REVIEW

The mammalian START domain protein family in lipid transport in health and disease

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Abstract

Lipid transfer proteins of the steroidogenic acute regulatory protein-related lipid transfer (START) domain family are defined by the presence of a conserved ~210 amino acid sequence that folds into an α/β helix-grip structure forming a hydrophobic pocket for ligand binding. The mammalian START proteins bind diverse ligands, such as cholesterol, oxysterols, phospholipids, sphingolipids, and possibly fatty acids, and have putative roles in non-vesicular lipid transport, thioesterase enzymatic activity, and tumor suppression. However, the biological functions of many members of the START domain protein family are not well established. Recent research has focused on characterizing the cell-type distribution and regulation of the START proteins, examining the specificity and directionality of lipid transport, and identifying disease states associated with dysregulation of START protein expression. This review summarizes the current concepts of the proposed physiological and pathological roles for the mammalian START domain proteins in cholesterol and lipid trafficking.

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Introduction

Lipid transport proteins play an important role in non-vesicular trafficking of cholesterol, phospholipids, and sphingolipids between biological membranes to help maintain the proper cholesterol:phospholipid:sphingolipid distribution (reviewed in Prinz (2007) and Lev (2010)). Cholesterol content is maintained at relatively low levels within the endoplasmic reticulum (ER) and mitochondrial membranes compared with the plasma membrane (PM; Mesmin & Maxfield 2009). The source of PM cholesterol is from both *de novo* synthesis in the ER and cellular uptake of low-density lipoprotein (LDL)-derived cholesterol. *De novo*-synthesized cholesterol is rapidly transferred to the PM by non-vesicular trafficking mechanism(s), implicating a role for soluble sterol transport proteins (Maxfield & van Meer 2010). Receptor-mediated endocytosis of LDL delivers lipoproteins to the late endosomes/lysosomes where free cholesterol is hydrolyzed from cholesterol esters (Goldstein & Brown 2009). The free cholesterol is recycled back to the PM or transported to the ER. In the PM, cholesterol can be found clustered with sphingolipids into detergent-resistant protein–lipid microdomains referred to as lipid rafts (Danielsen & Hansen 2003, Rajendran & Simons 2005, Hanzal-Bayer & Hancock 2007). Functionally, lipid rafts are proposed to provide an organized membrane region for signaling and other functions (Lingwood & Simons 2010). Changes in ER membrane cholesterol levels signal for changes in gene expression leading to altered cholesterol metabolism while transport of cholesterol to mitochondria is required for production of steroid hormones and bile acids. Thus, it has long been appreciated that maintaining proper cholesterol distribution within the cell is important for cholesterol homeostasis and membrane function (Qin *et al.* 2006, Maxfield & van Meer 2010). There are two major gene families for lipid transfer proteins with specificity for sterols: the steroidogenic acute regulatory protein (StAR)-related lipid-transfer (START) domain family and the oxysterol-binding protein (OSBP) family, which includes the OSBP-related proteins (ORPs). This review focuses on the role of the mammalian START domain family in lipid trafficking and the implications for dysregulation of START protein expression in disease states. The OSBP/ORP family has been reviewed by others (Prinz 2007, Ngo *et al.* 2010).

The START domain protein family

The START domain is defined by a conserved sequence of ~210 amino acids that folds into an α/β helix-grip structure

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forming a hydrophobic pocket for binding sterols and other lipids (Ponting & Aravind 1999, Iyer *et al.* 2001). The helix-grip fold is used to define a large superfamily of proteins that bind hydrophobic lipids, classified as the SRPBCC protein superfamily on NCBI's Conserved Domain Database (START/RHO_alpha_C/PITP/Bet_v1/CoxG/CalC, cl14643 NCBI; Marchler-Bauer *et al.* 2009). BLASTP searches have identified START domains in genomes from plants, bacteria, protists, and animals, but not in archaea or yeast (Schrick *et al.* 2004). START domains are relatively rare in bacteria and protist genomes, and to date, there is no evidence that these proteins are expressed. Proteins containing the START domain are most abundant in plants and are highly represented in proteins that contain a homeodomain, suggesting a role in transcription (Schrick *et al.* 2004). The homeodomain–START domain structure has only been found in plant proteins. Coupling the START domain with other motifs is, however, a common theme as START domains in other phyla are found in multi-domain proteins that provide additional functions such as protein localization, enzymatic activity, or signaling (Ponting & Aravind 1999, Iyer *et al.* 2001).

The mammalian START domain protein family is well characterized and is composed of 15 members that group into six subfamilies based on the sequence and ligand similarities (Ponting & Aravind 1999, Soccio *et al.* 2002; Table 1). In very general terms, the subfamilies can be classified into cholesterol- and oxysterol binding proteins (STARD1/D3 and STARD4/D5/D6 subfamilies), the phospholipid- and sphingolipid-binding proteins (STARD2 (phosphatidylcholine transfer protein, PCTP)/D7/D10/D11 subfamily), the multi-domain proteins containing either putative Rho-GTPase signaling function (STARD8/12/13 subfamily) or thioesterase activity (STARD14/15 subfamily), and the STARD9 subfamily composed of a single member of unknown function that is not further discussed (Soccio *et al.* 2002, Soccio & Breslow 2003, Alpy & Tomasetto 2005). The crystal structures for the START domains of hSTARD3/metastatic axillary lymph node 64 kDa protein (MLN64) and mSTARD4 were the first to be solved and showed an α/β helix-grip fold with a nine-stranded anti-parallel β-sheet forming a U-shaped hydrophobic cleft that binds the ligand and is flanked by amino- and carboxyl-terminal α helices (Tsujishita & Hurley 2000, Romanowski *et al.* 2002). The carboxyl-terminal α helix is proposed to serve as a 'cap' to the ligand-binding site, with lipid access to the binding pocket requiring a conformational change in the START domain and movement of the C-terminal helix (Baker *et al.* 2005, Bose *et al.* 2008*a*,*b*). To date, crystal structures for the START domains of hSTARD1, hSTARD5, hSTARD2/PCTP, STARD11/CERT, hSTARD13, and hSTARD14 have been reported and the data confirm the basic three-dimensional helix-grip fold structure across the five mammalian subfamilies that defines this family of proteins (Roderick *et al.* 2002, Kudo *et al.* 2008, 2010, Thorsell *et al.* 2011). Modeling of START domain conformational changes and mechanisms for cholesterol absorption/desorption have been reviewed elsewhere (Alpy & Tomasetto 2005, Miller 2007, Lavigne *et al.* 2010).

Cholesterol trafficking and homeostasis

Intracellular cholesterol levels are tightly regulated by controlling biosynthetic and degradation pathways. There are several excellent reviews on cholesterol homeostasis and cholesterol trafficking (Russell 2003, Soccio & Breslow 2004, Prinz 2007, Brown & Goldstein 2009, Mesmin & Maxfield 2009, Maxfield & van Meer 2010, Wollam & Antebi 2011), and only a brief overview of these topics is provided to set the cellular context for START protein function(s). The major regulatory step for cholesterol biosynthesis is the expression and activation of the enzyme HMG-CoA reductase (HMGR). HMGR transcription is controlled by sterol regulatory element-binding protein-2 (SREBP2), a member of the basic helix–loop–helix–leucine zipper (bHLH-Zip) transcription factor family that is encoded by the *SREBP2* gene (Hua *et al.* 1993, Yokoyama *et al.* 1993, Sakai & Rawson 2001). SREBP2, however, is a proteolytic fragment of a larger transmembrane protein of the ER that forms a protein complex composed of SREBP, SREBP-cleavage-activating protein (SCAP), and insulin-induced genes 1 and 2 (INSIGs) that functions as a cellular cholesterol sensor. When ER cholesterol levels decline below some threshold level, SREBP-SCAP dissociates from INSIGs and moves to the Golgi apparatus where two proteolytic cleavages releases a ~50 kDa N-terminal fragment that translocates to the nucleus and activates target gene transcription (Anderson 2003; Fig. 1). Major target genes induced by SREBP2 in the liver are within the cholesterol biosynthetic pathway including HMGR and the LDL receptor (LDLR) (Horton *et al.* 2003). A resulting increase in cholesterol synthesis (HMGR) and uptake (LDLR) increases intracellular cholesterol levels. The resulting increase in ER cholesterol stabilizes the INSIG-SCAP-SREBP2 complex within the ER and thereby suppresses SREBP2 processing and subsequent transcriptional function(s). Cholesterol is also converted to cholesterol esters by acyl-CoA:cholesterol acyl transferase activity (ACAT), an enzyme localized to the ER. An increase in ER cholesterol levels activates ACAT leading to increased cholesterol ester synthesis.

Cholesterol metabolism to bile acids in the liver represents the major route for cholesterol clearance. There are two pathways for bile acid biosynthesis, the classical pathway that is initiated in the cytosol and the alternative pathway in mitochondria (reviewed in Russell 2003). In liver mitochondria, cholesterol is hydroxylated on the side chain at position C27 or C25 by the cytochrome P450 enzyme CYP27A1 to produce the oxysterols 27-hydroxycholesterol (27HC) or 25-hydroxycholesterol (25HC) (Li *et al.* 2007). In addition to simply serving as intermediates in bile acid biosynthetic pathway, 27HC and 25HC are cellular signals

Table 1 Characteristics of the mammalian START domain protein family members

START subfamily	START protein	Other name(s)	Domain structure	Tissue distribution*,†	Cellular location	Lipid binding	Function/metabolic pathway
STARD1/D3	STARD1	StAR	—^{SS}START	Adrenal, ovary, testis, brain†	Mitochondria‡,a,b,c	Cholesterol[d,e]	Steroidogenesis[1]
	STARD3	MLN64	MENTAL-START	Placenta, breast, macrophages*	Transmembrane, late endosomes‡,a,b,c	Cholesterol[d,e]	endosomal cholesterol efflux[2]
STARD4	STARD4		START	Liver, macrophages, kidney†	Cytosolic[a] ≫ ER[a,b] mitochondria[b]	Cholesterol¥,d	ACAT activation[3]
	STARD5		START	Macrophage kidney proximal tubules†	Cytosolic ≫ ER, Golgi, PM[a]	Cholesterol, 25HC¥,d	ER stress response[4] ?
	STARD6		START	Testis germ cell†	Cytosolic[c], mitochondria[b]	Cholesterol[d,e]	?
STARD2	STARD2	PC-TP	START	Liver, lung*	ER/Golgi‡,b	PC[f]	Glycolysis[5], FA synthesis[5]
	STARD7 STARD7-I	GTT-1	—START	Liver*	Cytosolic[c] mitochondria[c]	PC[e]	?
	STARD10	PCTP like	—START—	Liver, kidney, testis, colon*	Cytosolic[c]	PC > PE[e]	?
	STARD11	CERT, GPBPΔ26, COL4A3BP	–PH—^{FFAT}—START	Liver*	ER/Golgi‡,b	Ceramide¥,e	ER → Golgi ceramide transport[6]
The RhoGAP multidomain proteins	STARD8	DLC-3	—RhoGAP–START	Cancer*	Focal adhesions‡	?	Tumor suppressor[7]
	STARD12	DLC-1	— SAM —RhoGAP–START	Cancer*	Focal adhesions‡ PM[a]	?	Cytoskeletal organization[8] tumor suppressor[8]
	STARD13	DLC-2	— SAM —RhoGAP–START	Endothelial cells*	Focal adhesions‡	Charged lipid[d] (?)	Tumor suppressor[9]
STARD9	STARD9		—START	?	Cytosolic[c] ?	?	?
The thioesterase multidomain proteins	STARD14	ACOT11_v2, BFIT2	Hotdog–Hotdog– START	Brown adipose tissue†	Cytosolic[c] ?	Fatty acid[d] (?)	Medium chain fatty-acyl-coA hydrolysis[10]
	STARD15	ACOT12	–Hotdog–Hotdog– START	Liver†	Cytosolic[c]	?	Acetyl-coA hydrolysis[11]

StAR, steroidogenic acute regulatory protein; START, StAR-related lipid transfer domain; MLN64, metastatic axillary lymph node 64 kDa protein; PC-TP, phosphatidylcholine transfer protein; *GTT1*, gestational trophoblastic tumor gene-1; CERT, ceramide transfer protein; GPBP, Goodpasture antigen-binding protein; COL4A3BP, collagen-type IV α3 binding protein; DLC, deleted in liver cancer; ACOT, acyl-CoA thioesterase; BFIT2, brown fat-inducible thioesterase-2; ss, signal sequence; MENTAL, MLN64-N terminal domain; PH, pleckstrin homology domain; FFAT, peptide EFFDAxE; SAM, sterile α domain; Hotdog, conserved domain structure for acyl-coenzyme A thioesterase family that has thioesterase activity; ACAT, acyl-CoA:cholesterol acyl transferase activity. Cellular location: ‡domains direct subcellular location; [a]based on immunohistochemistry data for endogenous protein expression; [b]based on *in vitro* activity; [c]based on structure. Lipid binding: ¥direct ligand binding assay; [d]modeled based on structure; [e]based on *in vitro* lipid extraction assay; [f]shown in crystal (Tsujishita & Hurley 2000, Roderick *et al*. 2002, Olayioye *et al*. 2005, Rodriguez-Agudo *et al*. 2005, Murcia *et al*. 2006, Bose *et al*. 2008*a*,*b*, Kudo *et al*. 2008, Rodriguez-Agudo *et al*. 2008, Barbar *et al*. 2009, Horibata & Sugimoto 2010, Thorsell *et al*. 2011). Function: [1](Stocco 2001); [2](Alpy & Tomasetto 2006); [3](Rodriguez-Agudo *et al*. 2011); [4](Soccio *et al*. 2005, Chen *et al*. 2009); [5](Scapa *et al*. 2008); [6](Hanada *et al*. 2009); [7–9](Durkin *et al*. 2007*a*,*b*); [10,11](Kirkby *et al*. 2010).

*Ubiquitous expression with the major tissues studied listed.

†Restricted expression (Adams *et al*. 2001, Stocco 2001, Soccio *et al*. 2002, Strauss *et al*. 2003, Gomes *et al*. 2005, Rodriguez-Agudo *et al*. 2005, Durkin *et al*. 2007*a*,*b*, Kanno *et al*. 2007*a*,*b*, Chen *et al*. 2009, Kirkby *et al*. 2010, Mencarelli *et al*. 2010, Rodriguez-Agudo *et al*. 2011). Note that *STARD4* and *STARD5* mRNA have been detected at low levels in heart; therefore, they may have a broader expression.

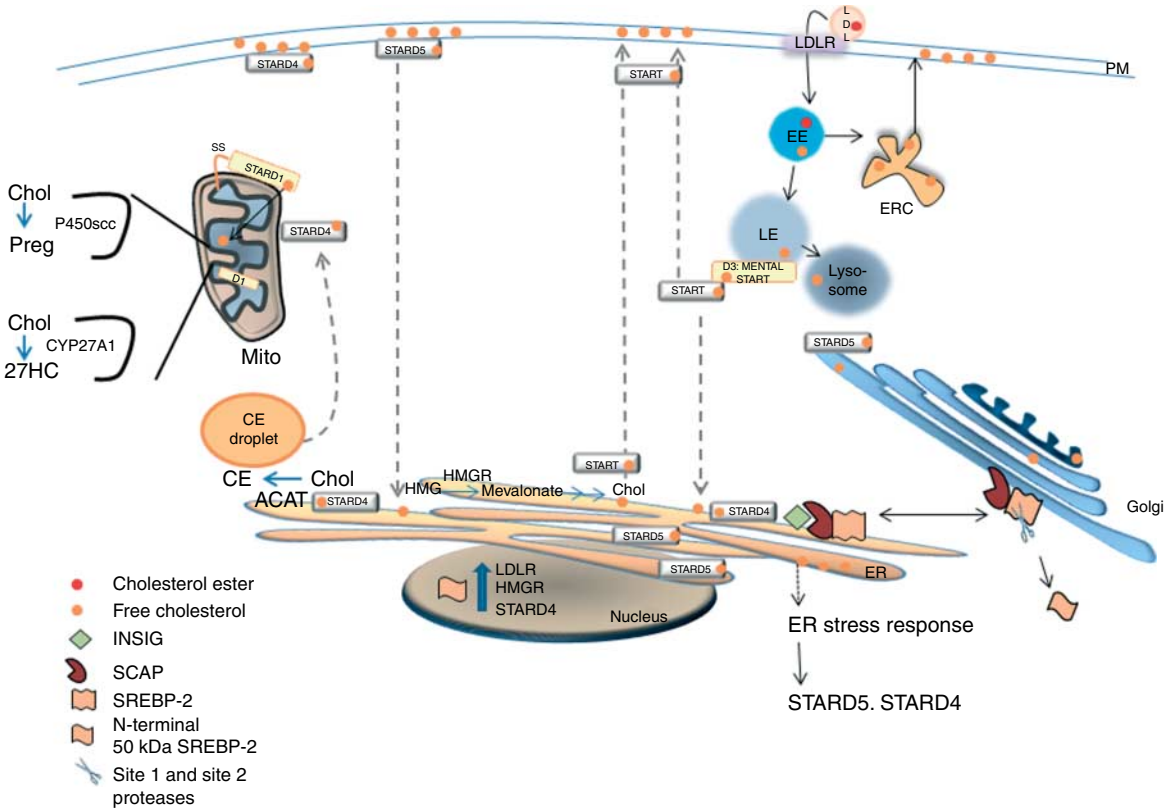

Figure 1 Model for non-vesicular cholesterol trafficking by the START domain proteins. The cholesterol-binding START proteins are shown at subcellular locations identified by immunohistochemistry (Alpy *et al.* 2001, Rodriguez-Agudo *et al.* 2005, Chen *et al.* 2009). STARD4 stimulates ACAT activity and increases CE synthesis and, therefore, is depicted in close proximity to ACAT at the ER membrane (Rodriguez-Agudo *et al.* 2011). STARD5 does not stimulate ACAT activity and is shown at different ER sites than STARD4 to indicate potential different functions. The SREBP2 pathway is cartooned to show activation of SREBP2 target genes HMGR, LDLR, and STARD4. The ER to PM and PM to ER cholesterol trafficking is shown to be mediated by a START protein. Although no data have directly demonstrated START proteins in this directional cholesterol trafficking pathway, STARD5 is presented as shuttling cholesterol from the PM to the ER as an example for a START protein in this process based on the apical membrane association renal proximal tubule cells (Chen *et al.* 2009). Cholesterol transport to mitochondria by STARD1 and STARD4. STARD1 binds cholesterol and facilitates its translocation into the matrix to the P450scc or CYP27A1 enzyme for steroid hormone or oxysterol synthesis respectively. Cholesterol in late endosome/lysosomes is transported to the PM and ER. STARD3/MLN64 in late endosomes is depicted as the intermediate between late endosome/lysosome cholesterol and a soluble cytoplasmic START protein, potentially STARD4 or STARD5. STARD3/MLN64 may obtain cholesterol from NPC2 or MENTHO directly or from NPC1 (see text for details). Cholesterol accumulation in the ER will promote ER stress that can induce STARD5 and STARD4 expression. The purpose for START protein induction upon ER stress is not known and future studies are required to determine whether they are protective or detrimental to the stress response. Solid arrows, vesicular transport; dashed arrows, non-vesicular transport.

that help control cholesterol homeostasis by repressing cholesterol synthesis and enhancing cholesterol efflux. 27HC and 25HC bind to INSIGs and block SREBP-SCAP translocation to the Golgi, thereby repressing SREBP2-dependent pathways (cholesterol synthesis; Radhakrishnan *et al.* 2007, Sun *et al.* 2007). In addition, 27HC and 25HC are ligands for the liver X receptor alpha (LXRα), a nuclear receptor that regulates lipid metabolism to help maintain cholesterol homeostasis. LXRα directly activates transcription of the gene encoding the ATP-binding cassette A1 (ABCA1), a cholesterol transporter located in the PM that is important for cholesterol efflux from extra-hepatic cells. Another LXRα target gene is *SREBP1* that encodes SREBP1c, another ER membrane-bound SREBP protein that has an N-terminal bHLH-Zip transcription factor that activates genes that encode enzymes in the fatty acid biosynthesis pathway.

Macrophages also convert cholesterol to 27HC in mitochondria using CYP27A1. In extra-hepatic cells, the C27 hydroxyl group of 27HC can be oxidized to a carboxylic acid generating 3β-hydroxy-5-cholestenoic acid, a soluble bile acid precursor that can be secreted and taken up by the liver where it can be further metabolized to bile acids (Babiker & Diczfalusy 1998, Bjorkhem *et al.* 1999). Thus, the action of CYP27A1 and production of 27HC in macrophages can decrease cellular cholesterol levels by two mechanisms; activation of the LXR-dependent pathway leading to enhanced cholesterol efflux via

ABCA1 and production of soluble 3β-hydroxy-5-cholestenoic acid. These are important adaptive responses since macrophages scavenge oxidized-LDL and accumulate lipids in the process of foam cell development. The mechanisms that modulate oxysterol production can have an impact on overall cholesterol homeostasis in liver and macrophages.

Disorders in cholesterol homeostasis are recognized as important contributors to disease states associated with dyslipidemia, e.g. atherosclerosis, fatty liver disease, diabetes, and cancer. This review highlights the current literature that implicates dysregulation of START protein expression in several of these disease states.

The cholesterol/oxysterol-binding START proteins

STARD1/STARD3 subfamily: the membrane-targeted START proteins

StAR is the founding member of the START domain protein family and is expressed predominantly in the adrenal and gonads where it functions to bind cholesterol and facilitate its transfer from the outer to the inner mitochondrial membrane to initiate steroid hormone biosynthesis (Clark *et al.* 1994, Stocco & Clark 1996). StAR is unique among START family members in that it contains a mitochondria-targeting sequence, a classical amino-terminal amphipathic helix that directs the protein to the mitochondria. It is a nuclear-encoded phosphoprotein that is synthesized in the cytosol as a 37 kDa precursor protein. Mitochondrial import and processing of the precursor produce a 32 kDa intermediate product and a mature 30 kDa form that is localized within the matrix (reviewed in Stocco (2001)). Phosphorylation of the 37 kDa StAR protein at Ser194/195 (mouse/human) by protein kinase A is required for maximal activity (Arakane *et al.* 1997, Fleury *et al.* 2004, Jo *et al.* 2005, Dyson *et al.* 2008). Although the detailed mechanism for StAR-mediated cholesterol transfer across the mitochondrial membranes is not established, structural, biophysical, and biochemical studies have provided significant insight into this process. One study has shown that the processing of newly synthesized 37 kDa STAR is important for cholesterol transfer function while other reports support that association of the START domain with the outer mitochondrial membrane is sufficient to promote cholesterol transfer (Arakane *et al.* 1996, 1998, Artemenko *et al.* 2001, Bose *et al.* 2002, Baker *et al.* 2007). Structural modeling and biophysical studies indicate that conformational changes, e.g. movement of the C-terminal α-helix, a pH-dependent molten globule transition, are important to promote cholesterol release and activate cholesterol transfer across the mitochondrial membranes (Bose *et al.* 1999, Baker *et al.* 2005, Yaworsky *et al.* 2005, Murcia *et al.* 2006, Barbar *et al.* 2009, Fluck *et al.* 2011). Cholesterol desorption into the intramembrane space for uptake by the inner membrane has been suggested for StAR functioning independent of cholesterol transfer (Christenson & Strauss 2001). However, more recent biochemical studies indicate functional interactions between StAR and components of a putative cholesterol transfer channel, suggesting a multi-protein complex transfer mechanism for cholesterol movement from the outer to the inner mitochondrial membrane (Hauet *et al.* 2005, Bose *et al.* 2008*a*,*b*, Rone *et al.* 2009). There is evidence to suggest that StAR phosphorylation occurs at the mitochondria and that StAR phosphorylation is important for processing to the 30 kDa protein (Artemenko *et al.* 2001, Bose *et al.* 2008*a*,*b*, Dyson *et al.* 2008). Once StAR has been imported into the mitochondrial matrix and processed to the 30 kDa mature form, it is no longer functional since it is no longer accessible to the mitochondrial outer membrane. A more in-depth description of these current models of StAR mechanism of action can be found in recent reviews (Miller 2007, Papadopoulos *et al.* 2007, Lavigne *et al.* 2010). Although a consensus for a mechanism for StAR-mediated cholesterol transport requires further study, all models are similar to the requirement for continual synthesis of the 37 kDa StAR in response to tropic hormone stimulation to maintain cholesterol transfer into the mitochondria.

Metastatic axillary lymph node protein 64 (MLN64) was identified by differential screening of a cDNA library for amplified products in breast cancer-derived MLN and was found to contain a domain that shared 33% sequence identity and 53% sequence similarity with the human StAR START domain (Tomasetto *et al.* 1995, Moog-Lutz *et al.* 1997). Thus, MLN64 was recognized as a START protein and named STARD3. STARD3/MLN64 is a transmembrane protein that is targeted to the late endosomes by an N-terminal MENTAL (MLN64-N-terminal) domain with a predicted membrane topology of four transmembrane helices that orients the C-terminal START domain facing the cytoplasm (Tomasetto *et al.* 1995, Alpy *et al.* 2001).

Overall, the two members of the STARD1/D3 subfamily are similar in that the START domain for both proteins binds only cholesterol and additional sequences or domains localize the proteins to specific subcellular compartments. The differential subcellular localization of these START proteins suggests different functions in cholesterol trafficking (Table 1).

STARD1 and cholesterol transport to mitochondria

Acute regulation of steroidogenesis Steroidogenic cells of the adrenal and gonads respond to tropic hormone stimulation by rapidly increasing the rate of steroid hormone biosynthesis. The first enzymatic reaction of steroidogenesis is the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage complex, which is localized to the mitochondrial inner membrane. This step requires the delivery of the substrate cholesterol from cellular stores to the inner mitochondrial membrane, a process that occurs rapidly in response to tropic hormone stimulation and requires *de novo* protein synthesis. Thus, a protein that is synthesized in response to hormone stimulation and promotes cholesterol

movement to the mitochondrial matrix would be a candidate for the acute regulation of steroidogenesis (reviewed in Stocco 2001).

StAR was first described as a 30 kDa protein in hormone-treated rat adrenal cortex and cell culture systems as a protein induced by tropic hormone stimulation. Its expression pattern was correlated with increased steroid hormone output (Krueger & Orme-Johnson 1983, Pon *et al.* 1986*a*,*b*, Alberta *et al.* 1989, Stocco & Chen 1991, Stocco & Sodeman 1991). Cloning the 30 kDa cDNA from the MA-10 mouse Leydig tumor cell line revealed a predicted protein of 284 amino acids and with no sequence similarities at either the nucleic acid or the protein level within the databases (GenEMBL and SWISS-PROT, GCG Package, University of Wisconsin) indicating, at that time, that the 30-kDa protein represented a novel protein (Clark *et al.* 1994). As stated above, in heterologous transient transfection experiments, expression of the cDNA-encoded 30 kDa protein resulted in an increase in steroid synthesis, and so was named the StAR (Clark *et al.* 1994). To date, the vast literature on StAR (herein referred to as STARD1) provides strong biochemical and genetic data that support STARD1's role in cholesterol transfer in regulated steroidogenesis (reviewed in Stocco 2001). Of particular importance was the finding that mutations in the *hSTARD1* gene are the most common basis for congenital lipoid adrenal hyperplasia (lipoid CAH; Lin *et al.* 1995, Bose *et al.* 1996, King *et al.* 2011), a disorder characterized by the inability to synthesize adrenal or gonadal steroid hormones due to the absence of cholesterol transport into mitochondria. Recently, new mutations have been identified in the *STARD1* gene that lead to partial loss of function and less severe lipoid CAH (Fluck *et al.* 2011). *Stard1* knockout mice confirmed that in the absence of the protein, the adrenal and gonads accumulated significant lipid deposits and the animals die shortly after birth due to the absence of adrenal hormones (Caron *et al.* 1997). Re-expression of a *Stard1* transgene in the knockout mice fully restored adrenal and gonadal steroidogenesis, as would be anticipated. However, mice that expressed an amino terminal truncated STARD1 that was not targeted to the mitochondria had partially restored steroidogenesis in a tissue- and gender-specific manner and retained lipid accumulation in the adrenal and gonads (Sasaki *et al.* 2008). These data support that STARD1 is capable of functioning without being targeted to the mitochondria but highlight the importance of correct and efficient subcellular localization of STARD1 for full function *in vivo*.

STARD1 and oxysterol production in non-steroidogenic tissues

In vitro protein overexpression studies demonstrated that STARD1 transports cholesterol across mitochondrial membranes in many cell types, potentially expanding STARD1 function outside of steroidogenic tissues (Sugawara *et al.* 1995). One of the first reported roles for STARD1 outside of steroidogenic cells was in cholesterol transfer across the mitochondrial membranes in the liver for initiation of bile acid synthesis by the alternative pathway. Overexpression of STARD1 significantly increases 27HC and bile acid synthesis in primary rat or mouse hepatocytes or human HepG2 hepatoma cells (Pandak *et al.* 2002, Ren *et al.* 2004*a*,*b*, Hall *et al.* 2005). Enhanced rates of bile acid synthesis also occur in both rats and mice after overexpression of STARD1 in the liver, providing evidence for an *in vivo* function (Ren *et al.* 2004*a*,*b*). A key finding of these studies is that the transport of cholesterol into the mitochondria is rate-limiting for bile acid synthesis by the CYP27A1 alternative pathway, suggesting that cholesterol transport into hepatic mitochondria may be regulated under normal or pathological conditions (Pandak *et al.* 2002). Importantly, STARD1 expression in human HepG2 hepatoma cells is increased by treatment with 27HC or by induced expression of CYP27A1, the enzyme that metabolizes cholesterol to 27HC. LXRα-dependent transactivation of *Stard1* has been established in mouse adrenocortical cells (Cummins & Mangelsdorf 2006, Cummins *et al.* 2006). Therefore, 27HC oxysterol activation of LXRα may account for the observed increase in STARD1 in HepG2 cells. Strikingly, overexpression of *Stard1* in liver of ApoE-deficient mice improved serum and liver lipid profiles and reduced lipid accumulation in aortic segments (Ning *et al.* 2009*a*,*b*). It has been proposed that the anti-atherogenic action of hepatic STARD1 expression is due to both increased oxysterol synthesis leading to LXR-mediated anti-atherogenic effects and increased bile acid synthesis leading to clearance of cholesterol (Ning *et al.* 2009*a*,*b*). Thus, mechanisms to enhance STARD1 expression in hepatocytes have been proposed as a target to help attenuate dyslipidemia and the development of atherosclerosis (Ning *et al.* 2009*a*,*b*). However, a potential beneficial role for STARD1 expression in hepatocytes is complicated by recent findings that show elevated STARD1 levels in liver are associated with non-alcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (discussed below).

STARD1 overexpression in human THP-1-derived macrophages also decreases total lipid and cholesterol levels (Bai *et al.* 2009, Ning *et al.* 2009*a*,*b*). The LXRα-dependent target genes *ABCG1*, *PPARγ*, *LXRα*, and *CYP27A1* are increased at the mRNA and protein levels in the STARD1 overexpressing cells. Treatment of THP-1 cells with the oxysterols 25HC or 27HC had similar effects on gene expression as STARD1 overexpression. These data suggest STARD1 overexpression functions in macrophages to supply cholesterol for CYP27A1-dependent oxysterol synthesis that promotes LXRα-dependent mechanism(s) to decrease cholesterol levels (Ning *et al.* 2009*a*,*b*). In support of this model, stable overexpression of STARD1 in RAW264.7 murine macrophages resulted in increased 27HC levels (Taylor *et al.* 2010). STARD1 overexpression also increased LXRα expression, LXRα-dependent reporter gene activity, ABCA1 expression, and cholesterol efflux, similar to STARD1 functioning within the LXR pathway. Suppression of the SREBP2 pathway by 27HC was indirectly indicated in these studies by decreased expression of the target genes

HMGR and *LDLR* (Taylor *et al.* 2010). Interestingly, overexpression of STARD1 in THP-1 macrophage cells attenuated ox-LDL-induced inflammatory cytokine release and apoptosis (Ning *et al.* 2009*a*,*b*). It remains to be determined whether this effect is mediated by elevated oxysterols generated in response to STARD1 overexpression.

Detection of STARD1 protein in liver has not been consistently reported and most of the functional link to bile acid synthesis has been proposed in systems where STARD1 is exogenously supplied. Therefore, it is not clear whether endogenous STARD1 expression in the liver is sufficient to contribute significantly to bile acid synthesis via the alternative pathway. There are no reports of either bile acid disorders or increased risk for cardiovascular disease in patients with lipoid CAH who lack a functional STARD1 protein, suggesting that STARD1 is not required for liver or macrophage cholesterol metabolism. However, this does not exclude the possibility that another START protein functions in this process or that aberrant overexpression of STARD1 may occur in pathological states (see below). An important question to address is whether STARD1 levels are induced in hepatocytes and macrophages in pathological states and to determine the mechanism(s) of regulation.

STARD1 in fatty liver disease

One example for increased STARD1 expression in disease states may be in NAFLD. NAFLD describes a group of disorders associated with an accumulation of lipids, mostly triacylglycerol, in the liver. NAFLD is a common consequence of obesity and type 2 diabetes mellitus that can lead to non-alcoholic steatohepatitis (NASH) and states of hepatic fibrosis and cirrhosis. The potential for cholesterol metabolism disorders in NAFLD disease progression is suggested by reports that hepatic cholesterol accumulation enhances the progression of NAFLD to NASH in mouse models (Van Rooyen *et al.* 2011). A similar result appears in humans where free cholesterol levels in the liver are elevated in patients with NASH relative to patients with NAFLD or without fatty liver disease (Puri *et al.* 2007, Caballero *et al.* 2009). HMGR and SREBP2 transcript levels in liver were elevated in patients with NAFLD and NASH, providing a rationale for the elevated hepatic free cholesterol levels (Caballero *et al.* 2009). In this cohort, *STARD1* mRNA was also increased in the liver with levels being highest in NASH patients. These data indicate a possible positive correlation between *STARD1* expression and hepatic cholesterol levels, although STARD1 protein levels in the NAFLD and NASH patients remain to be measured. SREBP2 and LDLR protein levels were increased and ABC transporters were decreased in an obese, diabetic mouse model that develops NASH when on a high-fat diet (Van Rooyen *et al.* 2011), and the authors proposed a central role for SREBP2 in disease progression (Van Rooyen & Farrell 2011). The lipotoxicity associated with increased hepatic cholesterol levels and disease progression from NAFLD to NASH has been proposed to be mediated at the level of mitochondria (reviewed in Montero *et al.* (2010)). Current data indicate that mitochondria are more susceptible to apoptotic stimuli due to glutathione depletion resulting in increased reactive oxygen species levels (Mari *et al.* 2006). Thus, the possible positive association for STARD1 and cholesterol in NASH patients would suggest a mechanism for increased mitochondrial cholesterol content. It would be interesting to examine additional START protein family members in this disease. In particular, STARD4, which is highly expressed in hepatocytes and is regulated by SREBP2, is a strong candidate for a START protein involved in fatty liver disease (see below).

STARD1 and hepatocellular carcinoma

An increase in *de novo* cholesterol synthesis in hepatocellular carcinomas has been a long-standing observation (Siperstein & Fagan 1964) and only recently studies have begun to address the potential mechanism(s) for increased cholesterol synthesis in liver cancers. As with NASH, one focus has been on mitochondria with early observations that mitochondria isolated from hepatoma xenografts have increased cholesterol:phospholipid ratio relative to mitochondria from normal rat liver (Feo *et al.* 1973, Crain *et al.* 1983). Increased mitochondrial cholesterol content in cancer cells has the potential for suppression of apoptosis by decreasing mitochondrial permeability and suppressing cytochrome *c* release. Mitochondrial permeability is regulated by a multi-protein complex that spans the inner and outer mitochondrial membranes termed the mitochondrial permeability transition pore (mPTP; Henry-Mowatt *et al.* 2004). The major components of the mPTP include the voltage-dependent anion channel, the adenine nucleotide translocase, and cyclophilin D (Alirol & Martinou 2006). Apoptosis can be induced by activation of either the extrinsic or intrinsic apoptotic pathways and activation of either pathway ultimately results in caspase activation leading to cell death (Riedl & Salvesen 2007). The intrinsic apoptotic pathway involves disruption of the mPTP resulting in release of cytochrome *c* and pro-apoptotic proteins into the cytoplasm (Henry-Mowatt *et al.* 2004). Expression of pro-apoptotic BCL2 proteins, BAX and BAK, forms homo-oligomers that insert into the mitochondrial outer membrane and disrupt mPTP leading to release of cytochrome *c* (Cory & Adams 2002).

Elevated mitochondrial cholesterol content in hepatocellular carcinoma has recently been linked to increased STARD1 expression. Rat H35 and human HepG2 hepatoma cell lines and human hepatocellular carcinoma samples have elevated mitochondrial cholesterol content relative to cholesterol content of normal rat and human liver (Montero *et al.* 2008). The cholesterol levels within these cell lines correlated with an increase in SREBP2 expression (Montero *et al.* 2008). Blocking cholesterol synthesis in the HepG2 cells increased sensitivity to agents that induce mPTP and apoptosis, indicating a link between mitochondrial

cholesterol content and chemoresistance. Cholesterol loading of isolated rat liver mitochondria increased membrane order and suppressed BAX-mediated release of cytochrome *c*, supporting the concept that cholesterol-enriched mitochondria are more resistant to apoptosis. STARD1 protein was highly expressed in the HepG2 cells and siRNA-mediated knockdown of STARD1 resulted in decreased mitochondrial cholesterol content and increased sensitivity to apoptosis-inducing agents. Thus, in hepatocellular carcinoma, overexpression of SREBP2 and increased cholesterol levels together with aberrant increased expression of STARD1 in the tumor may provide a mechanism for elevated mitochondrial cholesterol levels and increased resistance to apoptosis.

STARD3 and lysosomal cholesterol

STARD3/MLN64 is a transmembrane protein localized to the late endosomes by an N-terminal MENTAL (MLN64-N-terminal) domain with the C-terminal START domain facing the cytoplasm (Table 1; Tomasetto *et al.* 1995, Alpy *et al.* 2001). The location of STARD3/MLN64 to late endosomes led to studies on its potential role in Niemann Pick type C disease. Niemann Pick type C disease is a lipid storage disorder caused by mutations in genes encoding either NPC1 or NPC2 that result in accumulation of cholesterol in lysosomal storage organelles, which leads to neurological disorders and hepatosplenomegaly (reviewed in Rosenbaum & Maxfield 2011). In brief, free cholesterol that is generated by hydrolysis of LDL-derived cholesterol esters is bound by NPC2 (Niemann-Pick C2), a soluble late endosomal/lysosomal luminal protein, and transferred to the N-terminal cholesterol-binding domain of the late endosome transmembrane protein NPC1 (Niemann-Pick C1). NPC1 then transfers cholesterol across the membrane for release from the lumen by an undefined mechanism (Wang *et al.* 2010). The MENTAL domain of STARD3 is capable of binding cholesterol and is required for its dimerization with another endosomal membrane protein composed only of a MENTAL domain termed MENTHO (MLN64 N-terminal homolog; Alpy *et al.* 2005). In one model for trafficking of late endosome/lysosome cholesterol, STARD3/MLN64 acts as the cytosolic acceptor of NPC1-derived cholesterol (reviewed in Strauss *et al.* (2003)). Alternatively, STARD3/MLN64 and MENTHO may bind cholesterol via the MENTAL domains and independently move cholesterol across the membrane (Alpy *et al.* 2005, Alpy & Tomasetto 2006, Charman *et al.* 2009). The fate of cytosolic cholesterol bound by STARD3/MLN64 may be direct absorption by a closely associated membrane or transfer to another soluble cholesterol-binding protein, potentially a member of the STARD4 subfamily (Soccio & Breslow 2003, Alpy & Tomasetto 2006). However, it is not clear whether STARD3/MLN64 is required for cholesterol trafficking *in vivo* (Kishida *et al.* 2004). Homozygous STARD3/MLN64 mutant mice that express a STARD3/MLN64 protein containing the N-terminal MENTAL domain but lacking the START domain do not accumulate cholesterol in late endosomes/lysosomes and synthesize steroid hormones at wild-type levels (Kishida *et al.* 2004). *Stard4*, *Stard5*, *Npc1*, and *Npc2* mRNA levels were not changed due to loss of the START domain from STARD3/MLN64, suggesting that the lack of a phenotype was not due to compensatory increases of these cholesterol transporters. However, whether the intact MENTAL domain of STARD3/MLN64 may be responsible for the function of this transporter in late endosomes in the knockout mice, or whether STARD4 or STARD5 can act as the soluble cytoplasmic acceptor of cholesterol from STARD3/MLN64 or NPC1, remains to be determined (Fig. 1).

STARD4 subfamily: the soluble sterol-binding proteins

The STARD4 subfamily is composed of STARD4, STARD5, and STARD6 and is most closely related to the STARD1/D3 subfamily with ~20% sequence identity (Soccio *et al.* 2002; Table 1). STARD4 was identified as a novel EST in a cDNA microarray study designed to identify cholesterol-regulated genes in mouse liver (Soccio *et al.* 2002). Mice fed a high-cholesterol diet had reduced *Stard4* transcript levels with *Stard4* gene expression later shown to be regulated by a SREBP2-dependent mechanism (Soccio *et al.* 2002, 2005, Rodriguez-Agudo *et al.* 2011). STARD5 and STARD6 were identified from a BLAST search of the human genome against STARD4 (Soccio *et al.* 2002). Analysis of deduced amino acid sequences for the STARD4 family predicts ~22 kDa soluble proteins entirely composed of the START domain and lacking any membrane targeting sequence (Table 1). The soluble cytoplasmic localization for these START proteins has generated much speculation on their role in cholesterol trafficking.

As outlined above, cholesterol transport to mitochondria can result in oxysterol synthesis for bile acid metabolism or LXRα-dependent responses depending on the tissue and cell type. START proteins that function to traffic cholesterol to the ER would enhance ER cholesterol that would increase ACAT activity and cholesterol ester synthesis and potentially suppress SREBP2 processing leading to decreased cholesterol synthesis. Alternatively, accumulation of cholesterol in the ER may promote ER stress. All of these endpoints have been attributed to START proteins of this subfamily; the question is which START protein plays a physiological or pathological role in which cell type and under what conditions? To explore possibilities for the role of this subfamily of START proteins in cholesterol transport, the similarities and differences between the members are reviewed with a focus on the current proposed functions.

STARD4 and cholesterol transport to the mitochondria and ER

The potential for STARD4 to deliver cholesterol to both mitochondria and ER is based on several studies where

STARD4 is overexpressed in cell culture systems. Heterologous expression of STARD4 in COS-1 cells or addition of recombinant purified STARD4 to isolated mitochondria stimulated cholesterol transfer into mitochondria, although with lower efficiency relative to STARD1 (Soccio *et al.* 2005, Bose *et al.* 2008*a*,*b*). STARD4 overexpression in primary mouse hepatocytes increased bile acid synthesis and cholesterol ester synthesis (Rodriguez-Agudo *et al.* 2008), indicating increased cholesterol transport to mitochondria and ER (Fig. 1). Since the expression of CYP7A1, the enzyme that regulates the classical bile acid synthesis pathway, is lost in cultured primary mouse hepatocytes (Hylemon *et al.* 1992), these data suggest that STARD4 has the capability to increase cholesterol transport to mitochondria for the alternative pathway for bile acid synthesis. Importantly, endogenous STARD4 protein was detected in human liver by immunohistochemistry and was shown to be expressed in hepatocytes and Kupffer cells, i.e. macrophages within the liver reticuloendothelial system (Rodriguez-Agudo *et al.* 2011). Endogenous expression in mouse 3T3-L1 fibroblasts and human THP-1 macrophages was repressed by sterol treatment and induced by blocking cholesterol synthesis by treatment with a HMGR inhibitor. These data are consistent with a SREBP2-mediated regulation for STARD4 *in vivo* (Soccio *et al.* 2005). STARD4 colocalized with ER marker protein calnexin in 3T3-L1 cells and with ACAT1 in THP-1 macrophages, an association that was more pronounced after treatment with HMGR inhibitors to increase SREBP2-dependent increase in STARD4 (Rodriguez-Agudo *et al.* 2011). Cholesterol ester synthesis was increased by addition of recombinant, purified STARD4 to isolated microsomes, indicating that STARD4 positively affects ACAT activity *in vitro*. The association of STARD4 with ER membranes and ACAT1 and the direct effect on ACAT activity strongly support STARD4 functions to transport cholesterol to the ER and ACAT1 for cholesterol ester synthesis (Fig. 1). It remains to be determined whether STARD4-mediated cholesterol transport to the ER may also play a role in providing substrate for CYP7A1 and enhancing the classical pathway for bile acid synthesis in hepatocytes.

A role for STARD4 functioning at the ER is further supported by a recent study that demonstrated STARD4 overexpression in U2OS osteosarcoma cells enhances the transport rate of a fluorescent cholesterol analog, DHE, to the endosome recycling complex and the ER. However, the cholesterol redistribution in U2OS cells mediated by STARD4 can be mimicked with injection of the non-specific cholesterol-binding compound, methyl-β-cyclodextrin, suggesting that STARD4 may contribute to non-selective sterol transport that is required to maintain proper cholesterol distribution between cellular membranes (Mesmin *et al.* 2011). STARD4 overexpression also increased cholesterol ester levels and the responsiveness of SCAP-SREBP2 processing and trafficking to changes in cellular cholesterol levels (Mesmin *et al.* 2011). The authors propose that STARD4 represents a component of the cellular cholesterol sensing system wherein STARD4 would transport cholesterol to the ER membrane and help modulate the SREBP2 pathway and ACAT1 activity (Mesmin *et al.* 2011).

STARD4-mediated cholesterol transport to the ER may also promote ER stress. Although *Stard4*/*STARD4* is established as a SREBP2 target gene (Soccio *et al.* 2005, Rodriguez-Agudo *et al.* 2011), it is also an ER stress response gene. *STARD4* mRNA was increased in HeLa cells between 2 and 6 h treatment with the ER stress inducer tunicamycin and returned to control levels between 12 and 24 h (Yamada *et al.* 2006). Reporter gene activity assays identified an ATF6-dependent responsive element, confirming that *STARD4* promoter can be activated by transcription factors that are activated during the ER stress response. The significance for the transient *STARD4* mRNA expression during ER stress is not known, but an increase in STARD4 protein expression in disease states associated with dyslipidemia and ER stress may impact cholesterol homeostasis by dampening the ER cholesterol sensing system and promoting cholesterol ester formation.

Surprisingly, homozygous STARD4 knockout mice do not present with a strong lipid phenotype; the plasma and hepatic lipid content for both male and female STARD4 null mice is comparable to its wild-type counterparts (Riegelhaupt *et al.* 2010). However, female STARD4 knockout mice have decreased cholesterol and phospholipid content in gallbladder bile. Lipid profiles compared after 1 week on diets supplemented with high cholesterol revealed elevated plasma and hepatic lipids as expected, but the female STARD4 knockout mice had ∼20% lower plasma total cholesterol and cholesterol ester levels relative to the wild-type female mice with no differences in hepatic cholesterol, cholesterol ester, or triacylglycerol levels. There was no difference in the effect of lovastatin, a HMGR inhibitor that blocks cholesterol synthesis, on plasma or hepatic lipid profiles between the wild-type and STARD4 knockout mice. *Stard5* and *Stard3* mRNA expression appear to be repressed, although not significantly decreased, and *Stard1* undetectable in the liver of STARD4 knockout mice, suggesting that the other major START proteins are not responsive to loss of STARD4. It remains to be determined whether environmental, dietary, or disease stressors may highlight a phenotype resulting from loss of STARD4 on cholesterol ester or bile acid synthesis.

STARD5 and cholesterol transport to the ER and PM

STARD5 does not transfer cholesterol to mitochondria *in vitro* (Bose *et al.* 2008*a*,*b*) and transient overexpression in primary mouse hepatocytes has no effect on bile acid synthesis rates (Rodriguez-Agudo *et al.* 2005), indicating that STARD5 does not function to transport cholesterol to mitochondria. Overexpression of human STARD5 in primary rat hepatocytes, however, resulted in increased cellular-free cholesterol levels with possible increased ER cholesterol content (Rodriguez-Agudo *et al.* 2005).

The redistribution of cholesterol was measured as a threefold increase in cholesterol recovered with microsomes isolated from STARD5 overexpressing cells compared with controls. STARD5 binds both cholesterol and 25HC (Soccio *et al.* 2002, Rodriguez-Agudo *et al.* 2005, 2008) and is expressed predominantly in liver and kidney (Soccio *et al.* 2002, Chen *et al.* 2009). In liver, STARD5 is localized to the Kupffer cells and is not expressed in hepatocytes (Rodriguez-Agudo *et al.* 2006). This observation was confirmed by subcellular localization studies for STARD5 protein in cell lines from human macrophages and monocytes as well as mast, lymphoblast, and promyeloblast cells. Double immunofluorescence studies in human THP-1 macrophages revealed that STARD5 was localized to the perinuclear regions of the cell and colocalized with Golgi but not with endosome markers (Rodriguez-Agudo *et al.* 2006). Filipin staining to detect distribution of free cholesterol in the macrophages revealed high cholesterol concentration within the Golgi, suggesting localization of STARD5 with membranes enriched in free cholesterol. In mouse kidney sections, STARD5 protein was detected by immunohistochemistry in the proximal tubules, but not in the glomeruli (Chen *et al.* 2009). The staining pattern indicated diffuse cytoplasmic distribution with concentrated expression at the apical membrane. Greater resolution for STARD5 subcellular distribution by immunoelectron microscopy confirmed diffuse cytoplasmic distribution with enriched staining along the brush-border (apical) and rough ER membranes with no apparent association with mitochondria or Golgi apparatus. In HK-2 human proximal tubule cells, double immunofluorescence confocal microscopy showed that STARD5 had a punctate expression pattern that colocalizes with the ER but not endosome marker proteins. Together, the data indicate a potential broad cellular distribution, e.g. cytoplasm–PM-Golgi–ER, for STARD5 in macrophages and renal proximal tubules (Fig. 1). As a soluble sterol transporter, STARD5 may shuttle cholesterol between the Golgi, ER and PM, although STARD5's trafficking remains to be determined. However, unlike STARD4, STARD5 overexpression does not increase cholesterol ester synthesis rates (Rodriguez-Agudo *et al.* 2005, 2008) or ACAT activity (Rodriguez-Agudo *et al.* 2011), indicating a distinction for the fate of cholesterol transported to the ER by these two lipid transporters. Another distinction in overexpression systems is that STARD5 promotes an increase in free cholesterol levels while STARD4 has no effect on free cholesterol levels (Rodriguez-Agudo *et al.* 2005, 2008). In human proximal tubule cell lines, STARD5 expression is higher in the cells with greater cholesterol content, supporting a positive correlation between cellular free cholesterol content and STARD5 expression in the kidney (BJ Clark, unpublished observations). STARD5 overexpression in THP-1 macrophages markedly increases *SREBP2* mRNA levels, suggesting a potential for increased cholesterol synthesis (Borthwick *et al.* 2010). Therefore, STARD5 may contribute to determining the levels of cellular free cholesterol content.

Stard5/STARD5 mRNA expression is increased by agents that promote ER stress, such as in thapsigargin-treated NIH-3T3 and HK-2 cells or cholesterol-loaded mouse macrophages (Soccio *et al.* 2005, Chen *et al.* 2009). In HK-2 human proximal tubule cells, chemically induced ER stress promotes STARD5 redistribution from a diffuse to a more prominent perinuclear and cell membrane localization (Chen *et al.* 2009). The role for STARD5 during ER stress is not known but chronic ER stress and inflammation are underlying metabolic disorders in many disease states, including NAFLD, type II diabetes, and cancer (Tsai & Weissman 2010, Malhi & Kaufman 2011). There is one study looking at STARD5 expression in disease states associated with ER stress. In a diabetic mouse model, *Stard5* steady-state mRNA and STARD5 protein levels in kidney were shown to be significantly increased, as were free cholesterol levels, compared with wild-type control mice (Chen *et al.* 2009). Cholesterol accumulation in the ER is known to promote ER stress; however, the significance of the association between elevated renal cholesterol, ER stress, and STARD5 in diabetic kidney remains to be determined.

In summary, the differential regulation and distinct cell-type distribution help to control potential redundant actions of STARD4 and STARD5. STARD4 is expressed in hepatocytes and regulated by SREBP2 and activates ACAT. Therefore, in hepatocytes, STARD4 is a strong candidate for cholesterol transport to mitochondria for bile acid synthesis and to the ER for cholesterol ester synthesis. Both STARD4 and STARD5 are expressed in macrophages and both may function as cholesterol transporters that shuttle cholesterol to the ER. STARD4 would increase ACAT and cholesterol ester synthesis while STARD5 may promote an increase in free cholesterol level resulting in ER stress. Both STARD4 and STARD5, therefore, may promote foam cell development and increase the risk for atherosclerosis. Alternatively, STARD5 may serve as a cholesterol buffer to bind the free cholesterol and help suppress the potential lipotoxicity of excess free cholesterol in the cell. Similar functions proposed for STARD5 in macrophage would apply to renal proximal tubule cells. In addition, the prominent apical membrane localization in polarized epithelial cells of renal proximal tubules indicates a potential role for STARD5 in PM cholesterol (Fig. 1). Whether STARD5 contributes to lipid raft formation and stabilization or conversely, extraction of PM cholesterol remains to be determined. Finally, STARD5 binds 25HC and its role in oxysterol transport has yet to be examined.

STARD6 and cholesterol transport to the mitochondria

STARD6 was originally shown to be predominantly expressed in mouse testis and later specifically localized in rat testis to the germ cells with highest expression in round spermatids (Soccio *et al.* 2002, Gomes *et al.* 2005). The function of STARD6 in spermatogenesis is not known. However, *Stard6* was recently identified as a putative gene

required for mitochondrial NADH-dependent dehydrogenase activity (diaphorase) associated with sperm motility and quality (Golas *et al.* 2010). Using recombinant inbred mice strains, a quantitative trait loci approach identified three chromosomal regions, 19q43–19q47, 18q44, and 18q49–18q50, that segregated with diaphorase activity (Golas *et al.* 2010). *Stard6* was highlighted as a putative gene within 18q44, leading to the speculation that *Stard6* along with other genes may regulate activity of a mitochondrial enzyme. The significance of this observation may be linked to earlier work that showed addition of recombinant purified STARD6 to isolated mitochondria-stimulated cholesterol transfer as efficiently as the START domains of STARD1 and STARD3 (Bose *et al.* 2008*a*,*b*). Furthermore, STARD6 protein folding, cholesterol binding, and association with the mitochondrial outer membrane are all very similar to STARD1, suggesting that this protein may function at the mitochondrial level in male germ cells (Bose *et al.* 2008*a*,*b*). Similar to other members of the STARD4 subfamily, STARD6 lacks any organelle targeting sequence. To validate potential actions for STARD6 at the mitochondria, future studies are required to determine the subcellular localization of STARD6 in male germ cells. Expression has also been reported in rat brain and nervous system with potential regulation under neurotoxic conditions (Chang *et al.* 2009).

The phospholipid/sphingolipid-binding START proteins

STARD2/PCTP subfamily: the phosphatidylcholine and ceramide transporters

This subfamily is composed of STARD2, STARD7, and STARD10 that all bind phosphatidylcholine (PC) and STARD11 that binds ceramide (Table 1). STARD2/PCTP was purified from bovine liver and was shown to exchange specifically PC within a membrane or to shuttle PC from the ER to the PM. The crystal structure of STARD2 with bound PC shows the classical helix grip fold that forms a large hydrophobic tunnel. The choline head group provides the specificity of binding and the binding pocket can accommodate PC with saturated or unsaturated acyl groups of different lengths (Roderick *et al.* 2002).

STARD7 (also referred to as gestational trophoblastic tumor gene-1, *GTT1*) was first identified as a transcript that was overexpressed in JEG-3 choriocarcinoma cells (Durand *et al.* 2004). STARD7 shares 25% sequence identity with STARD2/PCTP and the purified protein extracts PC but not phosphatidylserine, phosphatidylethanolamine, or sphingomyelin from lipid vesicles *in vitro* (Horibata & Sugimoto 2010).

STARD10 was identified as a 35 kDa anti-phospho-FKHR immunoreactive band that was overexpressed in tumors of ErbB2 transgenic mice (Olayioye *et al.* 2004). Recombinant purified STARD10 specifically extracts PC and PE from reconstituted lipid vesicles and enhances PC and PE transfer from donor to acceptor vesicles *in vitro* (Olayioye *et al.* 2005). *In vivo*, PC and PE are recovered by immunoprecipitation of overexpressed STARD10 in HEK-293 cells. In both the *in vitro* and *in vivo* studies, PC appears to be the preferred lipid for STARD10 (Olayioye *et al.* 2005). Phosphorylation of STARD10 on Ser284 by casein kinase II decreases *in vitro* lipid transport activity, possibly by decreasing membrane association (Olayioye *et al.* 2007).

STARD11 is more commonly known as CERT, a ceramide transport protein shown to be a splice variant of the Goodpasture antigen-binding protein (GPBPΔ26). Within this subfamily, STARD2/PCTP and STARD11/CERT have been studied in more detail and have been recently reviewed by others (Kanno *et al.* 2007*a*,*b*, Hanada *et al.* 2009, Kang *et al.* 2010, Mencarelli *et al.* 2010). Therefore, only summaries of the recent data on the members of this subfamily are provided.

STARD2/PCTP and insulin resistance

The initial proposed functions for PCTP were in PC transport across the hepatic canalicular membrane and for lung surfactant synthesis, given that PC is the major phospholipid in bile and surfactant and the transporter is expressed in the hepatocytes and alveolar cells (van Helvoort *et al.* 1999). However, PCTP knockout mice ($Pctp^{-/-}$) have no apparent phenotype with normal levels of PC measured in the bile and lung surfactant (van Helvoort *et al.* 1999). Although the predicted phenotype(s) was not observed with the $Pctp^{-/-}$ mice, new data indicate STARD2/PCTP functions in insulin-regulated pathways to maintain glucose homeostasis (Scapa *et al.* 2008, Shishova *et al.* 2011). Fasting serum glucose and free fatty acid levels are significantly decreased in $Pctp^{-/-}$ mice compared with wild-type counterparts due to increased insulin sensitivity (Scapa *et al.* 2008). In addition, hepatic SREBP1c expression is decreased in $Pctp^{-/-}$ mice along with downstream target gene expression for enzymes within the fatty acid biosynthesis pathway. Isolated hepatocytes from $Pctp^{-/-}$ mice have decreased fatty acid synthesis rates, providing a functional readout to support the gene expression profile. Treatment of wild-type mice with a STARD2/PCTP small molecular inhibitor (compound A1) that displaces PC binding attenuates high-fat diet-induced increase in serum glucose levels (Shishova *et al.* 2011). Treatment of human hepatocytes and HEK-293 cells with compound A1 promotes activation of the insulin signaling pathway (Shishova *et al.* 2011). Together, the data indicate that blocking STARD2/PCTP function in the liver results in increased hepatic insulin sensitivity. The mechanism for STARD2/PCTP action in liver glucose metabolism, however, remains to be determined.

STARD2/PCTP was recently shown to interact with thioesterase superfamily member 2 (Them2) and the transcription factor paired box gene 3 (*PAX3*; Kanno *et al.* 2007*a*,*b*). The significance of these particular protein–protein

interactions is that other START family members, including mammalian STARD14 and STARD15, are multi-domain proteins that have amino-terminal thioesterase domains. In plants, a major START protein subfamily is characterized by homeobox domain(s) and function in DNA-binding and transcriptional regulation (Schrick *et al.* 2004). Interestingly, the thioesterase activity of recombinant Them2 in *in vitro* assays is enhanced by the presence of STARD2 as is the transactivation activity of PAX3 (Schrick *et al.* 2004). It has been speculated that during cold adaptation in mice, PCTP–Them2 interaction in brown fat increases and attenuates the uptake and oxidation of fatty acids within mitochondria (Kang *et al.* 2009, 2010). Although the mechanism(s) are not defined, it has been proposed that phosphorylation of STARD2/PCTP results in mitochondrial association and interaction with Them2. The PCTP–Them2 interaction increases Them2 activity resulting in a decrease in fatty acyl-CoA levels and thereby a decrease in fatty acid oxidation (Kang *et al.* 2009, 2010).

STARD7: a phosphatidylcholine-binding protein

A STARD7 variant, STARD7-I, was identified by a BLAST search and shown to have an extended amino terminal sequence that forms an amphipathic helix that functions as a mitochondrial targeting sequence (Horibata & Sugimoto 2010). Mitochondrial PC levels in the HEPA-1 mouse hepatoma cell line are increased after STARD7 overexpression but are unchanged by STARD7 silencing by siRNA. STARD7-I can be processed to a smaller protein, presumably by mitochondrial proteases that cleave the targeting sequence, but it remains sensitive to protease digestion when isolated mitochondria are treated with proteinase K (Horibata & Sugimoto 2010). Thus, STARD7 appears to remain on the cytoplasmic side of the mitochondrial outer membrane. This membrane association would be consistent with both the cytosolic and mitochondrial localization of endogenous STARD7-I in the HEPA-1 mouse hepatoma cell line and rat liver. Regulating mitochondrial PC levels would influence membrane structure and, as the authors of this study speculate, regulate acylation reactions (Horibata & Sugimoto 2010). Since STARD7 is expressed at relatively high levels in lung, colon, and liver cancer cell lines (Durand *et al.* 2004), it may play a more general role in proliferating cells, possibly for supply of PC for mitochondrial biogenesis.

STARD10: a phosphatidylcholine/ethanolamine-binding protein in breast cancer

STARD10 was originally reported to be co-expressed with ErbB2/HER2/neu in breast cancer cell lines and primary breast carcinomas (Olayioye *et al.* 2004). Functionally, the overexpression of STARD10 in NIH-3T3 fibroblast cells promoted anchorage-independent cell growth only if expressed together with ErbB2, suggesting that STARD10 may function within the ErbB2/HER2/neu receptor signaling pathway (Olayioye *et al.* 2004). However, *STARD10* and *HER2/neu* mRNA and protein expression levels were later shown to be inversely correlated when analyzed in breast tumors from a large cohort of patients (Murphy *et al.* 2009). Unexpectedly, loss of STARD10 expression was found to be an independent marker for poor patient outcome and may be used to identify a specific subgroup of patients at high risk (Murphy *et al.* 2009). Whether the beneficial effect for STARD10 expression in breast cancer is related to its PC binding/transport activity will require elucidating the biological functions of STARD10 in mammary tissues (Olayioye *et al.* 2005). This function most likely will be related to the phosphorylation state of STARD10, therefore elucidating the phosphatase(s) responsible for the dephosphorylation and activation of STARD10, and the pathways involved in STARD10 activation are important future studies (Olayioye *et al.* 2007).

STARD11/CERT: a ceramide-binding protein

STARD11 is unique within this subfamily in that the protein contains additional motifs that localize the START domain to its cellular sites of action. STARD11/CERT is responsible for the movement of ceramide from the ER to the Golgi membrane (Hanada *et al.* 2003). The protein has an amino terminal pleckstrin homology domain (PH), a middle region with a FFAT motif, and carboxyl terminal START domain (Table 1). The PH domain binds to phosphoinositides, specifically PI4P in the Golgi membrane, while the FFAT motif interacts with the ER resident protein VAP. STARD11/CERT phosphorylation is proposed to maintain the protein in a folded, inactive form with dephosphorylation resulting in a conformational change that exposes the PH and FFAT domains for membrane interaction and positions the START domain for ceramide transfer. A detailed description of the proposed model for ceramide transfer by STARD11/CERT has been presented (Hanada *et al.* 2009) and the basic concept is the orientation of the protein with the N-terminus bound to the Golgi membrane and the middle region bound to the ER would place the START domain in close proximity to both membranes to facilitate ceramide extraction from the ER and delivery to the Golgi. The crystal structure of the STARD11/CERT START domain confirmed the helix-grip fold structure for ceramide binding and supports a mechanism for membrane interaction and ceramide extraction/absorption (Kudo *et al.* 2008, 2010).

The multi-domain START proteins

STARD8/12/13: the SAM-RhoGAP-START subfamily

This subfamily is more commonly referred to as the deleted in liver cancer (DLC) family of proteins. The history, genomic structure, isoform expression, and known and potential

function(s) for STARD12/DLC-1, STARD13/DLC-2, and STARD8/DLC-3 have been reviewed (Durkin *et al.* 2007*a*,*b*) and only a few aspects are highlighted here. Members of this subfamily share the same multi-domain structure, an amino terminal sterile α motif (SAM; Ponting 1995) followed by a serine-rich region, a RhoGAP domain, and a carboxyl-terminal START domain (Table 1). *STARD12/DLC-1* was first isolated as a genomic clone that was localized on chromosome 8p21.3–22, a region associated with loss of heterozygosity in several cancers and shown to be deleted in 50% of primary human hepatocellular carcinoma tumor tissues (Yuan *et al.* 1998). Re-expression of DLC-1 in human liver, lung, breast, and ovarian cancer cell lines suppresses cell growth and increases apoptosis, supporting DLC-1 as a tumor suppressor. Protein kinase D phosphorylates multiple sites on STAR12/DLC-1 and the phosphoprotein has decreased activity (Scholz *et al.* 2009, 2011), suggesting a potential regulatory mechanism controlling STARD12/DLC-1 function. Targeted deletion of *Stard12/dlc-1* gene in mice results in embryonic lethality, most likely due to disruption of cytoskeletal organization. STARD12/DLC-1 has been shown to regulate RhoA activity via the RhoGAP domain, to colocalize with focal adhesions via binding to the SH2 domain of tensin 1, and to stimulate PLC-δ1 leading to IP3-dependent intracellular Ca^{2+} release (Durkin *et al.* 2007*a*,*b*). Any or all of these functions could disrupt/promote cytoskeletal organization.

STARD13/DLC-2 and STARD8/DLC-3 also have tumor suppressor activities when overexpressed in cancer cell lines (Ching *et al.* 2003, Durkin *et al.* 2007*a*,*b*) and localize to focal adhesions (Kawai *et al.* 2007, 2009), indicating similar activities as characterized for STARD12/DLC-1. However, STARD13/DLC-2 and STARD8/DLC-3 cannot compensate for loss of STARD12/DLC-1. Recent reports characterizing STARD13/DLC-2 knockout mice show that the mice are healthy and fertile with no overt phenotype (Yau *et al.* 2009, Lin *et al.* 2010). The knockout mice were not more susceptible to spontaneous tumors or induced hepatocarcinogenesis, indicating potential compensatory effects of the other DLCs for tumor suppressor activity or possible requirement for a 'second hit' to promote tumor formation (Yau *et al.* 2009, Lin *et al.* 2010). However, STARD13/DLC-2 may help suppress angiogenesis associated with tumor growth (Lin *et al.* 2010). STARD13/DLC-2 has widespread tissue distribution, with expression observed in CD31-positive cells of blood vessels, indicating endothelial specific expression. Based on this observation, matrigel-induced vascularization and B16 murine melanoma xenograft tumor cell growth assays were performed and angiogenesis was shown to be enhanced in the knockout mice compared with wild-type counterparts. Silencing STARD12/DLC-2 expression in HUVECs leads to increased cell migration in a RhoA-dependent manner, supporting the *in vivo* data for STARD12/DLC-2 promoting angiogenesis (Lin *et al.* 2010).

To date, the functions associated with this subfamily have been attributed to the RhoGAP domain. The role of the START domain in these proteins is not known. However, confocal fluorescent imaging demonstrated that endogenous STARD12/DLC-1 colocalizes with caveolin-1 in BHK cells and the two proteins co-immunoprecipitated, indicating that STARD12/DLC-1 is localized to cholesterol and sphingolipid-rich regions of the PM (Yamaga *et al.* 2004). The caveolin-1 interaction is dependent on the RhoGAP domain of STARD12/DLC-1 and the authors of this study speculated that the START domain may bind cholesterol and regulate STARD12/DLC-1 GAP function (Yamaga *et al.* 2004). Expression of a tagged STARD13/DLC-2 in a human hepatoma cell line was shown by confocal immunofluorescence imaging to be localized with mitochondria (Ng *et al.* 2006) and the mitochondrial association was dependent on the START domain. Expression of the START domain of STARD12/DLC-1 in a breast cancer cell line, on the other hand, did not show a pattern consistent with mitochondrial localization and the full protein was localized to the cytoskeleton and enriched at focal adhesions (Kim *et al.* 2008). The ligand(s) that bind the START domain(s) of this subfamily remain to be determined. New data on the crystal structure for STARD13/DLC-2, however, indicate that the STARD13/DLC-2 ligand-binding pocket is smaller and contains polar residues that make it different from the cholesterol and phospholipid START proteins. The authors propose that a charged lipid would be a likely binding candidate (Thorsell *et al.* 2011). Once the START domain ligands have been defined, this information will help to elucidate the biological significance for the START domain within this family of RhoGAP proteins. It will be interesting to see the effect of the lipid binding to the START domain on RhoGAP activity and whether subcellular localization and/or ligand binding affects function.

STARD14/15: the acyl-CoA thioesterase subfamily.

The acyl-coenzyme A thioesterase (ACOT) family of proteins hydrolyze the thioester bond of fatty acyl-CoAs to generate free fatty acids and coenzyme A (reviewed in Kirkby *et al.* (2010)). ACOT11_v2 and ACOT12 are unique within this family as they contain C-terminal START domains (Hunt *et al.* 2005, Kirkby *et al.* 2010) and are also known as STARD14 and STARD15 respectively (SRPBCC protein superfamily on NCBI's Conserved Domain Database). Human STARD14/ACOT11_v2 is a splice variant that is the ortholog of the mouse brown fat-inducible thioesterase (mBFIT2; Adams *et al.* 2001; Table 1). mBFIT2 is induced in brown adipose tissue of mice after exposure to cold temperatures and is expressed at higher levels in lean mouse models compared with obese mouse models. The data suggest an association between STARD14/ACOT11_v2/BFIT2 with increased metabolic activity in brown fat. STARD15/ACOT12 is a cytosolic acetyl-CoA thioesterase (hydrolase) that has been cloned from rat, mouse, and human (Suematsu *et al.* 2001, 2002, Suematsu & Isohashi 2006) and is highly

expressed in liver. The biological significance for the thioesterase activity of this enzyme should be implicit based on the fact that acetyl-CoA is the substrate, but this enzyme has been relatively understudied.

The crystal structure for the START domain of STARD14/ACOT11_v2/BFIT2 shows that the functionally critical C-terminal α helix is broken into two shorter helices (Thorsell *et al.* 2011). Electron density consistent with a fatty acid filled the ligand-binding cavity in the crystal, but the actual ligand and whether it is a fatty acid was not solved. It is tempting to speculate that fatty acid binding may regulate thioesterase activity of these enzymes; possibly, the START domain binds the free fatty acid product for transfer to fatty acid binding proteins, the soluble intracellular carriers of fatty acids.

Summary

One-third of the mammalian START domain proteins belong to the STARD1/D3 and STARD4 subfamilies and function to bind and transport cholesterol and oxysterols. While the biological function of STARD1 is established as the regulator of cholesterol transport across mitochondrial membranes for steroid hormone synthesis, the challenge remains to define the functions for the remaining members of the cholesterol-binding proteins. Current data suggest that both STARD4 and STARD5 associate with ER cholesterol yet serve unique roles at the ER membrane. The knockout mouse models that do not present with an apparent phenotype will require further study to determine whether exposure to environmental, dietary, or disease stressors may highlight a phenotype associated with loss of the START protein(s). Alternatively, a phenotype may only manifest upon aberrant overexpression of a START protein, as indicated by the studies that demonstrate overexpression of START proteins leads to disorders in cholesterol homeostasis in hepatocytes and macrophages. Therefore, it will be important to continue to identify START protein expression associated with different disease states that involve dyslipidemia, inflammation, and ER stress to help establish the biological significance of the *in vitro* data and to help distinguish unique functions from redundant functions for the STARD4 subfamily.

The phospholipid/sphingolipid-binding proteins of the STARD2/PCTP subfamily appear to have diverse functions, from modulating insulin sensitivity in liver to inter-membrane transfer of ceramide, and tumor proliferation. Although the ligands are known for this subfamily, it is not yet clear what the significance is for phosphatidylcholine binding. Similarly, the next question to address for the RhoGAP and thioesterase START protein subfamilies is whether lipid binding within the START domain affects the function of the protein. The first step, however, is to determine the ligands that bind to the START domains of members of these two subfamilies.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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