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## Chapter 6

# STARD3: A Lipid Transfer Protein in Breast Cancer and Cholesterol Trafficking

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**Abstract** STARD3 was isolated in the early 1990s in a study aimed at finding new genes implicated in breast cancer. The function of the STARD3 gene, referred to at that time as Metastatic Lymph Node clone number 64 (MLN64), remained a mystery until the discovery of the steroidogenic acute regulatory protein (StAR/STARD1). Indeed, homology searches showed a region of significant similarity between StAR and the carboxy-terminal half of STARD3. This homology proved to be functionally relevant with both proteins being cholesterol carriers; however, quite early it appeared that they were very distinct in terms of expression, subcellular localization, and function. It was then reported that STARD3 was part of a family of 15 human proteins that shared a conserved StAR-related lipid transfer (START) domain. Structurally, the STARD3 protein distinguishes itself by the presence of an additional conserved domain spanning the amino-terminal half of the protein that we named the MLN64-N-terminal (MENTAL) domain. This domain contains most of the functional properties that have been attributed to STARD3. This chapter will present our current understanding of STARD3 function in cancer, cell biology, and cholesterol trafficking.

### Abbreviations

CAB1: Co-Amplified with ERBB-2

CBP: Carotenoid binding protein

CHO: Chinese Hamster ovary

DARPP32: Dopamine and cAMP-regulated phosphoprotein

EGFR: Epidermal growth factor receptor

ER: Endoplasmic reticulum

FFAT: Diphenylalanine [FF] in an acidic tract

GRB7: Growth factor bound protein 7

HER2: Epidermal growth factor receptor 2

LE: Late endosome

LOF: Loss-of-function

LY: Lysosome

MCF-7: Michigan Cancer Foundation-7

MCS: Membrane contact site

MDA-MB-231: Monroe Dunaway Anderson- Metastatic Breast-231

MENTAL: MLN64-N-terminal domain

MENTHO: MLN64 N-terminal domain homologue

MLN64: Metastatic lymph node clone 64

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NPC1: Niemann-Pick C1

SRA: Smallest region of amplification

StAR: Steroidogenic acute regulatory protein

STARD3: START domain containing protein 3

STARD3NL: STARD3 N-terminal like

START: (StAR)-related lipid transfer

TEM : Transmission electron microscopy

VAP : VAMP (vesicle-associated membrane protein)-associated protein

## Introduction

### ***STARD3 Perspectives: How we Discovered the Second Member of the Steroidogenic Acute Regulatory Protein (StAR)-Related Lipid Transfer (START) Protein Superfamily by Catherine L. Tomasetto***

This is my personal recollection about the discovery of STARD3 more than 20 years ago. I remember it as a collective effort from several people including two young brilliant graduate students Dr. Catherine Regnier and Dr. Christel Moog. It all started in the early 1990s when I came back to Strasbourg after spending 3 years in Boston at Harvard University, in the Dana-Farber Cancer Institute. Under Dr. Ruth Sager's firm guidance, my colleagues and I chased tumor suppressor genes in mammary epithelial cells. There I learned new methods in cloning and manipulating genes; importantly, I became an expert in the subtractive hybridization method. In Strasbourg, I joined one of the best research institutes in Europe—the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC)—a pioneer in many aspects of molecular biology especially in the field of gene expression. The IGBMC was led by Dr. Pierre Chambon, one of the most prominent life scientists. In this large institute, I naturally joined the group working on breast cancer headed by Dr. Paul Basset and Dr. Marie-Christine Rio. My project was to find new genes important for breast cancer, which could be used as prognostic markers, therapeutic targets, and which would help to understand the biology of cancer. The idea was to isolate genes expressed in breast tumors but not in normal breast tissue. This was before the microarray era and only tedious methods were available. I chose subtractive hybridization and we decided to work on patient biopsies instead of established cell lines. Because we were interested in finding genes involved in aggressive tumors, we selected tumor samples using the following clinical criteria: young age of the patient at the onset of diagnosis, large tumor size, and elevated histological grade. After 2 years of screening, cloning, and sequencing, we narrowed down the number of positives hits from over 200 to 10 independent genes [1]. Among these 10 genes, the clone number 64 was intriguing because its sequence was novel, it was overexpressed in all breast tumors that were positive for the epidermal growth factor receptor 2 (HER2) oncogene and it was expressed at a basal level in all tissues and cell lines tested. Christel Moog undertook the challenge of characterizing this novel gene; she notably found the homology with steroidogenic acute regulatory protein (StAR) and produced the consensus sequence for this region of homology that she called the StAR homology domain (SHD; [2]). We tried to establish a link between STARD3 and endocrine-dependent breast cancer. One of the major subtypes of breast cancer expresses high levels of the estrogen receptor and can be treated with endocrine therapies. To our disappointment, Christel found that STARD3 did not promote steroidogenesis in breast cancer cells and was not localized in the mitochondria (unpublished, [2]). Then, fortunately, a dedicated and talented graduate student, Dr. Fabien Alpy, joined us, became interested in this peculiar protein, and took on the challenge of finding its function. His major contributions were to discover the subcellular localization of STARD3 in late endosomes and the definition of a novel conserved domain that he called MENTAL for MLN64-N-terminal domain [3]. Almost a decade later, Fabien is now a staff scientist on the team, remains very interested in STARD3 and is committed to the pathophysiological functional characterization of STARD3. I thank all the colleagues who have contributed to the work that is described in this chapter. I apologize for leaving out many names. I acknowledge funding from the French research agencies: Institut National de la Santé et de la Recherche Médicale (INSERM), Center National de la Recherche Scientifique (CNRS); the University of Strasbourg; and the charities: the Fondation pour la Recherche Médicale, the fondation ARC pour la recherche sur le cancer, and the Ligue nationale Contre le Cancer.

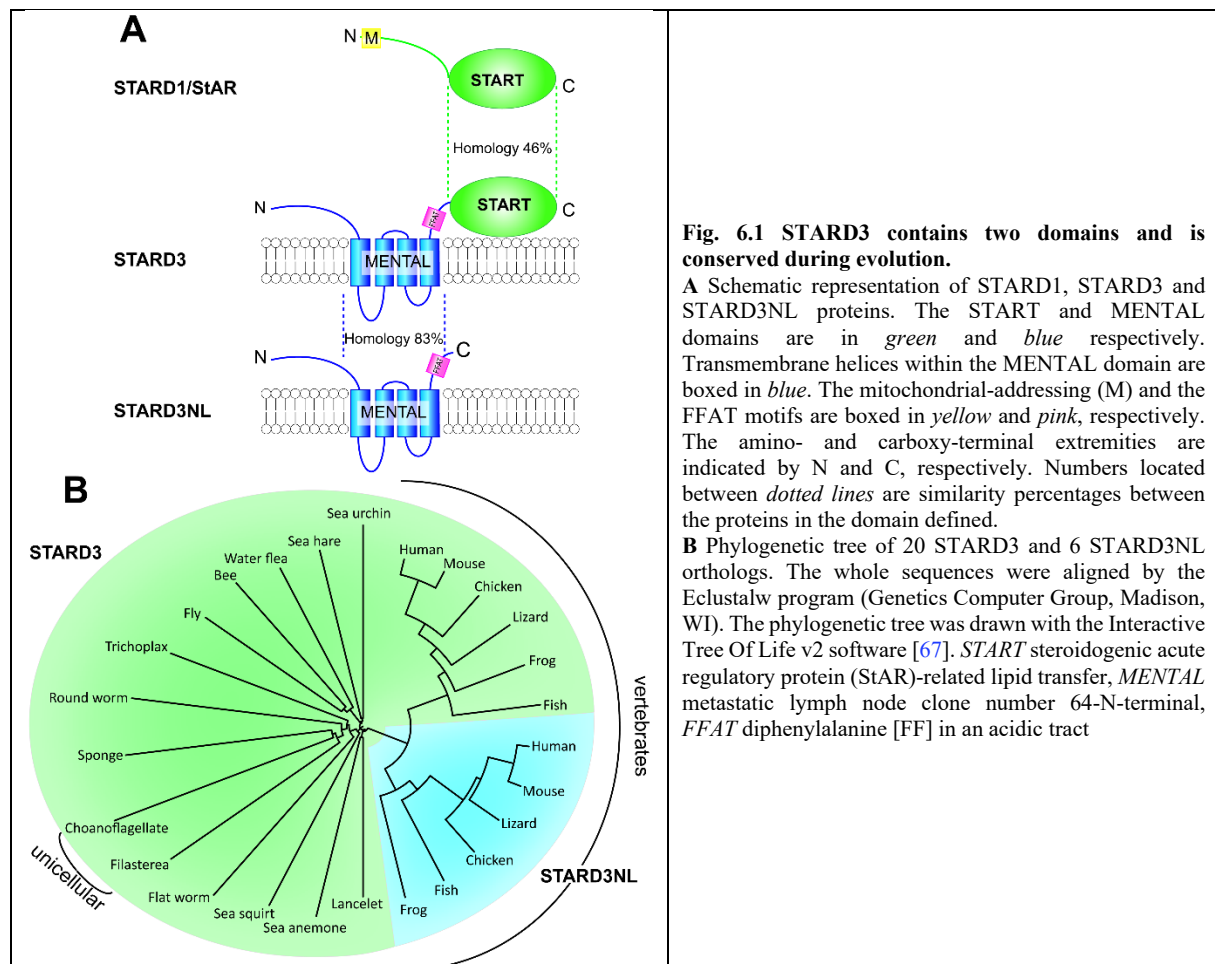
### ***STARD3 Overview***

STARD3 was first isolated in a screen designed to identify new genes implicated in breast cancer. Using subtractive hybridization as a screening method, several unknown complementary deoxyribonucleic acids (cDNAs) were isolated from a library prepared from a pool of metastatic lymph nodes derived from breast cancers. STARD3 was originally the 64<sup>th</sup> clone isolated in the screen and was therefore named metastatic lymph node clone 64 (MLN64; [1]). In this original publication, we showed that the STARD3 gene lied next to the oncogene HER2/ERBB2 on chromosome 17q11-12 and proposed that amplification of this chromosomal subregion was responsible for the observed co-expression between STARD3 and HER2 in breast cancer cells [1]. This hypothesis was substantiated using a series of about 100 human primary breast cancers which showed that STARD3 was indeed coamplified and co-expressed with HER2 in about 25 % of breast cancers [4]. Several years later, STARD3 was isolated again by a different group in the context of breast cancer and named CAB1 for co-amplified with ERBB-2 as a new gene co-amplified and overexpressed in breast cancer [5]. Soon after its identification, we noted that STARD3 shares a functionally conserved domain with the StAR protein [2, 6]; this finding provided the foundation for the definition of a novel conserved protein family called the StAR-related lipid transfer (START) protein superfamily ([7–11] and see Chaps. 1 and 2). A second insight into STARD3's function came from the identification of its subcellular localization at the membrane of late endosomes [12] and from its bi-functional protein structure [13, 14]. Over the last decade, efforts to understand the pathophysiological function of STARD3

have proceeded along two main research themes, cholesterol trafficking and cancer; these themes will be discussed in this chapter.

### STARD3 is a Bi-Functional Protein Conserved from Unicellular Organisms to Humans

STARD3 is characterized by the presence of two distinct structural domains separating the protein in two halves (Fig. 6.1a). The amino-terminal half of the protein is highly conserved during evolution; it is present in a second protein called STARD3 N-terminal like (STARD3NL) originally named MLN64 N-terminal homolog (MENTHO; [13]). As shown in Fig. 6.1a, the carboxy-terminal half contains a START domain conserved with STARD1 also known as StAR (see Chap. 1). Like STARD1, STARD3 can enhance steroidogenesis in cellular assays; removal of the conserved START domain resulted in the complete loss of steroidogenic activity; on the contrary, removal of the amino-terminal region of STARD3 increased this activity [6, 15]. In addition, the isolated recombinant START domain of STARD3 was shown to be a sterol transfer protein *in vitro* [16–18]. In early studies, the 3-dimensional structure of the START domain of STARD3 illustrated the presence of a deep lipid-binding pocket able to accommodate one molecule of cholesterol; consistently, *in vitro* the START domain bound cholesterol at an equimolar ratio [19]. Since the structure/function of the START domain will be extensively described in other chapters (see Chaps. 3 and 4), we will describe here mainly the structure/function of the MENTAL domain of STARD3.



**Fig. 6.1 STARD3 contains two domains and is conserved during evolution.**

**A** Schematic representation of STARD1, STARD3 and STARD3NL proteins. The START and MENTAL domains are in *green* and *blue* respectively. Transmembrane helices within the MENTAL domain are boxed in *blue*. The mitochondrial-addressing (M) and the FFAT motifs are boxed in *yellow* and *pink*, respectively. The amino- and carboxy-terminal extremities are indicated by N and C, respectively. Numbers located between *dotted lines* are similarity percentages between the proteins in the domain defined.

**B** Phylogenetic tree of 20 STARD3 and 6 STARD3NL orthologs. The whole sequences were aligned by the Eclustalw program (Genetics Computer Group, Madison, WI). The phylogenetic tree was drawn with the Interactive Tree Of Life v2 software [67]. *START* steroidogenic acute regulatory protein (StAR)-related lipid transfer, *MENTAL* metastatic lymph node clone number 64-N-terminal, *FFAT* diphenylalanine [FF] in an acidic tract

### The MENTAL Domain Distinguishes STARD3 from the Other START Proteins

The finding of STARD3NL, a second protein sharing a high homology with the N-terminal half of STARD3, provided the basis for the definition of a novel protein domain that we named MENTAL after the original designation of STARD3 as the MLN64 protein [13, 14]. The MENTAL domain is composed of four transmembrane helices with three short intervening loops (Fig. 6.1a). This organization resembles the structural organization of the protein from the Tetraspanin superfamily; however, no significant homology with Tetraspanin protein sequences or with other proteins containing four transmembrane helices was found. Thus, the MENTAL domain is unique to STARD3 and STARD3NL. To examine the evolution of STARD3, we performed a multi-

alignment analysis of STARD3 and STARD3NL primary sequences (Fig. 6.1b). Interestingly, STARD3 is present within all the animal kingdom as well as in unicellular organisms closely related to animals [20, 21]. Unlike STARD3, STARD3NL exists only in vertebrates (Fig. 6.1b). The restricted presence of STARD3NL in vertebrates as well as the identical gene organization between human STARD3 and STARD3NL genes [22] suggest that STARD3NL originates from a duplication event of the STARD3 gene.

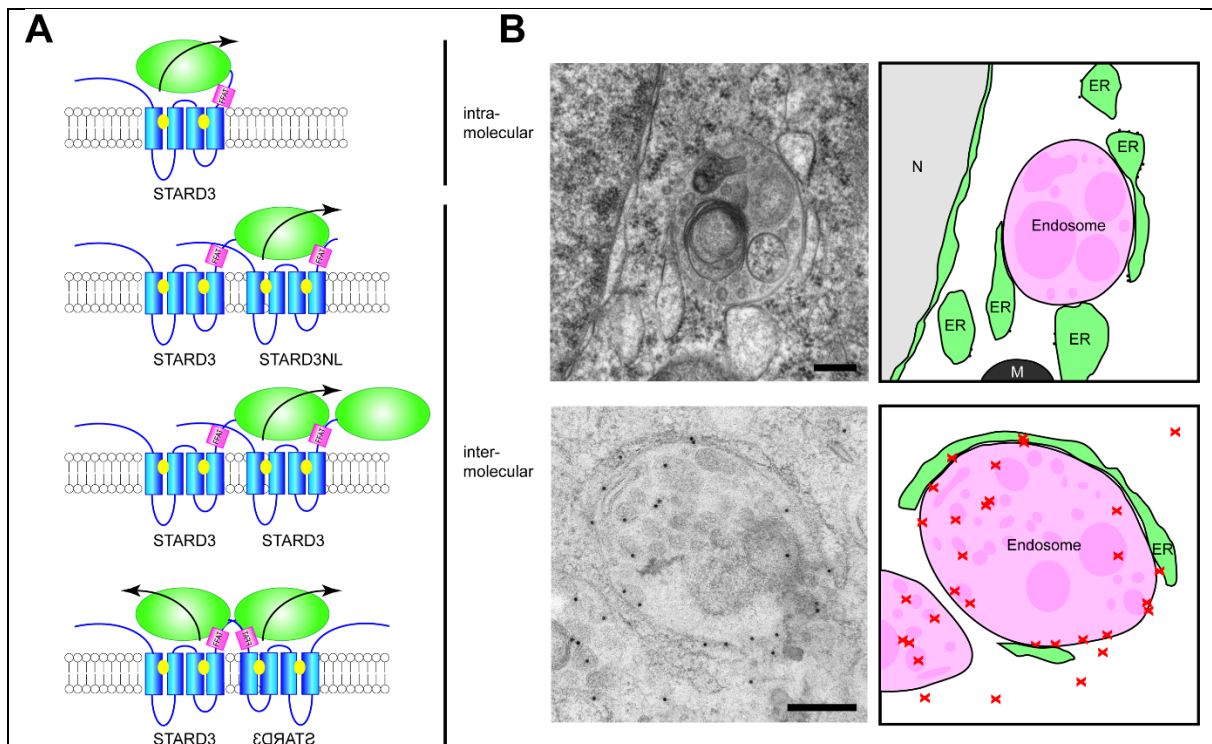
### **The MENTAL Domain Addresses STARD3 to Late Endosome (LE) and Promotes Homo and Hetero-Oligomerization**

STARD3 does not contain any of the typical late endosome (LE)-addressing motifs. However, by using a mutagenesis approach, it was demonstrated that the MENTAL domain was necessary and sufficient to target both STARD3 and STARD3NL to the endosomal limiting membrane of LEs [12, 13, 23]. In addition, using microinjection and endocytosis of specific antibodies, we showed that the MENTAL domain controls the orientation of the protein with respect to the endosomes and projects the START domain into the cytoplasm [12, 13]. Of note, utilizing electron microscopy, STARD3 was shown to mainly localize at the surface of late endosomes with an uneven staining pattern, suggesting that the protein could accumulate in discrete subregions of the membrane [12]. The structural resemblance of the MENTAL domain with Tetraspanins, which are known to associate with one another and to form a tetraspanin web [24] also supported the notion that STARD3 and STARD3NL could form protein complexes at the membranes of endosomes. Using classical biochemistry and imaging approaches, we demonstrated that STARD3 and STARD3NL homo- and hetero-oligomerize and that the MENTAL domain is instrumental in promoting this interaction [14].

### **The MENTAL Domain is a Cholesterol-Binding Domain**

To gain insight into the molecular mechanism implicated in the handling of cholesterol by STARD3, an *in vivo* cholesterol-binding assay was used in living cells. In this experiment, radiolabeled photoactivatable cholesterol was provided to living cells using low-density lipoprotein particles (LDL). After cross-linking and immunoprecipitation, it was shown that the MENTAL domain of STARD3 was a cholesterol-binding domain indicating that STARD3 contains two distinct cholesterol handling domains [14]. Moreover, a recent proteome-wide mapping of cholesterol interacting proteins substantiates this finding. Using the click chemistry methodology, the repertoire of cholesterol-bound peptides has been identified from HeLa cells by Hulse et al. [25]. Of interest, several cholesterol-bound peptides belonging to STARD3 (residues 243–260 and 286–307) and STARD3NL [23–37, 185–196] were identified. These novel findings support the notion that both STARD3 and STARD3NL, by using their MENTAL domains, trigger the formation of microdomains enriched in cholesterol. The exact molecular mechanism behind cholesterol transport mediated by STARD3 and STARD3NL is still unclear however as shown in Fig. 6.2a, several mode of action models can be proposed. In these models, the MENTAL domain may be regarded as a cholesterol reservoir for the cholesterol shuttling activity of the START domain (Fig. 6.2a).

*The MENTAL Domain has an Endoplasmic Reticulum (ER) Tethering Activity* We recently showed that the MENTAL domain contains a conserved diphenylalanine [FF] in an acidic tract (FFAT)-like motif ([26]; Fig. 6.1a). FFAT motifs were identified as signals responsible for targeting cytosolic proteins to the surface of the ER by directly interacting with vesicle-associated membrane protein (VAMP)-associated protein (VAP; [27]). For instance, some oxysterol-binding protein-related proteins (ORPs) contain conserved FFAT motifs that target them to the ER surface. Because STARD3 and STARD3NL are not free cytosolic proteins but LE-anchored proteins, the interaction between STARD3, STARD3NL and VAP proteins has a strong impact on the subcellular architecture. In cells expressing either STARD3 or STARD3NL, transmission electron microscopy pictures show that endosomes are covered by the ER (Fig. 6.2b). To gain more details on the localization of STARD3 with respect to subcellular architecture, immunogold labeling of STARD3 was performed on ultrathin sections using an antibody coupled to gold particles (Fig. 6.2b). Labeling of STARD3 was enriched at the outer periphery of LE in regions directly facing the ER (Fig. 6.2b; [26]) This endosome-ER tethering results in the formation of specific subcellular regions named membrane contact sites (MCSs) (Fig. 6.2b). The molecular identity of this LE-ER tethering complex is not exhaustive, but it contains STARD3 and/or STARD3NL and VAP proteins. In addition, extended endosome-ER contacts mediated by STARD3 alter the dynamics of the endosomal compartment by preventing vesicle-to-tubule transitions [26]. The functional significance of these specific endosome-ER MCSs remains to be addressed, but they probably represent specialized subcellular regions where signals and small molecules, such as lipids and calcium, are exchanged between organelles.



**Fig. 6.2 STARD3 belongs to an inter-organelle tethering machine.**

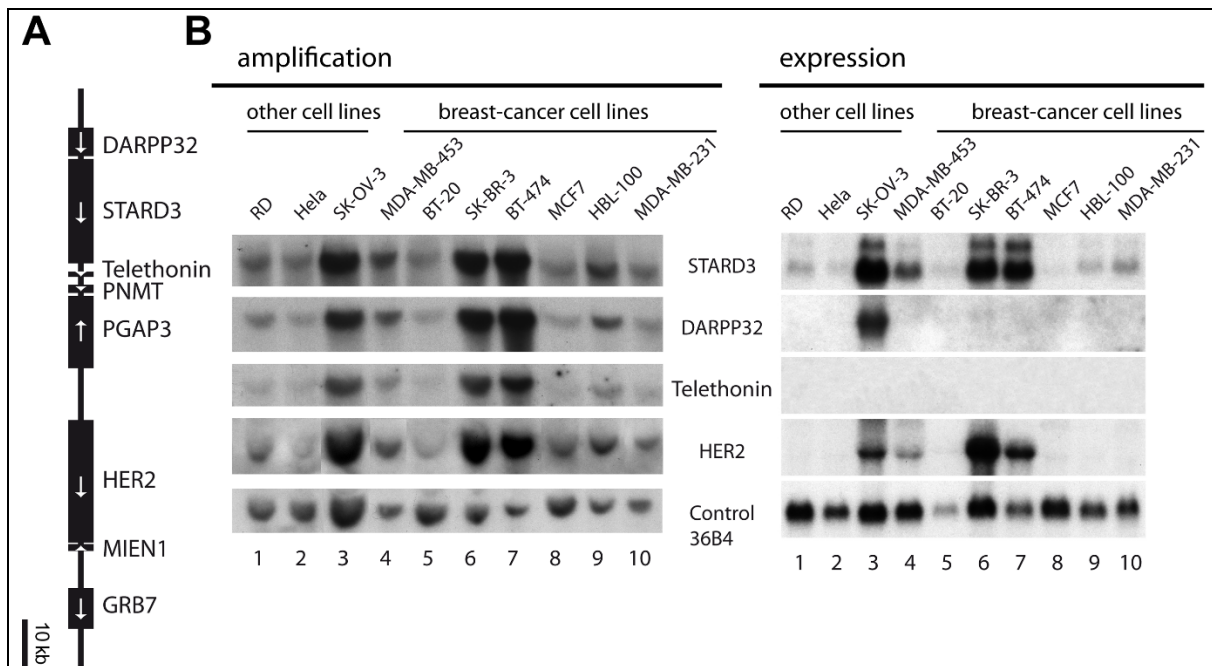
**A** Possible modes of action of STARD3 to transfer cholesterol. The MENTAL domain of STARD3 (blue) anchors the protein to endosome membranes, leaving its C-terminal START domain (green) in the cytoplasm. The START domain of STARD3 might work by extracting cholesterol (yellow) bound to its MENTAL domain from the late endosome membrane and transferring it to a closely positioned acceptor membrane or protein. STARD3 works as a monomer, oligomer, or inverted oligomer at the membrane of late endosomes making several cholesterol transfer scenarios possible. STARD3 may extract cholesterol using intramolecular or intermolecular mechanisms (see text for details).

**B** STARD3 expression induces the wrapping of the ER around endosomes. *Top panels: Left:* transmission electron microscopy image of HeLa cells overexpressing STARD3. *Right:* interpretation scheme of the TEM image: the ER and endosomes are green and pink, respectively; part of the nucleus and part of a mitochondria are gray and black, respectively; ribosomes are represented as small dots lining the ER. *Bottom panels: Left:* immunogold labeling of STARD3 positive endosomes. *Left:* gold-labeled STARD3 (black dots) is mainly found on the limiting membrane of endosomes. STARD3-positive endosomes are often tightly surrounded by ER-like structures. *Right:* Interpretation scheme of the TEM image with gold particles represented by red crosses; endosomes and ER are colored in pink and green, respectively. Bars represent 200 nm. *START* steroidogenic acute regulatory protein (STAR)-related lipid transfer, *MENTAL* metastatic lymph node clone number 64-N-terminal, *TEM* transmission electron microscopy, *ER* endoplasmic reticulum

## I. STARD3 is Implicated in the HER2 Molecular Subtype of Breast Cancer

*STARD3 Belongs to the Smallest Region of HER2 Amplification* Breast cancer is a heterogeneous disease at the genetic, molecular, and clinical levels. Studies reporting gene expression profiling revealed multiple tumor subtypes [28]. These distinct subtypes may reflect the variety of cell types found in the breast, their differentiation stages, and their phenotypic modifications via specific genomic alterations and key signaling pathways. In clinical practice, a basic molecular classification of breast cancer that considers three main subtypes is used to determine patient care: Luminal A and B, which express the estrogen receptor; HER2-positive tumors characterized by amplification and overexpression of the epidermal growth factor receptor 2 (ERBB2/HER2/neu); and triple-negative cancers, which do not express these receptors. This classification and the use of anti-hormonal and HER2-targeted therapy made a significant difference in clinical outcome [29]. Nevertheless, within these groups heterogeneity remains and, for instance, the molecular variations within the HER2 subgroup and their clinical implications remain largely unknown. HER2-positive tumors have higher levels of overall genomic instability than HER2-negative tumors. This observation supports the notion that HER2 amplification is functionally implicated in chromosomal instability on Chr17q [30]. Moreover, the complexity of the genomic alterations found at the HER2 locus highlights the diversity of HER2-amplified breast cancers. Since the identification of HER2 amplification in breast cancer, multiple genes have been reported to be co-amplified with HER2, supporting the idea that they contribute to the phenotype of individual tumors (reviewed in [31]). By identifying four new genes overexpressed and co-amplified with HER2 in breast cancer, we made one of the first observations in this area and proposed that these co-amplified genes—in particular STARD3— could possibly contribute to HER2-positive cancers [1, 4]. When STARD3 was identified, it was clear that its expression in breast cancer was linked with HER2 amplification. We demonstrated that the co-amplification of STARD3 and HER2 is due to the close association of their genes [1], an association also conserved in the mouse genome (F.A. and C.T., unpublished).

As shown in Fig. 6.3a, STARD3 and HER2 are located approximately 30–40 kb apart on chromosome 17q11-12. Several other genes are also found in this region that flank STARD3; on the centromeric side, the dopamine and cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein (DARPP32) gene is 0.5 kb upstream of the STARD3 transcriptional start site, whereas on the telomeric side, the Telethonin gene starts 2 kb downstream of the 3' end of the STARD3 gene [22]. However, unlike STARD3 and HER2, these two genes, although amplified, are not always expressed in HER2 breast cancer cells (Fig. 6.3b), indicating that amplification is not sufficient to drive overexpression [22]. Actually, the analysis of the STARD3 promoter region provided a clue regarding its overexpression with HER2. Indeed, both STARD3 and HER2 genes share Sp1 binding sites in their promoter regions supporting the notion that these genes are likely to be co-regulated by transcription factors belonging to the specificity protein/Krüppel-like factor (Sp/KLF) family that bind to these motifs [22]. To date, several studies using genome-wide microarray methods have defined the molecular identity of the HER2 amplicon in breast cancer [32–37]. Taken together, these studies indicate that the HER2 smallest region of amplification (SRA) is limited to a small number of genes, including STARD3 and growth-factor-bound protein 7 (GRB7). The data suggest that co-amplification and co-expression of various genes of the HER2-SRA is likely to influence the biology of these tumors, including the response to anti-cancer treatments. Therefore, it is necessary to understand the prognostic impact and function of STARD3 in HER2-positive cancers.



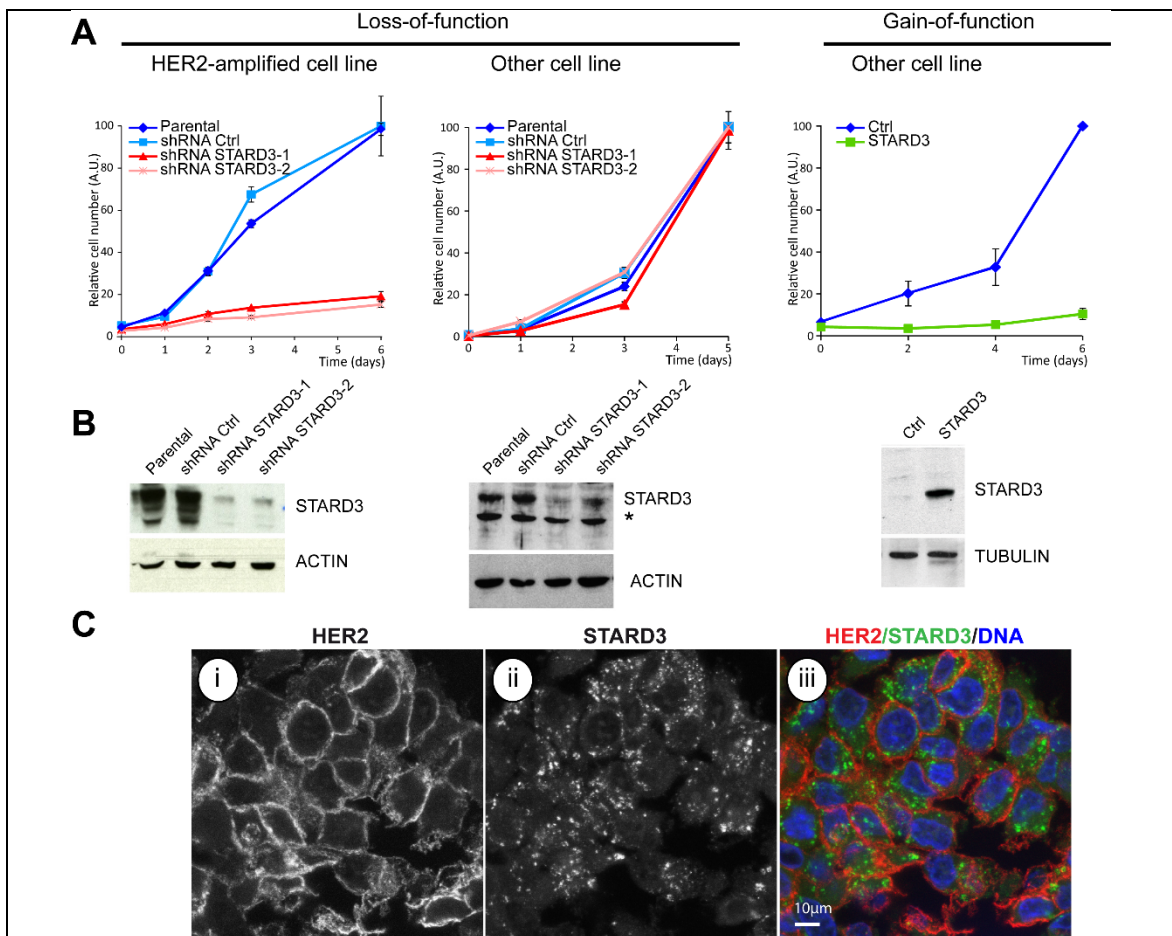
**Fig. 6.3 Co-amplification of STARD3 with HER2 in breast cancer cell lines. (Adapted from [22]).**

**A** Schematic representation of the genes present in the HER2 amplicon. This map was built using the human chromosome 17 sequence. DARPP32, dopamine, and cAMP regulated phosphoprotein (Accession no. AF233349); STARD3 alias metastatic lymph node 64 (Accession no. NM 006804.3); TCAP, telethonin or titin cap (Accession no. NM 003673); PNMT, phenylethanolamine N-methyltransferase (Accession no. NM 002686); PGAP3, post-GPI attachment to proteins 3 (Accession no. NM 033419); ERBB2, avian erythroblastic leukemia viral oncogene homolog 2 (Accession no. NM 004448); MIEN1, migration and invasion enhancer 1 (Accession no. NM 032339.3); GRB7, growth factor receptor-bound protein 7 (Accession no. XM 012695).

**B** Amplification and expression analysis of STARD3, DARPP32, Telethonin, and HER2 in cancer cell lines. A total of 10 µg of EcoRI-digested genomic deoxyribonucleic acid (DNA) (i) or 10 µg of total ribonucleic acid (RNA) (ii) extracted from various cancer cell lines were loaded in each lane as indicated. Hybridizations were carried out successively with probes corresponding to STARD3, dopamine and cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein (DARPP32), Telethonin, and epidermal growth factor receptor 2 (HER2). The loading control was the acidic ribosomal phosphoprotein P0, 36B4 probe

*STARD3* Contributes to the HER2-Amplicon Addiction Phenotype Some studies have addressed the prognostic significance of STARD3 co-amplification in patients with HER2-positive tumors; they all agree in showing that STARD3 amplification is associated with a shorter overall survival and disease-free survival in these patients [38–40]. The association of STARD3 with a poorer prognosis supported the idea that it has a direct influence on the aggressiveness of breast cancer cells. We originally proposed that STARD3 plays a synergistic role with HER2 during breast carcinogenesis. Consistent with this hypothesis, STARD3 downregulation in HER2 amplified cell lines resulted in reduced cell proliferation and increased cell death; on the contrary, STARD3 silencing in other cancer cells which are not HER2-amplified has no impact on cell growth and viability ([41]; Fig. 6.4a and b). Paradoxically, STARD3 forced expression in a non-HER2 cell line, like HeLa cells, compromises cell proliferation (Fig. 6.4a and b). The molecular mechanism by which STARD3 and HER2 may cooperate is still unclear. STARD3 and HER2 are highly conserved across different species and they are ubiquitously expressed but belong to distinct protein families. HER2 belongs to the epidermal growth factor receptor (EGFR) protein family that comprises four

members, EGFR, HER2, HER3, and HER4 [42]. Unlike the other member of the family, HER2 lacks ligand-binding activity. Moreover, compared to EGFR which undergoes a robust endocytosis and degradation upon activation, HER2 is slowly endocytosed and recycled to the cell surface [43]. Despite many attempts, we failed to find a direct interaction between STARD3 and HER2. Actually, in HER2-positive tumor samples, these proteins do not colocalize, with HER2 being predominantly present at the cell surface and STARD3 in the late endocytic compartment (Fig. 6.4c). Therefore, we reasoned that STARD3 might cooperate with HER2 by an indirect molecular mechanism which remains to be identified. Of interest, through loss of function studies (LOF), Cai et al. [39] showed that STARD3 contributes to proliferation in MCF7 cells and adhesion in MDA-MB-231 cells. This study pointed to a function of STARD3 in cell matrix adhesion and suggested a regulatory role for STARD3 on focal adhesion kinase (FAK; [39]). These cell models are, however, representative of luminal and basal breast subtypes and therefore, whether these functions are relevant to the HER2 subtype remains to be addressed. In HER2-amplified cells, Sahlberg et al. [44] did a systematic study of HER2 co-amplified genes by LOF and found that several genes of the amplicon, including STARD3 and GRB7, decreased cell viability. They also found that silencing of both STARD3 and HER2 had an additive effect on decreased cell viability and increased apoptosis [44]. These authors evoke the concept of “oncogene addiction” to explain this phenotype. This concept characterizes the dependency of some cancers on one or a few genes for the maintenance of the malignant phenotype [45].



**Fig. 6.4** HER2-amplified breast cancer cells are addicted to STARD3.

**A** Growth curves illustrating the paradoxical role of STARD3 on cell proliferation. Cell growth was analyzed using the MTT assay. *Left*: growth curves for the breast cancer HER2-amplified cell line SK-BR-3 after STARD3 silencing. Compared to the parental and control shRNA cell lines, clones silenced for STARD3 using two distinct silencing constructs have an impaired growth. *Middle*: cell growth curve for HeLa cells after STARD3 silencing. The same experiment was repeated in a HER2-non-amplified cell line. In HeLa cells, (cervical carcinoma cells), silencing of STARD3 using the same shRNA strategy does not compromise cell growth. *Right*: cell growth curve after STARD3 overexpression in HeLa cells. HeLa cells, expressing high levels of STARD3 have an impaired growth compared to control (empty vector) cells.

**B** STARD3 expression in the cell lines analyzed in **A**. Protein extracts (~10 µg) were analyzed by immunoblotting using anti-STARD3-, -Actin-, and -Tubulin-specific antibodies. In the middle panel a long exposure time was necessary to detect endogenous STARD3 expression in HeLa cells, the asterisk represents a non-specific band.

**C** HER2 and STARD3 do not co-localize in an invasive ductal carcinoma of the breast. Double immunofluorescence in the same tissue section shows: (i) HER2-plasma membrane staining; (ii) STARD3, cytoplasmic granular staining; (iii) merge image of HER2 (red), STARD3 (green) and nucleus (blue); note the absence of yellow staining revealing the lack of co-localization.

HER2 epidermal growth factor receptor 2, shRNA short hairpin ribonucleic acid



In conclusion, in cancer STARD3 is linked at the genetic and functional levels with HER2. HER2-amplified cancer cells are dependent on co-amplified genes including STARD3 for their growth and survival. In contrast, in other cancer cells STARD3 is dispensable for growth. Actually, STARD3-deficient mice are viable and do not show any growth defects indicating that STARD3 is not essential for cell survival under normal conditions [46]. Paradoxically, STARD3-forced expression is toxic in cancer cells which are not HER2-amplified. Moreover, acute STARD3 expression in the liver of mice-induced damage and apoptosis [47]. Cancer is a multistage process, and cancer cells (particularly HER2-amplified cancer cells) have unstable genetic material and they must continuously adapt to maintain their integrity, survive, and proliferate. The “oncogene addiction” concept suggests that signal transduction and gene expression are regulated in very different manners in cancer cells compared to normal cells, making the former more dependent on the activity of specific genes [45]. We propose that HER2-amplified breast cancers have evolved in a way that they are dependent on STARD3. To date, the molecular details of this dependency are unclear, but STARD3 likely represents an additional therapeutic target in HER2-amplified cancers.

## II. STARD3, a Carotenoid-Binding Protein

An unexpected function for STARD3 was revealed in silkworms (*Bombyx mori*). Silkworm larvae build cocoons which have a yellow color resulting from the presence of carotenoids [48]. Interestingly, animals cannot synthesize carotenoids *de novo* which means that silk carotenoids have a dietary origin, namely mulberry leaves. Therefore, carotenoids have to be absorbed by the gut, transported through the hemolymph to finally be delivered in the silk gland of the larvae where they will give the silk its color. This implies that there are transport mechanisms allowing carotenoids to be conveyed across epithelial cells from the gut and the silk gland to reach their final destination. Between the gut and the silk gland, carotenoids are transported within the hemolymph by lipoprotein particles named lipophorins. The color of carotenoids allowed for the purification of a carotenoid-binding protein, termed CBP, from larval silk glands that appeared yellow under non-denaturing conditions [49]. The main carotenoid bound to CBP was lutein, with  $\beta$ -carotene and  $\alpha$ -carotene being less frequently found. The molecular characterization of CBP cDNA showed that it belongs to the START protein family and is closely related to STARD1 and STARD3. However, unlike STARD1 and STARD3 which possess a mitochondria- and an endosome-targeting signal, respectively, CBP is a START-only protein. Intriguingly, the gene encoding CBP uses alternative promoter usage and splicing to produce an isoform closely resembling STARD3; indeed, this CBP isoform termed (BmStart1) possesses a MENTAL domain preceding the START domain [50]. Several silkworm mutants producing white cocoons have been collected over the years of sericulture. For instance, mutant silkworms for the *Y* (*Yellow blood*) gene display colorless hemolymph and silk resulting from an inefficient carotenoid absorption in the gut. Interestingly, in the *Y*-recessive strain, the locus encoding BmStart1/CBP is altered due to a retrotransposon insertion in a CBP-specific exon [51]. As a consequence, while BmStart1 protein is normally present in this strain, CBP is absent, thus suggesting that it is the CBP isoform that is responsible for the silk color. Accordingly, transgenic re-expression of CBP in the *Y* mutant strain restores a yellow color to the silk. To conclude, the STARD3 gene has evolved in silkworms to produce two different proteins, CBP which is involved in carotenoid transport and consequently silk color and BmStart1, which is similar to STARD3 found in other animals and is likely involved in cholesterol transport. In humans, lutein and zeaxanthin are two carotenoids that accumulate specifically in the central region of the retina named the macula lutea, the part of the retina that is responsible for central vision and is named for its yellow color resulting from the presence of carotenoids. The specific accumulation of these lipophilic molecules in the retina is most probably induced by the action of a molecular transporter. Accordingly, a carotenoid-binding protein was purified from human retina [52]. This protein of ~ 50 KD binds lutein with a  $K_d$  of 0.45  $\mu$ M and is detected with antibodies raised against the silkworm CBP. Considering the molecular weight of this transporter and its recognition by the anti-CBP antibodies [52], these data suggest that the protein involved in lutein accumulation in human retina is STARD3. In accordance with this idea, the recombinant START domain of STARD3 binds lutein with a  $K_d$  of 0.45  $\mu$ M [53]. These data raise an intriguing question: Is STARD3 responsible for lutein accumulation in the human retina in a manner similar to the silk-worm ortholog allowing lutein deposition in silk? This would imply that the START domain of STARD3 can bind different ligands; it may have a specialized function in lutein handling in some organs and a more general function in cholesterol transport in most cells.

## III. STARD3 and Cholesterol Accumulation Disorders

*STARD3 as a Surrogate for STARD1-Independent Steroidogenesis* STARD1 plays a central role in *de novo* steroidogenesis, however, STARD1 is not expressed in some steroidogenic organs such as the placenta and was long thought to be absent in the brain, suggesting that an alternative cholesterol transport pathway into the mitochondria might exist. Early on, STARD3 was proposed to act as a STARD1-like protein in the placenta and the brain and many lines of evidence supported this hypothesis. STARD3 is expressed in the placenta and the brain [2, 6, 54]. In isolation, the START domain of STARD3 has a StAR-like activity in *in vitro* steroidogenic assays

[6, 16]. Consistent with its tight attachment to the LEs membrane, the full-length protein is, however, poorly active in steroidogenic assays [6, 15, 17]. Of interest, in the placenta, a truncated STARD3 protein leading to the release of an active START domain of about 30 KDa has been described by several groups [6, 17, 55]. The presence of a cleaved form corresponding to the isolated STARD3-START domain supports the notion that STARD3 might act in placental steroidogenesis. However, the generation of mouse models deficient for STARD3 did not substantiate its role in steroidogenesis, at least in the mouse. Indeed, mice lacking STARD3 appear normal and show no reproductive defects [46]. However, the placenta can make estrogens from an alternative pathway which is not initiated by cholesterol conversion into pregnenolone in the mitochondria. The placenta can use steroids produced by the fetal adrenal and convert them to estrogens [56]. Whether this pathway is favored in STARD3-deficient mice remains to be addressed. Alternatively, other START domain containing proteins like the widely expressed STARD4 and STARD5 proteins might compensate for the lack of STARD3. Besides the placenta, the gonads, and the adrenal glands, steroidogenesis occurs in the brain. While STARD3 is well expressed in the brain, both in neurons and in glial cells, it does not co-localize with P450<sub>scc</sub> which makes it unlikely to be involved in neurosteroid synthesis [54, 57].

*STARD3 in Niemann-Pick Type C-Deficient Cells* The position of STARD3 at the surface of LEs suggests that it contributes to the distribution of cholesterol from this organelle. Besides *de novo* synthesis in the ER, cells can obtain cholesterol from circulating LDL. The ApoB protein component of LDL binds to LDL receptor family members, the ligand-receptor complex is taken up by clathrin-mediated endocytosis and is dissociated in endosomes. While the receptor is recycled back to the cell surface, the lipid component of LDL is targeted to LEs/Lysosomes (LE/Ly) where cholesterol becomes redistributed inside the cell [58]. How exactly cholesterol is trafficked from endosomes to distinct intracellular regions remains unclear. Studies of Niemann-Pick type C disease, a lipid storage disorder which leads to neurological disorders and hepatosplenomegaly (reviewed in [59–61]), revealed the instrumental role of two proteins, Niemann-Pick C1 (NPC1) and Niemann-Pick C2 (NPC2), on cholesterol egress from LE/Ly. NPC proteins are both LE resident proteins acting in concert; NPC2, a soluble LE/Ly luminal protein binds cholesterol in the lumen, and exchanges cholesterol with the N-terminal cholesterol-binding domain of NPC1 [62]. To address the potential role of STARD3 in cholesterol clearing from LE, STARD3 was overexpressed in NPC-deficient fetal fibroblasts but no correction of the cholesterol accumulation phenotype was observed [12, 63]. Mechanistic insights explaining the lack of connection between the NPC proteins and STARD3 were proposed recently [64]. According to this study, endosomal cholesterol would be trafficked in a sequential manner, first from LE to the plasma membrane through endosomes containing STARD3 and the cholesterol transporter ABCA3 and second from LE to the ER through a distinct set of endosomes positive for the oxysterol-binding protein-related protein 1L (ORP1L) and NPC1 [64]. This model is notably based on the finding that STARD3 and NPC1 mark two distinct endosome pools and is consistent with the fact that overexpression of STARD3 does not significantly increase ACAT-mediated cholesterol esterification in the ER [23]. Further, STARD3 overexpression mimics some of the features of NPC-deficient cells. In all cell types tested STARD3 overexpression was associated with sterol deposition in LE [12, 23, 63]. Moreover, acute overexpression of STARD3 in mouse liver was associated with increased cholesterol content, biliary bile acid concentration, and impaired bile flow and caused severe liver damage and apoptosis [47]. All these studies argue against a role of STARD3 in cholesterol egress from LE/LY. Other studies have, however, provided evidence that STARD3 promotes cholesterol transfer presumably from LDL to the mitochondria. Charman et al. [65] showed an increase transport of cholesterol to mitochondria in NPC1-deficient Chinese Hamster ovary (CHO) cells that is mediated by STARD3. Consistent with this, Zhang et al. [66] proposed the involvement of STARD3 in the transfer of cholesterol to the mitochondria. Indeed, using time lapse imaging techniques, these authors found that STARD3 LE tubules aligned parallel to mitochondria and reported the presence of transient contact between these two organelles. The fact that the efforts made to clarify the function of STARD3 in cholesterol trafficking have used many cancer cells models may explain some of the discrepancies found in the literature. While the role of STARD3 in cholesterol transfer from LE is still unclear, our new finding that STARD3-positive endosomes are tethered with the ER, argues for its participation in cholesterol egress from LE to the ER [26]. However, experimental data do not support this model. The cholesterol transfer function of STARD3 appears to be different from this tethering activity. The trafficking of endosomal cholesterol is probably complex and sequential, it may be regulated by redundant mechanisms, is probably cell-type specific and may be altered differently in cancer cells. In the future, mechanistic analysis of the function of STARD3 in cholesterol trafficking should use both *in vitro* and *in vivo* models that are physiological and highly relevant to human disease.

## Summary

The cloning of STARD3 was fairly rapid and easy; the functional characterization of this protein has been difficult and is far from being achieved. To date, an understanding of how STARD3 handles cholesterol has been elusive. From its homology with STARD1, it was proposed that STARD3 functions to transport cholesterol from endosomes to the ER. Many studies including those using NPC-deficient cells and the generation of STARD3-deficient mice did not support this hypothesis and it is clear that additional studies are necessary to uncover the

role of STARD3 in cholesterol trafficking. STARD3 is overexpressed in the HER2-subtype of breast cancer, it belongs to the smallest region of amplification involving HER2 and its presence in breast cancer is associated with a poor prognosis. Consistent with this, STARD3-silencing results in cell growth arrest and apoptosis in HER2-positive cell lines. How STARD3 contributes to the phenotype of HER2 cancers is still unclear. Unlike STARD1, STARD3 is not essential for steroidogenesis; however, STARD3 is a lipid-binding protein involved in cholesterol trafficking at the level of LEs. Remarkably, STARD3 modifies the intracellular distribution and morphology of LEs and moreover STARD3 forms membrane contact sites between LEs and the endoplasmic reticulum. These organelles are central for cholesterol homeostasis. The ER is the site of cholesterol biosynthesis while LEs ensure LDL-derived cholesterol distribution. Thus, the physical location of STARD3 between these two organelles places the protein at a unique position at the interface of two major cholesterol pathways. Moreover, extended ER-endosome contacts regulate the dynamics of the endocytic compartment probably by reducing the maturation of LEs to lysosomes. As the ER and endosomes are the sites of synthesis and degradation of membrane receptors, respectively, we can speculate that STARD3 acts on cancer cells by modifying the lipid composition of cellular membranes. Indeed, membranes are not only barriers, they also function as interfaces at which numerous cellular processes, including signaling and cell death mechanisms, are concentrated and regulated. Despite all these uncertainties, we have made considerable progress in clarifying the function of STARD3. It is without doubt that this fascinating protein will keep us busy for the coming years.

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