. Intriguingly, although the ORPphilins and related compounds have been reported to display similar mechanisms of action in cells, their effect on OSBP and ORP4L protein levels can be markedly different. For example, cephalostatin and OSW-1 cause a dose-dependent proteasomal degradation of OSBP but not of ORP4L, while Schweinfürthin A and LYZ-81 do not degrade either.[76,99] The structural basis for these differences remains to be determined.

Importantly, OSBP/ORP4L inhibitors including OSW-1 have shown very potent anti-viral activity,[103] along with their antiproliferative activity in specific cancer cell lines.[104] In addition to the diverse set of natural product STP inhibitors, a series of synthetic compounds have also been shown to bind OSBP and ORP4L and produce similar phenotypes (Fig. 5A). These include the anti-fungal itraconazole (ITZ), which has potent activity against enteroviruses, mediated by its inhibition of OSBP.[105] Unfortunately, this compound has a broad target profile, including the inhibition of hedgehog (Hh) signalling.[106] However, SAR analysis led to the synthesis of an analogue with improved enteroviral potency, and abolished Hh activity.[107] Similarly, the known enterovirus inhibitor TTP-8307 was also shown to target OSBP both in vitro and in cells, albeit with lower potency than both ITZ and OSW-1.[108] Besides OSBP/ORP4L inhibitors, the only other ORP to have been inhibited with a small molecule is ORP7, which was identified as a target of Cpd G in a phenotypic screen measuring upregulation of ABCA1-dependent cholesterol efflux (Fig. 5B).[109] As with all ORP inhibitors to date, selectivity across the remainder of the ORPs and against other STP families would need to be evaluated, before the compounds can fulfil their potential to be excellent tools for the study of ORPs.

While a significant number of small modulators have been reported for the ORP family of STPs, the same cannot be said for the sterol-binding STARDs. From a virtual screen a compound has previously been identified as a weak inhibitor of STARD3, however validation and optimisation experiments remain to be carried out.[110] The only other reported inhibitors of STARDs target phosphatidylcholine transfer protein (PC-TP, STARD2)[111] and ceramide transfer protein (CERT, STARD11).[112–114] As such, there is a significant gap in the STP field that remains to be filled.

Of all the STP families, the Asters have been targeted extensively by small molecules. From a phenotypic screen for new small molecule autophagy inhibitors, several acyl aminothiazole-based compounds, later termed autogramins, were identified as inhibitors of Aster-A (Fig. 5C).[72] These compounds display excellent selectivity towards Asters –B and –C, as well as STARD1 and −3, though their activity at other STPs has not been measured yet. In addition to this, oxysterol-based Aster inhibitors have also recently been disclosed (Fig. 5D), though further optimization is required to improve their selectivity and remove their inhibition of hedgehog signaling.[115] Recent work published from our group has identified inhibitors of the Aster/GRAMD1 proteins through the synthesis of a sterol-inspired compound collection.[116] We designed and synthesised a sterol-inspired screening collection featuring fusions of a steroidal scaffold **1** to a series of heterocyclic scaffolds (Fig. 5E). Screening of the compounds against the Aster proteins by differential-scanning fluorimetry (DSF), fluorescence polarisation (FP), and fluorescence resonance energy transfer (FRET) assays culminated in the identification of several hit compounds. The pyrazolopyrimidine-fused series **2** was identified as a new chemotype of Aster-A inhibitors, complementing the previously reported Autogramin-2, and inhibiting Aster-A with micromolar potency. A second pyrazole-fused series, the most potent of which we name (-)-Astercin 1, were identified as hits against Aster-C. (-)-Astercin 1 potently inhibited Aster-C with greater than 30-fold and 50-fold selectivity over both Aster-B and Aster-A respectively, making it the most potent and selective Aster-C inhibitors known to date.

5. Outstanding questions and outlook

Work surrounding the structure, function, and modulation of STPs has grown considerably in recent years, spurred by advances in structural, chemical and cell biology. Despite this, several important issues remain to be addressed. The specific lipid binding and transport activity has not been conclusively ascertained for all STPs. One of the main challenges is the differentiation of in vitro transport activity between liposomes carried out with recombinant protein (domains), with the activity in cells. In this regard, robust cellular assays to precisely measure inter-organellar lipid transport are still lacking. Furthermore, the majority of STPs contain a variety of different domains in addition to the SBD, the precise function of which is not always clear. While some, including the PH-GRAM domain, are associated with specific membrane tethering, others are also predicted to participate in important protein–protein interactions. Additionally, membrane localisation is also governed by additional protein-lipid interactions, the specificity of which is also not always clear. Finally, while x-ray structures of the ORD, StART or ASTER domains are more prevalent, liganded structures and those of other domains are less common.

Within the context of small molecule modulators, significant advances are still possible, with many STPs not yet targeted. For example, there are no well-validated inhibitors of the cholesterol-binding STARDs (1, 3, 4–6), and known ORP inhibitors have not been profiled for selectivity against other ORPs, STARDs or Asters. Despite this, recent efforts in unbiased screening as well as targeted design approaches are highly promising, as exemplified by the recent identification of several Aster inhibitors. When coupled to the growing number of STP x-ray structures, the outlook for the development of a greater number of potent and selective STP modulators and tool compounds is positive.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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