

Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis

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Summary

The prevailing hypothesis of lipid droplet biogenesis proposes that neutral lipids accumulate within the lipid bilayer of the ER membrane from where they are budded off, enclosed by a protein-bearing phospholipid monolayer originating from the cytoplasmic leaflet of the ER membrane. We have used a variety of methods to investigate the nature of the sites of ER–lipid-droplet association in order to gain new insights into the mechanism of lipid droplet formation and growth. The three-dimensional perspectives provided by freeze-fracture electron microscopy demonstrate unequivocally that at sites of close association, the lipid droplet is not situated within the ER membrane; rather, both ER membranes lie external to and follow the contour of the lipid droplet,

enclosing it in a manner akin to an egg cup (the ER) holding an egg (the lipid droplet). Freeze-fracture cytochemistry demonstrates that the PAT family protein adipophilin is concentrated in prominent clusters in the cytoplasmic leaflet of the ER membrane closely apposed to the lipid droplet envelope. We identify these structures as sites at which lipids and adipophilin are transferred from ER membranes to lipid droplets. These findings call for a re-evaluation of the prevailing hypothesis of lipid droplet biogenesis.

Key words: Lipid droplet formation and growth, Freeze-fracture immunocytochemistry, PAT proteins

Introduction

Recent studies on lipid droplets and their associated proteins have led to a new appreciation of the importance of these previously under-researched components of the cell (Murphy, 2001). Lipid droplets are now envisaged not merely as storage depots for superfluous intracellular lipids, but as metabolically active organelles, participating in a range of functions other than lipid homeostasis (Wang et al., 1999), notably cell signalling (Umlauf et al., 2004), intracellular vesicle trafficking (Liu et al., 2003) and the pathogenesis of diseases including atherogenesis, diabetes and obesity (Martinez-Botas et al., 2000; Mishra et al., 2004; Larigauderie et al., 2004).

Structurally, the lipid droplet consists of a core of apolar lipids (triacylglycerol and cholesterol esters) enveloped by a single monolayer of phospholipids (Tauchi-Sato et al., 2002; Ozeki et al., 2005). Formation of lipid droplets is widely believed to occur by accumulation of neutral lipid within the bilayer of the endoplasmic reticulum (ER) membrane. Distension of the membrane and separation of its component monolayers leads to budding of the droplet surrounded by an envelope consisting of the former cytoplasmic leaflet of the ER membrane. In this way, lipid-droplet-associated proteins are assumed, at least in part, to be derived from proteins resident in the ER membrane (Brown, 2001).

Contemporary advances in our understanding of the metabolic functions of lipid droplets have been stimulated by studies on the lipid droplet-associated proteins, particularly the PAT family proteins, perilipin, adipophilin and TIP47

(Fujimoto et al., 2001; Fujimoto et al., 2004; Ostermeyer et al., 2004; Pol et al., 2004; Liu et al., 2003; Ohashi et al., 2003; Wolins et al., 2001; Robenek et al., 2004; Cole et al., 2002; Londos et al., 1999). Crucial to understanding the role of the PAT family proteins is determination of their exact cellular distribution and intracellular itinerary. The widely promoted view is that PAT family proteins are confined to the lipid droplet surface and are not present in any other cellular compartment (Londos et al., 1999; Miura et al., 2002). However, a number of other studies present a more confusing and contradictory picture. One study reported that adipophilin in transfected murine 1246 cells is preferentially associated with the plasma membrane and the nucleus (Gao and Serrero, 1999), although subsequent studies failed to confirm this finding (McManaman et al., 2003). Although past studies have relied largely on low resolution immunofluorescence microscopy and biochemical analysis of subcellular fractions, our recent findings from freeze-fracture immunocytochemistry indicate that PAT family proteins are not restricted to the lipid droplet surface as previously maintained, but also pervade the droplet core (Robenek et al., 2005a; Robenek et al., 2005b). Moreover, we demonstrated unequivocally with this approach that PAT family proteins are indeed integral components of the plasma membrane (Robenek et al., 2005c) where they appear to play a role in the accumulation of fatty acids into lipid droplets that are juxtaposed to the plasma membrane. Another key issue centers on localization of PAT family proteins in the ER versus the cytosol. Whereas Heid et al. (Heid et al., 1996)

reported the presence of adipophilin in ER fractions of lactating mammary glands, Brasaemle et al. (Brasaemle et al., 1997) reported that sedimentable membranes do not contain this protein. Others favour the notion that adipophilin and the other members of the PAT family are cytosolic proteins synthesized on free ribosomes (Londos et al., 1999; Diaz and Pfeffer, 1998). If this is correct, then an ER-derived mechanism would not be essential for the acquisition of PAT family proteins by lipid droplets.

In view of these conflicting data, we have now sought detailed evidence for or against the presence of adipophilin in the ER membranes of cultured human macrophages by applying freeze-fracture immunocytochemistry, in parallel with standard immunofluorescence confocal light microscopy, thin-section electron microscopy, immunogold cryo-thin-section electron microscopy and western blot analysis of subcellular membrane fractions. Freeze-fracture immunocytochemistry is uniquely equipped to discriminate, at high resolution, the spatial relationship between closely associated membranes and lipid-containing organelles, and to define the precise location of proteins within them. Our results provide evidence that, contrary to popular belief, lipid droplets develop alongside, not within, the ER membrane. The ER membrane appears uniquely specialized at sites of lipid droplet biogenesis, with high concentrations of adipophilin in the leaflet closest to the nascent lipid droplet.

Results

Accumulation of lipid droplets

Standard thin-section electron microscopy reveals only a few small lipid droplets in macrophages cultured under normal conditions. Treatment with AcLDL, however, induces production of large numbers of medium-to-large-sized lipid droplets (Fig. 1A). In many instances, ER-membrane cisternae are seen closely apposed to lipid droplets, partially encircling them (Fig. 1B,C). The lumen of the ER is reduced in thickness and ribosomes are absent from these sites of close apposition.

Western blot detection of adipophilin

The antibody to adipophilin detected single bands at 52 kDa, the expected molecular mass for adipophilin, in lysates of whole cultured macrophages (Fig. 2A), confirming its specificity for adipophilin. AcLDL-laden macrophages contained more adipophilin than nonladen cells. Adipophilin was also readily detectable in western blots of fractions containing lipid droplets and membranes isolated from macrophages (Fig. 2B). Comparison of the adipophilin signal between fractions showed that in cells treated with AcLDL, the membrane fraction contained less adipophilin than the lipid fraction. The reverse was the case in the fractions derived from cells not treated with AcLDL. However, the membrane fraction from AcLDL-treated macrophages showed weaker adipophilin labeling than that from untreated macrophages. Conversely, the lipid droplet fraction from treated macrophages showed more intensive adipophilin labeling than that from untreated macrophages. Using calnexin as an ER

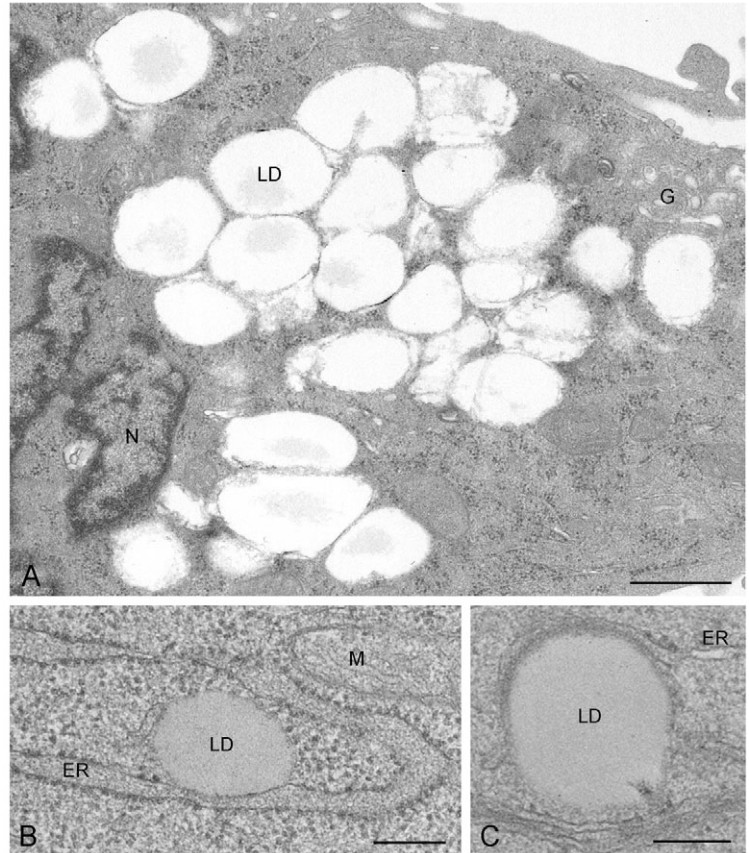


Fig. 1. Conventional thin-section electron microscopy of macrophages lipid laden by treatment with AcLDL for 24 hours. (A) The cells contain accumulations of lipid droplets (LD) in the cytoplasm. (B,C) Examples of close association between lipid droplets (LD) and ER membranes. At the sites of association with the lipid droplet, the ER lumen becomes narrowed and ribosomes are absent. G, Golgi apparatus; M, mitochondrion; N, nucleus. Bars, 1 μ m (A); 0.2 μ m (B,C).

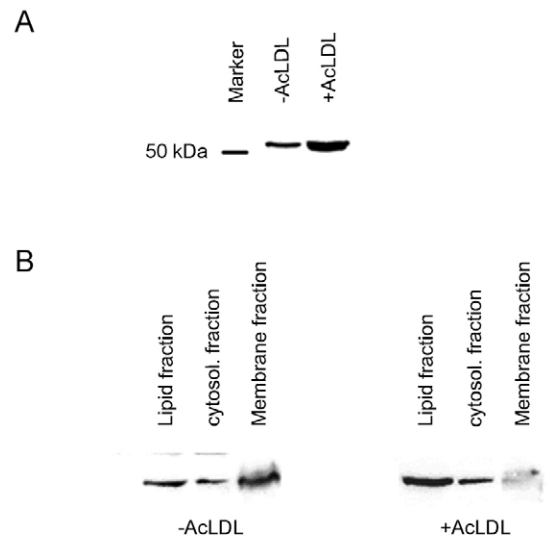


Fig. 2. Western blot analysis of adipophilin in whole nonlipid-laden (-AcLDL) and lipid-laden (+AcLDL) macrophages (A) and in isolated fractions from cells (B).

marker and flotillin-2 as a plasma membrane marker, it was demonstrated that the membrane fractions analyzed contained both ER and plasma membranes. Some flotillin-2 was consistently detected in the lipid fraction indicating the co-isolation of portions of plasma membrane adherent to lipid droplets (see Discussion).

Immunodetection of adipophilin by fluorescence microscopy and cryothin-section electron microscopy

Immunofluorescence confocal microscopy of lipid-laden macrophages stained for neutral lipids (Fig. 3A) and adipophilin (Fig. 3B) confirmed that adipophilin is associated with lipid droplets containing neutral lipids. The adipophilin immunolabeling and neutral lipid staining were colocalized. Lipid droplets typically showed a distinct ring of adipophilin labeling surrounding the neutral lipid core (Fig. 3C).

Cryothin sections viewed by electron microscopy revealed the lipid droplets as essentially electron-lucent bodies with little internal structure. Immunogold labeling for adipophilin was seen predominantly at the surfaces of the droplets (Fig. 3D).

Despite the western blot data indicating the presence of adipophilin in membrane fractions, the limitations of immunofluorescence microscopy and cryothin-section electron

microscopy did not permit the detection of adipophilin in any membrane systems of the cells.

Application of freeze-fracture electron microscopy

Freeze-fracture immunocytochemistry was applied to overcome the limitations inherent in the above localization techniques. Freeze-fracturing enables the visualization of planar views of intracellular membranes and lipid droplets, greatly enhancing the opportunities for detecting positive labeling compared with techniques in which membranes are viewed edge-on in sections. Moreover, freeze-fracture has the spatial resolution to allow closely associated membranes and organelles to be discriminated from one another, and even to ascribe any positive label to one or other of the two half-membrane leaflets of which biological membranes are comprised. Interpretation of freeze-fracture images depends critically on an understanding of how membranes are fractured and of the nomenclature of the resultant fracture faces.

When frozen cells are fractured, the fracture splits membranes into their two constituent monolayers along a plane between the hydrophobic tails of the phospholipids in the bilayer. In the case of the plasma membrane, one monolayer remains attached to the extracellular space (E-half or exoplasmic leaflet) whereas the other leaflet remains attached to the cytoplasm or protoplasm (P-half or cytoplasmic leaflet). The view of the E-half revealed by freeze-fracture is termed the E-face; that of the P-half is termed the P-face (Branton et al., 1975).

A corresponding nomenclature is applied to intracellular membranes. The fracture faces of the cytoplasmic leaflets of the ER and nuclear membranes are designated as P-face views, and those of the endoplasmic leaflets adjacent to the ER lumen and perinuclear space are the complementary E-face views. In this way, a consistent terminology describes structurally and functionally equivalent portions of the different membrane systems of the cell. The envelope surrounding the lipid droplet presents a special case because it is not part of a classic bilayer, but a phospholipid monolayer apposed to the neutral lipids of the core. The fracture plane often exposes the interface between the hydrophobic aspect of the monolayer and the core. The fracture face of the monolayer revealed in concavely fractured lipid droplets is considered to be a P-face view, and the complementary aspect seen in convexly fractured lipid droplets (which actually represents a view of the outermost layer of the neutral lipid core) is referred to as the E-face equivalent.

Rather than removing all cellular material from the replicas with strong oxidants (the standard procedure in freeze-fracture replica preparation) replicas for immunolabeling were washed with SDS. This preserves molecules adhering directly to the replicas, while the remaining cellular material is flushed away. Integral membrane proteins may then be labeled using immunocytochemical techniques. Labeling of the SDS-treated freeze-fracture replicas was carried out using primary antibodies against adipophilin followed by matching secondary antibodies coupled to colloidal gold. Viewed in the electron microscope, the electron-dense gold particles clearly mark the positions of this protein, superimposed upon the en face membrane views and other ultrastructural features of the cell.

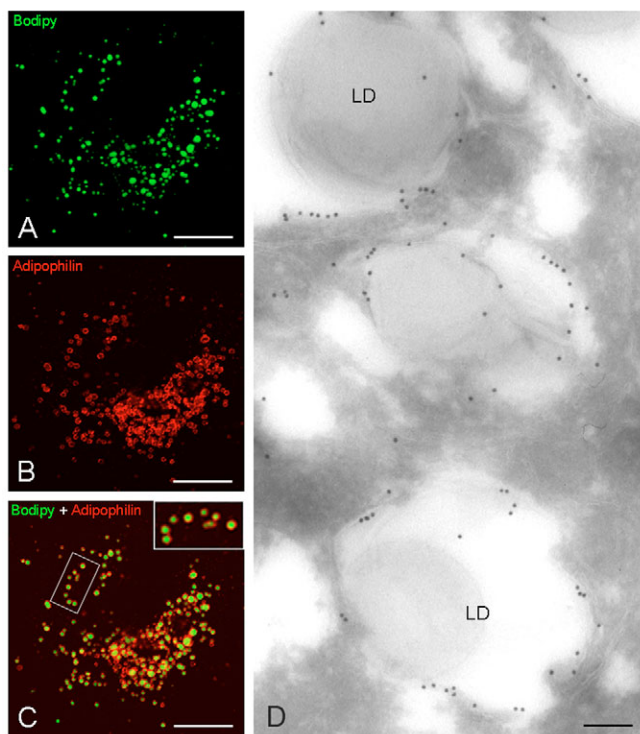


Fig. 3. Immunofluorescence confocal microscopy and cryothin-section immunogold label electron microscopy of adipophilin in lipid droplets of AcLDL-treated macrophages. (A) BODIPY staining for neutral lipids. (B) Immunofluorescence labeling of adipophilin. (C) Merged image of A and B. High magnification views (inset in C) show a rim of adipophilin labeling around the neutral lipid core of the droplet. Immunogold labeling (18 nm gold) for adipophilin in cryothin sections examined by electron microscopy reveals prominent labeling at the surface of the lipid droplet (LD), consistent with the immunofluorescence results. Bars, 5 μm (A-C); 0.2 μm (D).

Localization of adipophilin by freeze-fracture immunocytochemistry

Fig. 4 illustrates a freeze-fracture view of a macrophage after immunogold labeling for adipophilin. Apart from positive labeling in lipid droplets and on the plasma membrane P-face in accordance with our previous observations (Robenek et al., 2005c), prominent label is apparent on ER membranes. The ER labeling is confined to the P-face; no label was detected on the E-face. As described elsewhere (Robenek et al., 2003), P-faces and E-faces can be distinguished on the basis of their content of intramembranous particles: P-faces are studded with substantially more particles than E-faces. The membranes of the Golgi apparatus, mitochondria and vesicles were consistently devoid of label.

The presence of label on the ER membrane P-face and the absence of label on the ER membrane E-face was a consistent and reproducible observation (Fig. 5). Adipophilin label was also seen on the P-face of the outer nuclear membrane (Fig. 6),

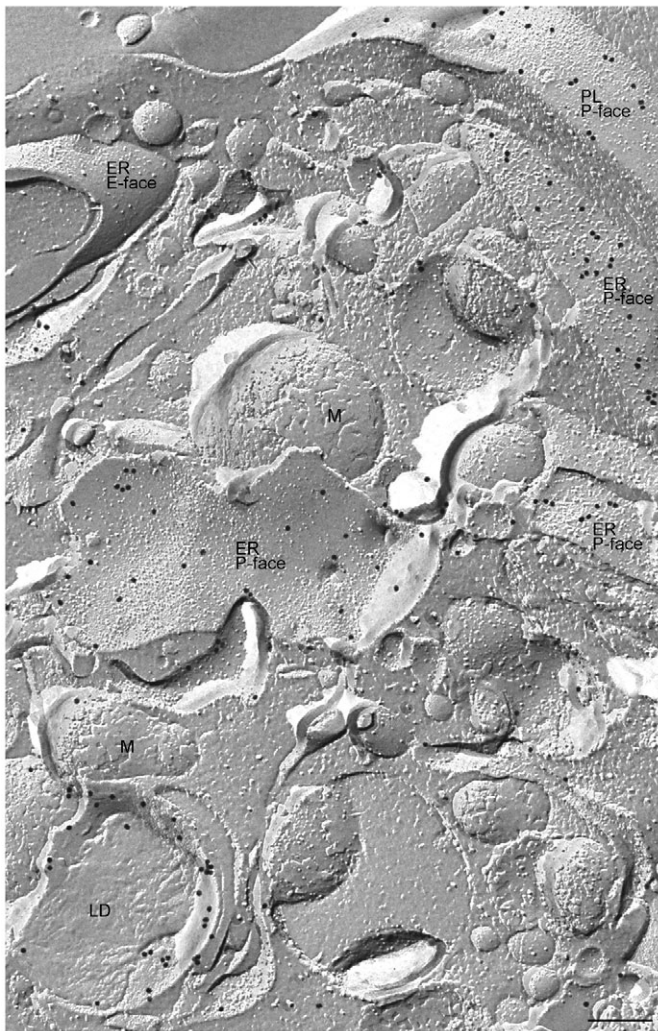


Fig. 4. Survey freeze-fracture view of a lipid-laden macrophage immunogold labeled (18 nm gold) for adipophilin. Prominent labeling is seen on the P-faces of plasma membrane (PL) and ER membranes (ER), and on lipid droplets (LD). The E-face of the ER membranes, mitochondrial membranes (M) and vesicle membranes are devoid of label. Bar, 0.2 μ m.

with which the ER membrane P-face is continuous. No label was apparent on the E-face of the outer nuclear membrane or on either fracture face of the inner nuclear membrane.

Morphology of sites of ER–lipid-droplet interaction revealed by freeze-fracture

Apart from disclosing differential labeling on the leaflets of the various membrane systems of the cell, freeze-fracture reveals three-dimensional aspects of intracellular membranes, permitting unique views of the spatial relationships of these membranes. Such perspectives (Fig. 7) enable the nature of the intimate association between lipid droplets and ER segments, apparent from thin sections (Fig. 1B,C), to be fully appreciated.

Fig. 7 presents two freeze-fracture images of the sites of ER–lipid-droplet association. Fig. 8 is a corresponding schematic diagram to aid interpretation. In Fig. 7A, a convexly fractured lipid droplet is seen projecting from a segment of ER. Both membranes of the ER lie external to and follow the contour of the lipid droplet, enveloping and partially enclosing it in a manner that may be likened to an egg cup (the ER) holding an egg (the lipid droplet). Where the ER lumen narrows in the cup region, the fracture skips back and forth between the two ER membranes, revealing portions of the P-face of the membrane closest to the lipid droplet and of the E-face of the membrane nearest to the observer. This view demonstrates unequivocally that the lipid droplet lies next to the two closely apposed membranes of the ER, not within one of the membranes of the ER. To the lower right of the image, the ER membranes can be seen extending away from the lipid droplet.

In Fig. 7B, the fracture plane has scooped out the lipid droplet, providing a bird's-eye view looking down into the ER cup. As in the previous example, the fracture plane has skipped back and forth between the two membranes of the ER in the zone adjacent to the lipid droplet. In this concave fracture, the uppermost ER membrane, adjacent to the position of the lipid droplet, is seen in E-face view, whereas the second ER membrane, distant from the lipid droplet, is seen in P-face view. Again, the space between the ER membranes becomes markedly wider as the ER sweeps away from the cup.

With these images in mind, fractures that leave less-extensive fragments of ER membrane associated with the lipid droplet are readily identified in the freeze-fracture images (Figs 9, 10).

Patterns of adipophilin labeling at sites of ER–lipid-droplet interaction

Freeze-fracture immunocytochemistry reveals distinctive labeling patterns for adipophilin at the sites of lipid-droplet association (Fig. 9). Abundant labeling for adipophilin is seen on the P-face of the ER membrane that lies closest to the lipid droplet; no label is detected on the E-face of the partner ER membrane distant from the droplet (Fig. 9A,B). Prominent adipophilin labeling is also seen on the P-face of the lipid droplet envelope, i.e. on the outermost surface of the envelope closest to the ER, with relatively little label apparent on the complementary E-face aspect (Fig. 9C,D).

In interpreting differences in adipophilin-labeling patterns in the views of ER membranes at sites of lipid-droplet ER association, it must be emphasized that as both ER membranes may be fractured to reveal a P-face or an E-face, a total of four

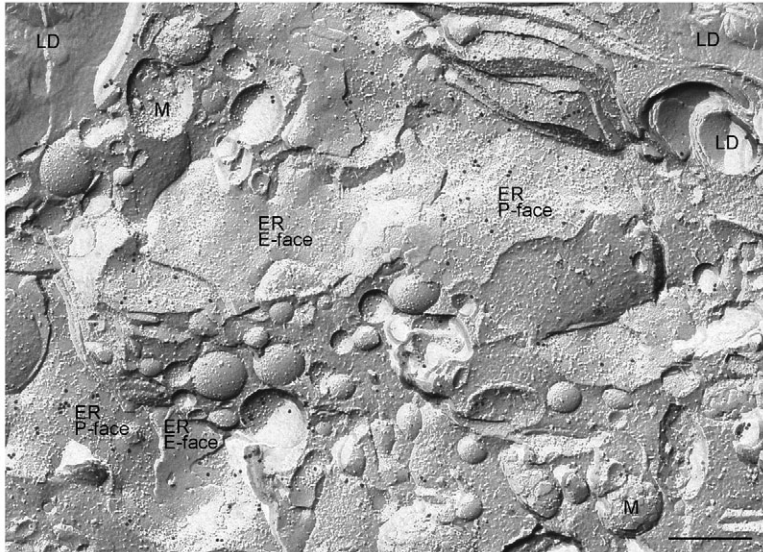


Fig. 5. Freeze-fractured ER membranes in a lipid-laden macrophage after immunogold labeling (18 nm gold) for adipophilin. This example illustrates the consistency of labeling on the P-face, and its absence from the E-face. LD, lipid droplet; M, mitochondria. Bar, 0.5 μ m.

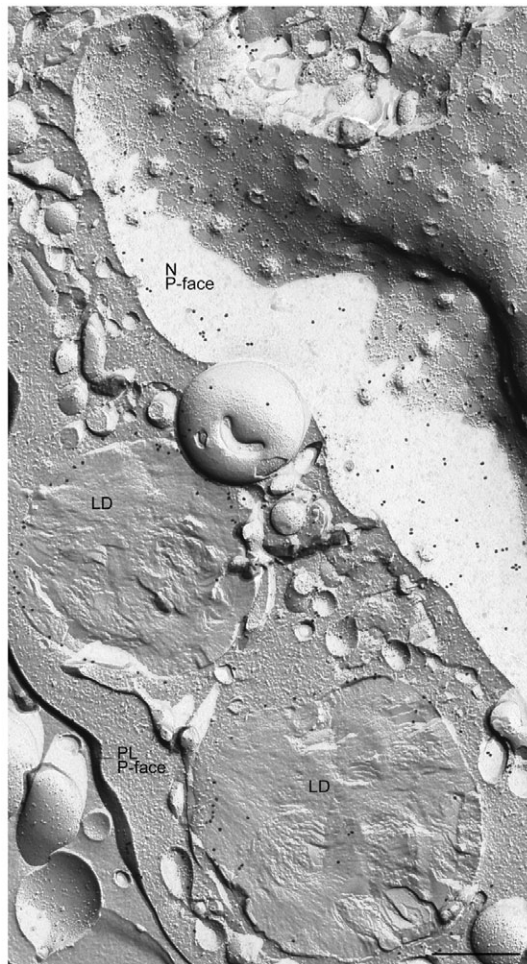


Fig. 6. Freeze-fractured nuclear membrane in a lipid-laden macrophage after immunogold labeling (18 nm gold) for adipophilin. The P-face of the outer nuclear membrane (N) is prominently labeled. The E-face of the outer nuclear membrane, and both fracture faces of the inner nuclear membrane are typically devoid of label. The periphery of cross-fractured lipid droplets (LD) and the P-face of the plasma membrane (PL) are also labeled. Bar, 0.5 μ m.

different ER membrane aspects may be disclosed in different images (Fig. 8). Examination of differences in labeling density between these views ideally requires side-by-side views of convexly and concavely fractured ER 'cups', as illustrated in Fig. 10. In the concave fracture (Fig. 10, upper example), the ER membrane immediately adjacent to the lipid droplet is revealed as an E-face, together with portions of the P-face of the partner ER membrane that is distant from the lipid droplet. In the convex fracture, portions of the P-face of the ER membrane that is adjacent to the lipid droplet as well as the E-face of the ER membrane that is distant from the lipid droplet, are revealed (Fig. 10, lower example).

The P-faces of both ER membranes in the cup-shaped domains show positive labeling for adipophilin (Fig. 9). However, the P-face of the ER membrane adjacent to the lipid droplet consistently reveals more intense labeling than does the P-face of the more distant partner membrane. This means that the most abundant labeling, in the form of prominent clusters, is restricted to the immediately apposing membrane leaflets of the outer surface of the lipid droplet and of the ER membrane area immediately adjacent to it. The E-faces of both ER membranes are always devoid of label.

Discussion

The obvious associations of lipid droplets, adipophilin clusters and ER membranes revealed in this study indicate that these features play important roles in lipid droplet biogenesis. The associations are ideally configured to function in lipid droplet growth, the transfer of lipids into the droplet, and the incorporation of lipid-droplet-associated proteins into lipid droplets. Whether they are also involved in the initial formation of lipid droplets has to be ascertained.

The prevailing hypothesis of lipid droplet biogenesis proposes that neutral lipids accumulate between monolayers of the ER membrane bilayer. As the droplet grows, the ER membrane progressively distends, a process culminating in the budding of the droplet into the cytoplasm surrounded by an envelope of the former cytoplasmic leaflet of the ER membrane. Paradoxically, lipid droplet budding from the ER has never been unequivocally observed. Lipid droplet-associated proteins are assumed to originate largely from proteins initially resident in this ER-derived monolayer (Brown, 2001), although some such proteins, notably including adipophilin, are envisaged as cytosolic shuttling proteins (Londos et al., 1999). The proposed mechanism accounts for initial formation and enlargement of the lipid droplet, but not for the incorporation of adipophilin into the droplet envelope. Despite their wide acceptance, these ideas are indeed more hypothetical than based on firm evidence.

Our present findings represent a significant challenge to the prevailing hypothesis of lipid droplet biogenesis. Freeze-fracture images disclose that whereas lipid droplets and ER membranes are often seen in close association, lipid droplets

always lie adjacent and apposed to paired membranes of the ER; lipid accumulations are never seen within ER membranes. The ER membranes partially enclose the lipid droplet in a manner akin to an egg cup holding an egg. It is important to

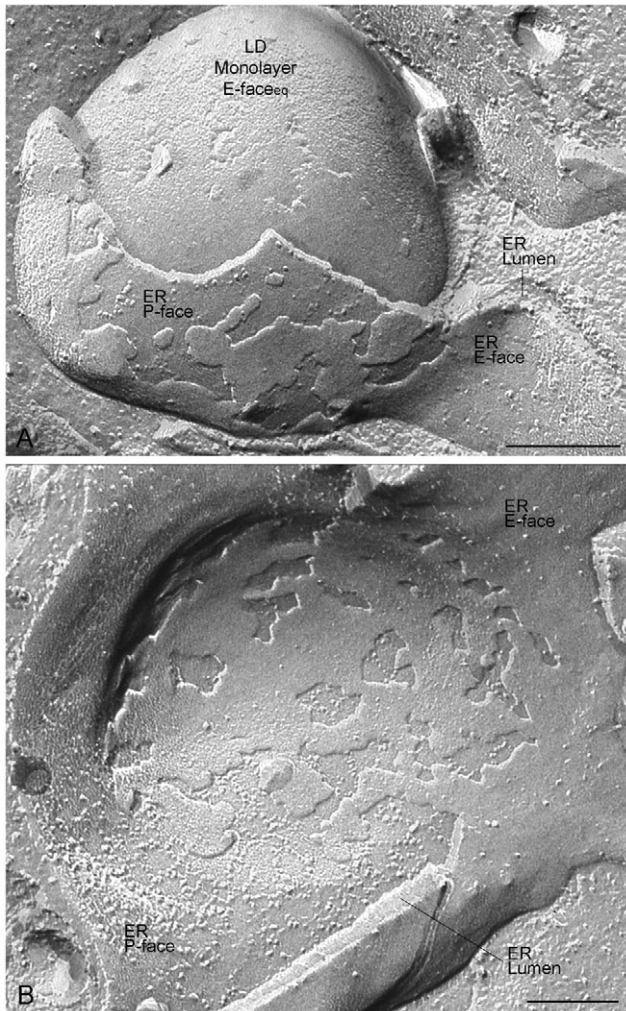


Fig. 7. Freeze-fracture views of lipid droplet-ER membrane associations in lipid-laden macrophages. (A) Lipid droplet (LD) situated in a cup formed from ER membranes. The lipid droplet has been convexly fractured to reveal the E-face equivalent (eq) of its outermost monolayer. Portions of the P-face and the E-face of the ER membranes are revealed; in this instance, the membrane adjacent to the lipid droplet is seen in P-face view, and portions of the partner ER membrane in E-face view. Note that both ER membranes constitute the cup that partially envelops the lipid droplet. The lipid droplet extends beneath both the ER membranes at the bottom of the field. As the ER membranes extend from the area of association with the lipid droplet, the lumen of the ER becomes larger. (B) 'Bird's-eye' view into an ER cup at a site of lipid droplet association. The fracture plane has followed the plane of the ER membranes within a cup, making a concave fracture, and scooping away the lipid droplet so that only the ER membranes remain. Portions of the E-face of the ER membrane closest to the lipid droplet, and of the P-face of the partner membrane distant, are revealed. This image again confirms that at the site of lipid-droplet association, both ER membranes participate in the cup, with the lipid droplet itself lying close to but external to them. As in A, the two membranes of the ER are much closer together at the site of apposition to the lipid droplet. Bars, 0.2 μm .

note that we found adipophilin concentrated in clusters in the cytoplasmic leaflet of the ER lining the inside of the ER cup, immediately adjacent to the lipid droplet envelope, in which high concentrations of adipophilin are likewise found. The pertinent features are shown diagrammatically in Fig. 11.

It is widely held that adipophilin and other PAT family proteins occur exclusively in lipid droplets and not in any other subcellular compartment (Londos et al., 1999; Miura et al., 2002). The present findings refute this view by demonstrating prominent labeling of adipophilin in the membranes of the ER.

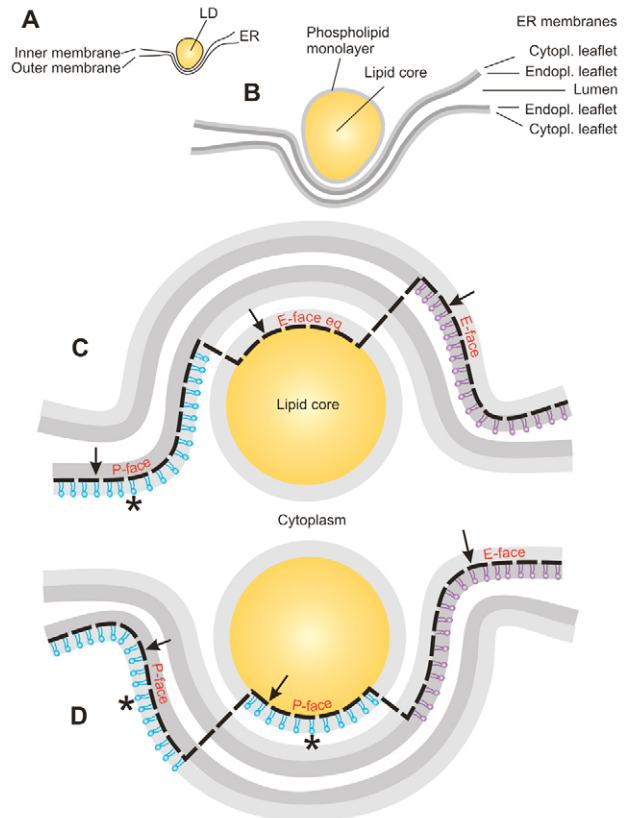
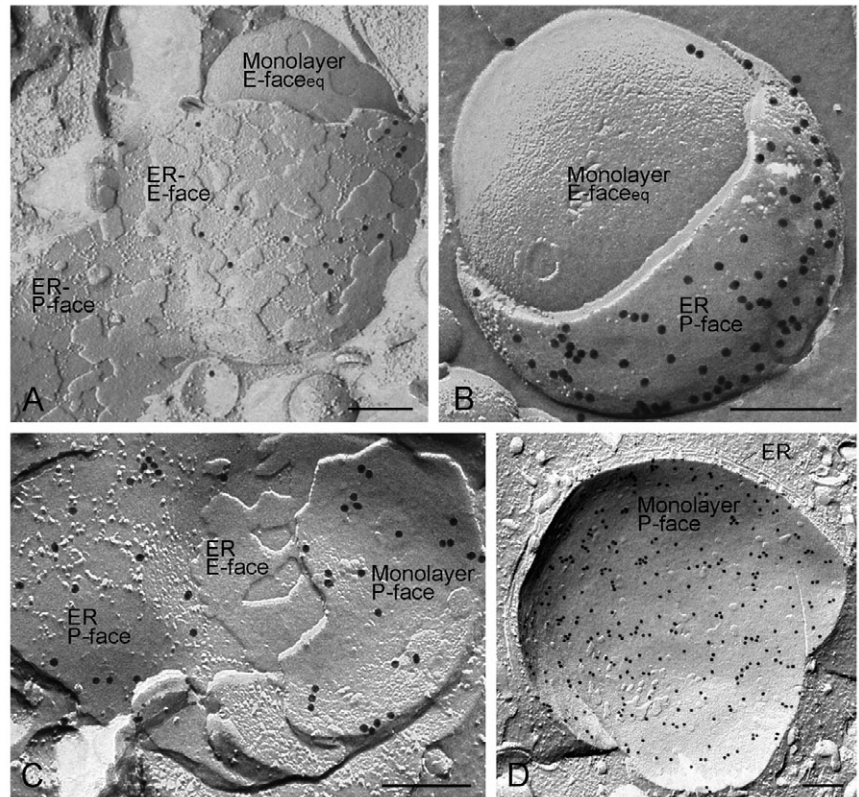


Fig. 8. Schematic diagram of fracture planes through ER membranes and lipid droplets. (A) In the electron microscope lipid droplets (LD) are frequently seen cupped by the ER. (B) One ER membrane lies adjacent to the droplet whereas the other membrane is farther removed. At the molecular level both membranes are comprised of cytoplasmic and endoplasmic leaflets of phospholipids, and the lumen of the ER intervenes between the two membranes. The lipid droplet core is enveloped by a phospholipid monolayer. (C) Upon fracturing between membrane leaflets as indicated by dashed lines, P-faces of the inner ER membrane, E-faces of outer ER membrane and the E-face equivalent of the lipid droplet core are revealed together with convexly fractured droplets. (D) Concave (E-face_{eq}) fractures of droplets, by contrast, are accompanied by E-faces of the inner ER membrane, P-faces of the outer ER membrane and the P-face of the lipid droplet monolayer. Phospholipid molecules (and immunoreactive integral membrane proteins, not shown) remaining on the replicas after SDS washing are depicted in blue (P-face) and purple (E-face). Gray areas represent residual membrane monolayers removed by SDS washing. Asterisks represent the specific locations of gold particles in the replicas marking specific membrane proteins following immunogold labeling. Arrows indicate the direction of viewing of the replicas in the electron microscope.

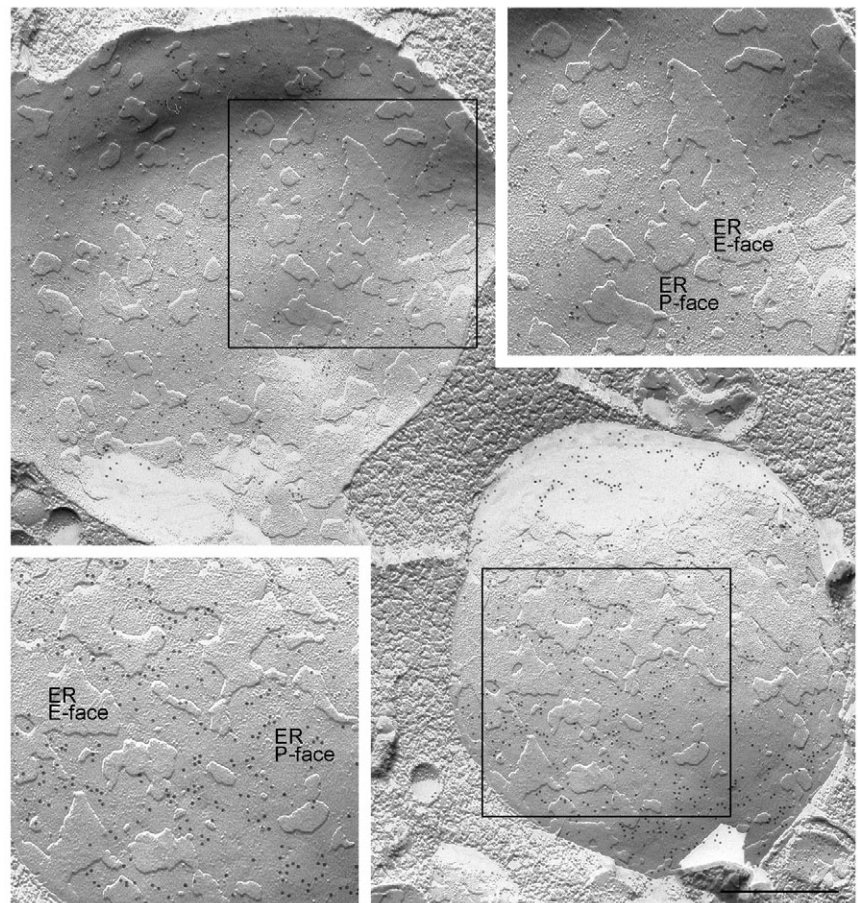
Fig. 9. Freeze-fracture views of lipid droplet-ER membrane associations after immunogold labeling (18 nm gold) for adipophilin in lipid-laden macrophages. (A,B) In convex fractures of lipid droplets and associated ER membranes, prominent labeling for adipophilin is visible on the P-face of the ER membrane adjacent to the lipid droplet, whereas the partner membrane, seen in E-face view in A is devoid of label. The view of the lipid droplet is the E-face equivalent (eq) of the monolayer; this aspect is devoid of label. (C,D) Concave fractures of the lipid droplet revealing the P-face of its outermost monolayer show prominent adipophilin labeling. The E-face of the ER membrane immediately adjacent to the lipid droplet is unlabeled, whereas the P-face of the partner ER membrane shows substantial labeling. In D, the ER cup associated with the lipid droplet has been cross-fractured. Bars, 0.2 μm .



Localization of adipophilin to ER membranes is of particular significance given that foci within the ER are hypothesized to be the sites of lipid droplet formation. Our findings indicate clearly that adipophilin is involved at least in the growth if not in the initial formation of lipid droplets.

Despite the well-recognized ubiquitous association of adipophilin with lipid droplets, the role of this protein in lipid droplet biogenesis is unknown. Adipophilin is considered to be an efficient transporter of free fatty acids (Gao and Serrero, 1999; Serrero et al., 2000; Atshaves et al., 2001), although it is also reported to bind cholesterol with high enough affinity to displace fatty acids (Atshaves et al., 2001). Proposed roles for adipophilin include neutral lipid packaging within lipid droplets (Brasaemle et al., 1997) and shuttling of lipid substrates to lipid droplets (Gao and Serrero, 1997). Clusters of adipophilin in the cytoplasmic leaflet of the ER membrane next to the lipid droplet, as demonstrated in

Fig. 10. Adipophilin immunogold labeling (12 nm gold) in side-by-side convex and concave fractures of ER cups. The example at the top left of the field (magnified at top right) has been concavely fractured, showing the E-face of the ER membrane adjacent to the lipid droplet and the P-face of its partner membrane. The example at the bottom right (magnified at bottom left) has been convexly fractured to reveal the P-face of the membrane closest to the lipid droplet and the E-face of its partner membrane. No labeling is observed on either E-face view. The P-face of the ER membrane closest to the lipid droplet (lower example) is more heavily labeled than that of the partner membrane (upper example). Bar, 0.5 μm .



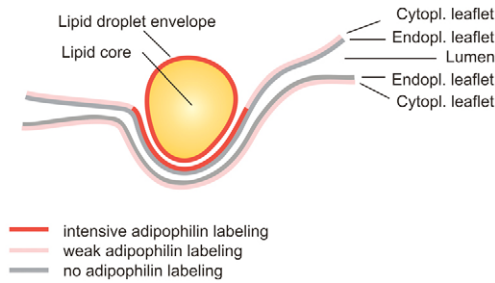


Fig. 11. Summary of principal features of putative sites of lipid droplet biogenesis in ER. Lipid droplet biogenesis takes place at specialized cup-shaped regions of the ER. The lipid droplet is cupped by closely apposed ER membrane outside the ER. Adipophilin (dark red) is highly concentrated in the cytoplasmic leaflet of the ER membrane of the cup immediately adjacent to the lipid droplet, and in the envelope of the lipid droplet. Lower concentrations of adipophilin (pink) are found in the cytoplasmic leaflets of ER membranes, but not in endoplasmic leaflets (gray).

the present study, are thus ideally situated to perform these functions. Furthermore, so situated, adipophilin could be transferred to the growing lipid droplet envelope from the ER. This is more a plausible route than cytoplasmic shuttling of this protein proposed by others (Londos et al., 1999). Adipophilin in the clusters and in the lipid droplet envelope would also be in an ideal position to interact directly with known ER-membrane-resident mediators of lipid metabolism like the isoenzymes of acyl-coenzyme A: cholesterol acyltransferase (ACAT). Chang et al. (Chang et al., 2001) have postulated recently that the catalytic site for ACAT may reside within the ER lipid bilayer and that cholesterol esters may burst from there directly into lipid droplets with an envelope of phospholipids. Thus, the necessity for lipid accumulation and assembly within the membrane bilayer of the ER for lipid droplet formation and enlargement would be obviated. Once the lipid droplet reaches a critical mass, it would be released from the ER cup. If the structural features we have identified also represent sites of lipid droplet origin as well as enlargement, formation of the droplet envelope from a budded-off cytoplasmic leaflet of the ER membrane would be precluded.

Our present findings on lipid droplet formation in the ER are remarkably similar to features we recently identified in the plasma membrane of cells subjected to lipid loading (Robenek et al., 2005c). Using freeze-fracture immunocytochemistry, we demonstrated that adipophilin and other PAT family proteins are dispersed in the plasma membrane of macrophages and adipocytes under normal culture conditions. However, when the cells are stimulated to accumulate large amounts of lipid, a dramatic reorganization of these proteins is seen; they aggregate to form prominent clusters in regions of the plasma membrane to which lipid droplets are juxtaposed. Combining these observations with our present findings suggests that a similar mechanism is involved in lipid transfer into the droplet at the ER and at the plasma membrane, the latter perhaps coming into operation only as a reserve mechanism under conditions of extreme lipid accumulation.

Our findings on the plasma membrane areas apposed to lipid droplets help explain some of the western blot data on the

fractions isolated from lipid-laden cells. The tight association between the plasma membrane areas enriched in adipophilin and underlying lipid droplets, manifested by plasma membrane apposition and bulging at the lipid droplet, would be expected to lead to significant numbers of droplets being isolated with attached plasma membrane domains. Accordingly, the plasma membrane marker, flotillin-2, becomes detectable in the lipid droplet fraction, as we observed. A further implication of this situation would be an overestimate of the amount of adipophilin in the lipid droplet fraction and an underestimate of adipophilin in the plasma membrane, thus explaining the apparent lack of adipophilin in sedimentable membranes (Londos et al., 1999; Diaz and Pfeffer, 1998).

Finally, the observation that adipophilin is associated with the cytoplasmic leaflet of the outer nuclear membrane and, in addition with the cytoplasmic leaflet of the ER membrane, may be explained in relation to the well documented connections between the two membrane systems. The rough ER is connected to and physically continuous with the outer nuclear membrane, and so those nuclear and ER membrane proteins that are free to diffuse in the plane of the membranes intermix in the two membrane systems. Thus, when adipophilin is present in ER, its detection in the nuclear membrane is not surprising. Although the inner and outer nuclear membranes are in continuity at the margins of nuclear pores, the nuclear pore complexes probably present a barrier to further lateral mobility, thus explaining the absence of adipophilin in P-face views of the inner nuclear membrane. The presence of adipophilin in the outer nuclear membrane suggests that this site might also have the potential to participate in lipid droplet formation or growth in the same manner as the ER. However, no lipid droplets have yet been seen opposite adipophilin clusters in the outer nuclear membrane, indicating that additional proteins, which are locally restrained in the ER, are probably involved in lipid droplet biogenesis.

In summary, the present observations suggest an entirely different mechanism of lipid droplet biogenesis to that currently held. This involves the elaboration of lipid droplets within ER cups, rather than between the leaflets of the ER membrane. Adipophilin clusters in the cytoplasmic leaflet of the ER adjacent to the forming lipid droplet appear to function in transferring lipids from the ER to the droplet surface. Incorporation of adipophilin into lipid droplets may also occur directly from the ER rather than from the cytoplasm. Contrary to the prevailing model, the envelope of the lipid droplet may not necessarily arise from a budded-off portion of the cytoplasmic leaflet of the ER membrane.

Materials and Methods

Antibodies

Adipophilin was immunolabeled using well-characterized mouse monoclonal antibody to a synthetic peptide representing the N-terminus (amino acids 5-27) of human adipophilin (AP125, Progen Biotechnik, Heidelberg, Germany) (Robenek et al., 2005b; Robenek et al., 2006). Irrelevant antibodies against Lamp-1, connexin43 and the inner nuclear membrane proteins LAP2 β and emerlin were used as controls.

Cell culture

Human THP-1 monocytes from the American Type Culture Collection (Manassas, VA) were cultured in suspension in RPMI 1640 medium containing the supplements recommended by Iwashima et al. (Iwashima et al., 2000), and differentiated to adherent macrophages by adding 100 μ M phorbol 12-myristate 13-acetate to the medium for 3 days. The cells were induced to accumulate lipid droplets by the

addition of 50–100 µg/ml acetylated low density lipoprotein (AcLDL) at day 2 for 24–48 hours as described by Hara et al. (Hara et al., 1987) and Gaus et al. (Gaus et al., 2004).

Isolation of lipid droplet and membrane proteins

Lipid-laden macrophages were digested by sonication in 200 mM phosphate-buffered saline (PBS) containing 0.25 M sucrose, 80 mM KCl, 5 mM 2-mercaptoethanol and protease inhibitor cocktail (Roche complete, Roche Diagnostics, Mannheim, Germany). Centrifugation of the cell lysate at 100,000 g for 1 hour resulted in a sedimented membrane pellet, a cytosolic fraction and a floating lipid fraction. The floating lipids were separated from the cytosolic fraction by cutting the centrifuge tube between the cytosolic and the lipid fraction. The proteins of the separated fractions were precipitated overnight with ice-cold acetone (1:6).

Western blotting

The proteins were solubilized in PBS and separated by SDS-PAGE. They were transferred for 1 hour and incubated with anti-adipophilin, anti-Flotillin-2 or anti-Calnexin antibodies for 1 hour. Subsequently, the membranes were washed in PBS containing 0.5% skimmed milk and 0.05% Tween 20 for 1 hour and incubated with peroxidase-conjugated secondary antibodies for 1 hour. Protein bands were detected using chemiluminescence reagents from Amersham Biosciences (Piscataway, USA).

Immunofluorescence light and confocal microscopy

Macrophages were cultured and loaded with lipids in chamber slides. They were rinsed with PBS and fixed in 4% paraformaldehyde at room temperature for 30 minutes. After extensive washing, the cells were incubated for 1 hour in PBS containing 1% bovine serum albumin (BSA) to block non-specific binding and 0.05% Tween 20 for permeabilization. Macrophages were immunolabeled with anti-adipophilin antibodies for 1 hour, followed by washing and incubation with anti-mouse Cy2-conjugated secondary antibodies (Dianova, Hamburg, Germany) for 1 hour at room temperature.

To visualize lipid droplets, the fluorophore BODIPY 493/503 (Invitrogen, Karlsruhe, Germany), which specifically stains neutral lipids (Goetze and Freeman, 1994), was dissolved in ethanol at 1 mg/ml and added to the secondary antibody solution to a final concentration of 20 µg/ml. Nuclei were stained with Hoechst 33528 dye (Sigma Aldrich, Hamburg, Germany). The preparations were mounted in fluorescent mounting medium (DakoCytomation, Hamburg, Germany) and examined in a fluorescence microscope or confocal laser-scanning microscope (Zeiss, Jena, Germany).

Thin-section electron microscopy

Lipid-laden macrophages were grown in flasks, fixed with 2% glutaraldehyde and 0.5% osmium tetroxide in PBS, and dehydrated with ethanol using standard procedures. A small volume of propylene oxide was added to the flasks, and the cells were removed by gentle swirling and embedded in Epon using standard procedures. Thin sections were cut using an ultramicrotome and contrasted with uranyl acetate and lead citrate.

Cryothen-section electron microscopy

Lipid-laden macrophages were fixed in 1% paraformaldehyde in PBS, scraped from the culture vessels, prepared further for cryoimmunoelectron microscopy basically as outlined by Tokuyasu (Tokuyasu, 1980) and immunogold labeled for adipophilin as described below.

Freeze-fracture replication

Unfixed cells were scraped from the culture vessels, centrifuged to remove excess medium, re-centrifuged briefly (30 seconds) in 30% glycerol, fixed in Freon 22 cooled with liquid nitrogen, and freeze-fractured in a BA310 freeze-fracture unit (Balzers AG, Balzers, Liechtenstein) at –100°C. Replicas of the fractured cells were immediately made by electron beam evaporation of platinum-carbon and carbon at angles of 38° and 90° and to thicknesses of 2 and 20 nm, respectively. The replicas were incubated overnight in 5% sodium dodecyl sulphate (SDS) to remove cellular material except for those molecules adhering directly to the replicas (Fujimoto, 1995). They were then washed in distilled water and incubated briefly in 5% BSA before immunolabeling.

Immunolabeling of freeze-fracture replicas and cryosections

Freeze-fracture replicas of macrophages were immunogold labeled with anti-adipophilin antibodies followed by a goat anti-mouse 12 or 18 nm gold conjugate (both conjugates from Jackson ImmunoResearch, West Grove, VA).

Cryothen sections of macrophages were immunolabeled with anti-adipophilin followed by a goat anti-mouse 12- or 18-nm gold conjugate. Control specimens, prepared without the primary antibodies, were essentially free of gold particles. Antibodies against Lamp-1, connexin43, LAP2β and emerin did not bind to replicas or cryosections of lipid droplets and ER in macrophages.

Electron microscopy

Examination of thin sections, immunogold-labeled cryothen sections, and immunogold-labeled freeze-fracture replicas was carried out using a Philips 410 transmission electron microscope. Observations on freeze-fracture immunogold replicas were based on examination of >200 cells from three separate experiments.

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References

- Atshaves, B. P., Storey, S. M., McIntosh, A. L., Petrescu, A. D., Lyuksyutova, O. I., Greenberg, A. S. and Schroeder, F. (2001). Sterol carrier protein-2 expression modulates protein and lipid composition of lipid droplets. *J. Biol. Chem.* **276**, 25324–25335.
- Branton, D., Bullivant, S., Gilula, N. B., Karnovsky, M. J., Moor, H., Mühletahler, K., Northcote, D. H., Packer, L., Satir, B., Satir, P. et al. (1975). Freeze-etching nomenclature. *Science* **190**, 54–56.
- Brasaemle, D. L., Barber, T., Wolins, N. E., Serrero, G., Blanchette-Mackie, E. J. and Londos, C. (1997). Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J. Lipid Res.* **38**, 2249–2263.
- Brown, D. A. (2001). Lipid droplets: proteins floating on a pool of fat. *Curr. Biol.* **11**, R446–R449.
- Chang, T. Y., Chang, C. C. Y., Lu, X. and Lin, S. (2001). Catalysis of ACAT may be completed within the plane of the membrane: a working hypothesis. *J. Lipid Res.* **42**, 1933–1938.
- Cole, N. B., Murphy, D. D., Grider, T., Rueter, S., Brasaemle, D. and Nussbaum, R. L. (2002). Lipid droplet binding and oligomerization properties of the Parkinson's disease protein α -synuclein. *J. Biol. Chem.* **277**, 6344–6352.
- Diaz, E. and Pfeffer, S. R. (1998). TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. *Cell* **93**, 433–443.
- Fujimoto, K. (1995). Freeze-fracture replica electron microscopy combined with SDS digestion for cytochemical labeling of integral membrane proteins. Application to the immunogold labeling of intercellular junctional complexes. *J. Cell Sci.* **108**, 3443–3449.
- Fujimoto, T., Kogo, H., Ishiguro, K., Tauchi, K. and Nomura, R. (2001). Caveolin-2 is targeted to lipid droplets, a new “membrane domain” in the cell. *J. Cell Biol.* **152**, 1079–1085.
- Fujimoto, Y., Itabe, H., Sakai, J., Makita, M., Noda, J., Mori, M., Higashi, Y., Kojima, S. and Takano, T. (2004). Identification of major proteins in the lipid droplet-enriched fraction isolated from the human hepatocyte cell line HuH7. *Biochim. Biophys. Acta* **1644**, 47–59.
- Gao, J. and Serrero, G. (1999). Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake. *J. Biol. Chem.* **274**, 16825–16830.
- Gaus, K., Kritharides, L., Schmitz, G., Boettcher, A., Drobnik, W., Langmann, T., Quinn, C. M., Death, A., Dean, R. T. and Jessup, W. (2004). Apolipoprotein A-1 interaction with plasma membrane lipid rafts controls cholesterol export from macrophages. *FASEB J.* **18**, 574–576.
- Goetze, P. M. and Freeman, D. A. (1994). Factors underlying the variability of lipid droplet fluorescence in MA-10 Leydig tumor cells. *Cytometry* **17**, 151–158.
- Hara, H., Tanishita, H., Yokoyama, S., Tajima, S. and Yamamoto, A. (1987). Induction of acetylated low density lipoprotein receptor and suppression of low density lipoprotein receptor on the cells of human monocytic leukemia cell line (THP-1 cell). *Biochem. Biophys. Res. Commun.* **146**, 802–808.
- Heid, H. W., Schnölzer, M. and Keenan, T. W. (1996). Adipocyte differentiation-related protein is secreted into milk as a constituent of milk lipid globule membrane. *Biochem. J.* **320**, 1025–1030.
- Iwashima, Y., Eto, M., Hata, A., Kaku, K., Horiuchi, S., Ushikubi, F. and Sano, H. (2000). Advanced glycation end products-induced gene expression of scavenger receptors in cultured human monocyte-derived macrophages. *Biochem. Biophys. Res. Commun.* **277**, 368–380.
- Larigauderie, G., Furman, C., Jaye, M., Lasselin, C., Copin, C., Fruchart, J. C., Castro, G. and Rouis, M. (2004). Adipophilin enhances lipid accumulation and prevents lipid efflux from THP-1 macrophages: potential role in atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **24**, 504–510.
- Liu, P., Ying, Y., Zaho, Y., Mundy, D. I., Zhu, M. and Anderson, R. G. W. (2003). CHO K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. *J. Biol. Chem.* **279**, 3787–3792.
- Londos, C., Brasaemle, D. L., Schultz, C. J., Segrest, J. P. and Kimmel, A. R. (1999). Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin. Cell Dev. Biol.* **10**, 51–58.
- Martinez-Botas, J., Anderson, J. B., Tessier, D., Laipillone, A., Chang, B. H., Quast, M. J., Gorenstein, D., Chen, K. H. and Chan, L. (2000). Absence of perilipin results in leanness and reverses obesity in Lep(db/db) mice. *Nat. Genet.* **26**, 476–479.
- McManaman, J. L., Zabaronick, W., Schaack, J. and Orlicky, D. J. (2003). Lipid droplet targeting domains of adipophilin. *J. Lipid Res.* **44**, 668–673.
- Mishra, R., Eminacipator, S. N., Miller, C., Kern, T. and Simonson, M. S. (2004).

- Adipose differentiation related protein and regulators of lipid homeostasis identified by gene expression profiling in murine db/db diabetic kidney. *Am. J. Physiol. Renal Physiol.* **286**, F913-F921.
- Miura, S., Gan, J. W., Brzostowski, J., Parisi, M. J., Schultz, C. J., Londos, C., Oliver, B. and Kimmel, A. R.** (2002). Functional conservation for lipid storage droplet association among perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, *Drosophila*, and *Dictyostelium*. *J. Biol. Chem.* **277**, 32253-32257.
- Murphy, D. J.** (2001). The biogenesis and function of lipid bodies in animals, plants and microorganisms. *Prog. Lipid Res.* **40**, 325-438.
- Ohashi, M., Mizushima, N., Kabeya, Y. and Yoshimori, T.** (2003). Localization of mammalian NAD(P)H steroid dehydrogenase-like protein on lipid droplets. *J. Biol. Chem.* **278**, 36819-36929.
- Ostermeyer, A. G., Ramchasan, L. T., Zeug, Y., Lublin, D. M. and Brown, D. A.** (2004). Role of the hydrophobic domain in targeting caveolin-1 to lipid droplets. *J. Cell Biol.* **164**, 69-78.
- Ozeki, S., Cheng, J., Tauchi-Sato, K., Hatano, N., Taniguchi, H. and Fujimoto, T.** (2005). Rab18 localizes to lipid droplets and induces their close apposition to the endoplasmic reticulum-derived membrane. *J. Cell Sci.* **118**, 2601-2611.
- Pol, A., Martin, S., Fernandez, M. A., Ferguson, C., Carozzi, A., Luetterforst, R., Enrich, C. and Parton, R. G.** (2004). Dynamic and regulated association of caveolin with lipid bodies: modulation of lipid body motility and function by a dominant negative mutant. *Mol. Biol. Cell* **15**, 99-110.
- Robenek, M. J., Schlattmann, K., Zimmer, K.-P., Plenz, G., Troyer, D. and Robenek, H.** (2003). Cholesterol transporter caveolin-1 transits the lipid bilayer during intracellular cycling. *FASEB J.* **17**, 1940-1942.
- Robenek, M. J., Schlattmann, K., Zimmer, K.-P., Plenz, G., Troyer, D. and Robenek, H.** (2004). Lipids partition caveolin-1 from ER membranes into lipid droplets: updating the model of lipid droplet biogenesis. *FASEB J.* **18**, 866-868.
- Robenek, H., Robenek, M. J. and Troyer, D.** (2005a). PAT family proteins pervade lipid droplet cores. *J. Lipid Res.* **46**, 1331-1338.
- Robenek, H., Lorkowski, S., Schnoor, M. and Troyer, D.** (2005b). Spatial integration of TIP47 and adipophilin in macrophage lipid bodies. *J. Biol. Chem.* **280**, 5789-5794.
- Robenek, H., Robenek, M. J., Buers, I., Lorkowski, S., Hofnagel, O., Troyer, D. and Severs, N. J.** (2005c). Lipid droplets gain their PATs by interaction with specialized plasma membrane domains. *J. Biol. Chem.* **280**, 26330-26338.
- Robenek, H., Hofnagel, O., Buers, I., Lorkowski, S., Schnoor, M., Robenek, M. J., Heid, H., Troyer, D. and Severs, N. J.** (2006). Butyrophilin controls milk fat globule secretion. *Proc. Natl. Acad. Sci. USA* **103**, 10385-10390.
- Serrero, G., Frolov, R., Schroeder, F., Tanaka, K. and Gehlaar, L.** (2000). Adipose differentiation related protein: expression, purification of recombinant protein in *Escherichia coli* and characterization of its fatty acid binding properties. *Biochim. Biophys. Acta* **1488**, 245-254.
- Tauchi-Sato, K., Ozeki, S., Houjou, T., Taguchi, R. and Fujimoto, T.** (2002). The surface of lipid droplets is a phospholipid monolayer with a unique fatty acid composition. *J. Biol. Chem.* **277**, 44507-44512.
- Tokuyasu, K. T.** (1980). Immunocytochemistry on ultrathin frozen sections. *Histochem. J.* **12**, 381-403.
- Umlauf, E., Csaszar, E., Moertelmaier, M., Schuetz, G., Parton, R. G. and Prohaska, R.** (2004). Association of stomatin with lipid droplets. *J. Biol. Chem.* **279**, 23699-23709.
- Wang, X., Reape, T. J., Li, X., Rayner, K., Webb, C. L., Burnand, K. G. and Lyso, P. G.** (1999). Induced expression of adipophilin mRNA in human macrophages stimulated with oxidized low-density lipoprotein in atherosclerotic lesions. *FEBS Lett.* **462**, 445-450.
- Wolins, N. E., Rubin, B. and Brasaemle, D. L.** (2001). TIP47 associates with lipid droplets. *J. Biol. Chem.* **276**, 5101-5108.