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Understanding the distinct subcellular trafficking of CD36 and GLUT4 during the development of myocardial insulin resistance

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A B S T R A C T

CD36 and GLUT4 are the main cardiac trans-sarcolemmal transporters for long-chain fatty acids and glucose, respectively. Together they secure the majority of cardiac energy demands. Moreover, these transporters each represent key governing kinetic steps in cardiac fatty acid and glucose fluxes, thereby offering major sites of regulation. The underlying mechanism of this regulation involves a perpetual vesicle-mediated trafficking (recycling) of both transporters between intracellular stores (endosomes) and the cell surface. In the healthy heart, CD36 and GLUT4 translocation to the cell surface is under short-term control of the same physiological stimuli, most notably increased contraction and insulin secretion. However, under chronic lipid overload, a condition that accompanies a Western lifestyle, CD36 and GLUT4 recycling are affected distinctly, with CD36 being expelled to the sarcolemma while GLUT4 is imprisoned within the endosomes. Moreover, the increased CD36 translocation towards the cell surface is a key early step, setting the heart on a route towards insulin resistance and subsequent contractile dysfunction. Therefore, the proteins making up the trafficking machinery of CD36 need to be identified with special focus to the differences with the protein composition of the GLUT4 trafficking machinery. These proteins that are uniquely dedicated to either CD36 or GLUT4 traffic may offer targets to rectify aberrant substrate uptake seen in the lipid-overloaded heart. Specifically, CD36-dedicated trafficking regulators should be inhibited, whereas such GLUT4-dedicated proteins would need to be activated. Recent advances in the identification of CD36-dedicated trafficking proteins have disclosed the involvement of vacuolar-type H$^+$-ATPase and of specific vesicle-associated membrane proteins (VAMPs). In this review, we summarize these recent findings and sketch a roadmap of CD36 and GLUT4 trafficking compatible with experimental findings.

1. Introduction

Overconsumption of energy-rich or fatty foods predisposes to a high risk of developing insulin resistance and eventually progresses towards type-2 diabetes in many subjects. Cardiovascular disease, including myocardial infarction and heart failure, is the most common cause of death among diabetic patients. The cardiac dysfunction that develops as a result of intramyocardial accumulation of lipid metabolites (diacylglycerols and/or ceramides) follows an increasingly understood pattern of changes including insulin resistance, decreased glucose uptake and structural remodeling in both humans and rodent models of lipid overfeeding [1,2]. At present it is recognized that changes in myocellular fatty acid uptake underly the progressive lipid accumulation in the heart, which then triggers further pathophysiological changes [3].

In the healthy heart, long-chain fatty acids (hereafter referred to as fatty acids) and glucose are the major energy substrates required to maintain contractile function. Both substrates first are transported

across the sarcolemma by facilitated diffusion, and then rapidly converted into 'activated metabolites' (acyl-coenzyme A and glucose-6-phosphate, respectively), resulting in their intracellular trapping so as to retain a steep substrate gradient, which is the driving force for substrate uptake. Depending on the metabolic state of the heart, fatty acids and glucose can be stored intracellularly but only to a limited extent, so that the bulk will be destined immediately for mitochondrial oxidation and ATP generation to sustain contractile activity. There are several control points in the metabolism of both substrates. For fatty acids, entry of fatty acyl-CoA into the mitochondria via carnitine-palmitoyltransferase-1 as part of the carnitine shuttle is a key regulatory site [4]. For glucose, the regulatory sites are phosphofructokinase (control of glycolysis) and pyruvate dehydrogenase (control of glucose oxidation) [4]. Yet, for both substrates their transsarcolemmal uptake has been indicated to exert most of the flux control [5,6]. For instance, at high extracellular substrate concentration at which the uptake is saturated, intracellular concentrations are retained at a low level, indicating that saturation of uptake is not due to saturation of metabolism [5,7]. Hence, for both substrates the cellular uptake process is a major rate-governing step, implicating that this step is strictly regulated. Below, we will briefly describe for both substrates how the uptake process is regulated (Sections 1.1 and 1.2) and how this regulation is altered during oversupply of lipids to the heart (Section 1.3) Thereafter, we will integrate this knowledge to formulate the aims of this review in relation to construction the roadmap of CD36 and GLUT4 trafficking (Section 1.4).

1.1. Regulation of glucose uptake

Uptake of glucose into the heart is mediated by GLUT1 and GLUT4, whereby GLUT1 is responsible for most of the basal uptake and GLUT4 for most of stimulus-inducible glucose uptake [8]. Concurrently, GLUT1 is mainly localized to the sarcolemma, while GLUT4 is mainly stored in intracellular compartments such as endosomes. Exposure of cardiac cells to appropriate stimuli then results in translocation of GLUT4 to the sarcolemma. It needs to be stressed that the term GLUT4 translocation reflects the net result of a change in balance between GLUT4 exocytosis and GLUT4 endocytosis. Both processes are constantly occurring, and most of the research supports the notion that GLUT4 endocytosis is relatively constant and slow, whereas GLUT4 exocytosis is highly variable: low under basal conditions and inducible by stimuli [9,10]. Moreover, exocytosis is a rapid process with changes occurring on a time scale of minutes after the onset of stimulation, and involves excision of transport vesicles from these intracellular GLUT4 compartments. On the other hand, alternative views have been described implicating decreased GLUT4 endocytosis as an important regulator of GLUT4 translocation [11]. However, for the remainder of this review we stay away from this controversy and will use the term translocation to indicate the net trafficking of GLUT4 to the sarcolemma. In heart, the main physiological stimuli inducing GLUT4 translocation are hormonal and mechanical triggers, i.e., an increase in circulating levels of insulin and an increase in contractile activity [8,12,13]. Both stimuli activate distinct signaling pathways to induce GLUT4 translocation, but converge at the level of Rab GTPase-activating proteins (RabGAPs) via Ser/Thr phosphorylations. The main RabGAPs involved in GLUT4 translocation are AS160 and TBC1D1, which are inhibited upon Ser/Thr phosphorylation leading to subsequent de-inhibition of Rab proteins [14]. Rab proteins are essential drivers of vesicular trafficking of GLUT4 via GTP/GDP cycling. Fig. 1 depicts the specific pathways for insulin and contraction-stimulated GLUT4 translocation.

1.2. Regulation of fatty acid uptake

Myocardial uptake of fatty acids has long been considered to be mediated by passive diffusion, but kinetic and inhibitor studies showed that passive diffusion only contributes to a minor extent to fatty acid

uptake [15]. The bulk of fatty acid uptake is mediated by the fatty acid transporters CD36 (officially designated SR-B2; [16]) and plasma membrane-localized fatty acid binding protein (FABPpm), which operate in a coordinated fashion [17]. FABPpm is a peripheral membrane protein localized at the extracellular leaflet, and may scavenge fatty acids from the extracellular medium to be transferred to CD36, which then governs the transsarcolemmal transport, likely via facilitating flip-flop of fatty acids across the lipid bilayer [18]. In addition, localization of CD36 to lipid rafts and binding to caveolins may further optimize cellular fatty acid uptake [19]. Similarly to GLUT4, a large portion of CD36 (about half of the total pool) is stored in intracellular compartments, while the remainder is present at the sarcolemma to mediate basal uptake. Evidence from subcellular fractionation experiments and immunohistochemistry pinpointed the (recycling) endosomes as intracellular compartment for CD36 [20,21]. Hence, the subcellular CD36 localization partially overlaps with that of GLUT4 (see previous section and Section 2.6). The similarity between CD36 and GLUT4 trafficking even goes further. The same physiological stimuli inducing vesicle-mediated GLUT4 translocation, i.e., insulin and contraction, also induce vesicle-mediated CD36 translocation [6]. Still there are subtle differences in the signaling pathways needed for CD36 translocation when compared to GLUT4 translocation (Fig. 1). Subsequently, the newly arrived CD36 at the sarcolemma partners up with FABPpm. In contrast to CD36, FABPpm does not recycle in the heart and is permanently present at the sarcolemma, waiting for CD36 to arrive from its intracellular location [7,18]. Upon termination of the stimuli, GLUT4 and CD36 are re-internalized via vesicle-mediated transport to their storage compartments, whereas FABPpm remains at the cell surface.

1.3. The heart during lipid overload

How does lipid overconsumption impact on myocellular fatty acid uptake? As mentioned above (Section 1), the rate of myocellular fatty acid uptake is determined by the fatty acid gradient across the sarcolemma, with low intracellular fatty acid concentrations maintained due to their rapid enzymatic conversion by long-chain acyl-CoA synthetase (ACSL) into acyl-CoA. When the fatty acid concentration in the circulation rises, such as upon increased lipid consumption, the fatty acid gradient increases because the ACSL capacity is amply sufficient to maintain a low intracellular fatty acid concentration [6,22]. As a result, fatty acid uptake increases, which is enabled mainly through increased flux through CD36. In line with this, cardiomyocytes pretreated with CD36-blocking antibodies [23] as well as hearts from CD36 knockout mice [24,25] are protected from lipid overload in vitro and in vivo, respectively, further demonstrating CD36 as being a key player in increased fatty acid uptake in lipid overload conditions.

How does increased myocellular fatty acid uptake affect cardiac function? The consequence of chronically increased fatty acid uptake is an increase in fatty acid oxidation and a progressive accumulation of fatty acid metabolites. Increased fatty acid oxidation leads to increased levels of acetyl-CoA and of protein acetylation. On its turn, protein acetylation inhibits insulin signaling at multiple levels as well as insulin-stimulated GLUT4 translocation via unknown mechanisms [26]. The fatty acid metabolites are mostly made up of triacylglycerols stored in lipid droplets. However, there is also an increase in intracellular concentrations of less inert lipid metabolites such as diacylglycerols and ceramides. These metabolites induce insulin resistance via inhibition of insulin signaling at distinct steps [27]. Besides induction of insulin resistance, the lipid accumulation leads to other lipotoxic effects, such as lipid-induced apoptosis [28]. Ultimately, the pleiotropic actions of lipotoxicity in the heart contribute for a significant portion of clinical heart failure [29]. In conclusion, increased fatty acid flux via CD36 is a key early process involved in the development of lipid-induced cardiomyopathy.

One important remark needs to be made regarding the mechanism of CD36-mediated myocellular lipid accumulation. Chronical
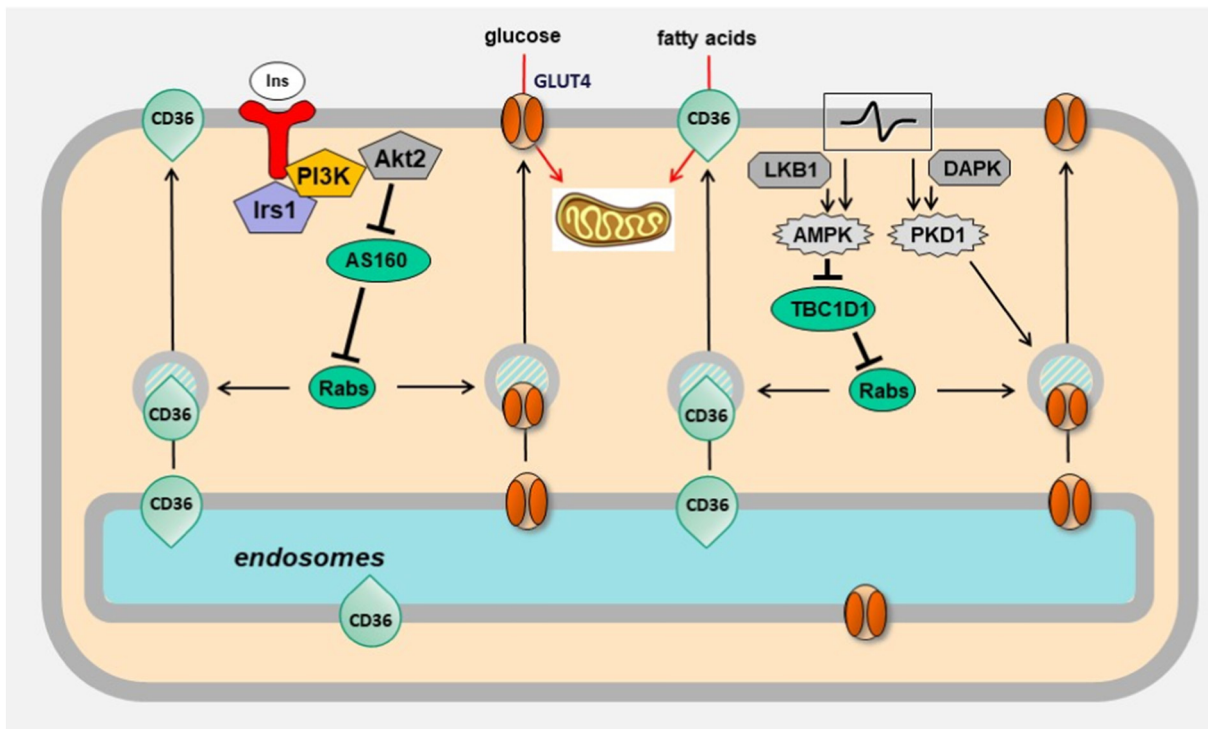
Fig. 1. Signaling mechanisms involved in translocation of CD36 and GLUT4 in cardiomyocytes under non-pathological conditions. The main physiological stimuli for CD36 and GLUT4 translocation from endosomes to the cell surface are increased circulatory insulin levels and an increase in contractile activity (indicated by a biphasic electrical pulse). Insulin binding to its receptor activates insulin receptor substrate-1 (IRS1), phosphatidylinositol-3 kinase (PI3K) and Akt2 in sequential order, which is known as the canonical insulin signaling pathway. Contraction induces activation of AMP-activated protein kinase (AMPK) and protein kinase-D1 (PKD1) in separate pathways. Full activation of AMPK and PKD1 additionally requires phosphorylation by LKB1 and death-activated protein kinase (DAPK), respectively. Contraction-induced CD36 translocation is mediated by AMPK activation, and contraction-induced GLUT4 translocation additionally requires PKD1 activation. For review, see [92]. Activation of Akt2 and AMPK leads to an inhibitory phosphorylation of the RabGAP proteins AS160 and TBC1D1. Please note that the involvement of AS160 in insulin signaling and of TBC1D1 in contraction signaling is an oversimplification as both RabGAPS might also contribute to regulation of the respective other signaling pathway. For an excellent review on this topic, see [85]. Deactivation of both RabGAPs results in liberation of the activity of several Rabs. A number of Rabs is involved in GLUT4 translocation [12], and their involvement has also been proven in CD36 translocation [91]. Finally, upon termination of the stimuli, both GLUT4 and CD36 return to the endosomes.

overexposure to lipids not only increases the entracellular-to-intracellular fatty acid gradient, but also elevates the presence of CD36 at the sarcolemma, as seen both in vitro and in vivo [22,23]. This combined impact of lipid overload on the transmembrane fatty acid gradient and CD36 transport capacity is part of a lipid-mediated feedforward cycle underlying excessive myocelular lipid accumulation, which will be further detailed in Section 2.5. The increased sarcolemmal presence of CD36 in lipid-overloaded hearts is the result of a CD36 translocation from endosomes to the sarcolemma rather than changes in CD36 expression [22,23]. Accordingly, in hearts from rats fed a high fat diet [22], from obese Zucker rats [30] or from diabetic db/db mice [31], the endosomal pool of CD36 becomes depleted with a concomitant increase in the sarcolemmal pool. In contrast, in the lipid-overloaded heart GLUT4 subcellular distribution shows a completely opposite picture, i.e., with decreased sarcolemmal abundance and concomitantly increased presence in intracellular membrane compartments [6,22]. Hence, also the GLUT4 translocation machinery undergoes alterations leading to the intracellular imprisonment of GLUT4 in the lipid-overloaded heart. Importantly, when the time course of lipid-induced metabolic alterations was studied in detail in palmitate-overexposed cardiomyocytes, the changes in CD36 traffic were already visible within 1 h, and preceded the changes in GLUT4 traffic by > 10 h, further indicating changes in CD36 translocation as a key early event in lipid-induced cardiomyopathy [32].

1.4. Purpose of review

Whereas the GLUT4 trafficking machinery has been the subject of intensive investigations [33], the CD36 trafficking machinery is relatively under-investigated. Our current understanding is merely that CD36 and GLUT4 traffic share the involvement of endosomes as intracellular storage compartment and the involvement of members of the VAMP family for mediating the translocation upon different stimuli [34]. An important feature of the endosomes is their intraluminal acidification, which is maintained by the endosomal/lysosomal proton pump vacuolar-type H^+-ATPase (v-ATPase). Recent research revealed that v-ATPase is a key regulator of both subcellular CD36 and GLUT4 localization [32,35]. In this review, we aim to integrate the novel literature evidence on the role of v-ATPase in the subcellular localization of CD36 and GLUT4 together with the existing knowledge on the role of VAMP members in their subcellular recycling. This leads us to the drafting of a first roadmap of CD36 traffic with specific focus on the similarities and differences with GLUT4 traffic. Such roadmap may provide novel insights and could initiate the design of novel therapies to redirect cardiac substrate uptake in the lipid-overloaded heart towards increased glucose use. For this purpose, we will first highlight the mechanistic role of v-ATPase in CD36 and GLUT4 traffic (Section 2), then we present the published data on the role of VAMP members in CD36 and GLUT4 traffic (Section 3), after which we will integrate v-ATPase and VAMPs into a roadmap of CD36 traffic (Section 4). Finally, based on this roadmap, we will discuss possible strategies to combat lipid-induced cardiomyopathy (Section 5).

2. v-ATPase regulates CD36 and GLUT4 traffic

Given that changes in CD36 traffic precede changes in GLUT4 traffic upon the onset of lipid overload, we start out with discussing CD36 followed by GLUT4. The mechanism by which lipids (especially palmitate) increase CD36 translocation is incompletely understood, but recently it became evident that excess lipids interfere with one of the hallmark properties of endosomes, i.e., maintaining intra-endosomal acidification. More specifically, lipid-induced loss of endosomal acidification was observed in vitro in cardiomyocytes cultured in high palmitate-containing media, as well as in vivo upon feeding rats a high fat diet [32]. Proper endosomal acidification appeared essential for retaining CD36 within the endosomes. For instance, interfering with endosomal acidification by treatment of cardiomyocytes with monensin, a proton ionophore, or with bafilomycin-A, an inhibitor of proton pumping into endosomes (and other acidic vesicles), causes expulsion of CD36 from the endosomes and subsequent translocation to the cell surface [35]. We currently do not know why endosomal acidification is important for CD36 retention, but it is speculated that a low endosomal pH keeps the primary amine groups of specific phospholipids (e.g., phosphatidylethanolamine) in a protonated form, which favours stabilization of the bilayer (because of ion pairing with negatively charged phosphate groups of adjacent phospholipids). Such bilayer stabilization would impair membrane curving events [36], so that budding of (CD36-containing) transport vesicles will become more difficult.

2.1. v-ATPase pumping retains CD36 within the endosomes

Proton pumping into the lumen of endosomes and into other acidic compartments is carried out by v-ATPase, a large multi-subunit complex. This function makes v-ATPase an essential player in the regulation of cellular compartmentalization. Structurally and functionally, the v-ATPase complex can be divided into two sub-complexes: a membrane-associated sub-complex (named V_0) of 6 subunits forming the proton translocation channel, and a cytoplasmic sub-complex (V_1) of 8 subunits forming the ATP-driven rotor [37,38]. Several subunits consist in multiple isoforms, which are expressed in a tissue-specific manner. This implies that v-ATPase has a unique subunit isoform composition in each tissue, giving it tissue-specific functional properties. For example, in kidney and osteoclasts v-ATPase also co-localizes with the plasma membrane for acid secretion and extracellular pH regulation [39]. In the heart, less information is available on the isoform-specific expression of the v-ATPase subunits. With respect to the V_0 subunit a, the a2 isoform is abundantly expressed in the heart [40], and has been associated with an endosomal/Golgi-location [41,42]. Of note, the a4 isoform, which is expressed in kidney but not in heart [40,42], is associated with a plasmalemmal localization of v-ATPase.

Given that in the heart v-ATPase is a protein resident to endosomes, it can be inferred that CD36 colocalizes with v-ATPase, particularly under conditions of low exogenous lipids. Accordingly, in an endosomally enriched membrane fraction from cardiomyocyte lysates from rats on a low fat diet, CD36 co-immunoprecipitates with v-ATPase subunits a2 and B2 under non-detergent conditions, indicating that v-ATPase and CD36 are present on the same subcellular (presumably endosomal) vesicles [32]. In line with this, microscopical studies show extensive co-localization of CD36 with a2 and B2 in cardiomyocytes cultured under low palmitate conditions [43]. Additional evidence for the involvement of v-ATPase in intracellular retention of CD36 stems from genetic manipulation of v-ATPase via siRNA-mediated silencing of the B2 subunit, which causes CD36 to lose its intracellular localization and translocate to the cell surface [32].

2.2. v-ATPase regulation by assembly/disassembly

v-ATPase activity is regulated by various molecular mechanisms including phosphorylation [37], but with respect to CD36 retention the main mechanism of regulation involves cycles of disassembly and (re-)assembly of the V_0 and V_1 sub-complexes [37,38]. V_0V_1 assembly/disassembly is rapid and reversible, enabling a rapid modulation of v-ATPase activity. The number of stimuli identified to alter v-ATPase assembly status is increasing. This list includes several nutrients including glucose [38]. Glucose re-addition upon withdrawal leads to v-ATPase re-assembly via a mechanism whereby aldolase binds to v-ATPase [44]. This aldolase binding is dependent on ongoing glycolysis, a condition in which this enzyme is supplied with substrate, and hence in an active mode. Interestingly, binding of another glycolytic enzyme, phosphofructokinase, also has been reported to support v-ATPase assembly [45].

2.3. Disassembly of v-ATPase by lipids

The number of metabolic signals able to regulate v-ATPase assembly is expanding. Our current research indicates that also lipids, in particular fatty acids, are regulators of v-ATPase assembly [32]. Thus, we observed that short-term (< 1 h) exposure of cardiomyocytes to excess palmitate induces the disassembly of v-ATPase into its two sub-complexes. This lipid-induced disassembly was accompanied by inhibition of v-ATPase activity resulting in decreased endosomal acidification [32]. Hence, v-ATPase regulation by lipids is opposite to that of glucose, so that it would be predicted that the assembly state of v-ATPase reflects the ratio between cardiac uptake of glucose and lipids at any given nutritional condition. Taking this a step further, the ability of v-ATPase to sense alterations in both glucose and lipid concentrations makes v-ATPase a central regulator of energy substrate metabolism in heart and presumably also in any other tissue for which glucose and lipids are key substrates.

2.4. v-ATPase disassembly and lipid-induced insulin resistance

Prolonged exposure of cardiomyocyte cultures to high palmitate causes CD36 to be more permanently located at the sarcolemma since the de-acidified endosomes are unable to harbor CD36 (see introductory paragraph of this section). The consequently increased rate of lipid uptake will progressively lead to intracellular accumulation of lipid metabolites, among which ceramides and diacylglycerols, both of which directly interfere with the canonical insulin signaling pathway (involving IRS1, PI3K and Akt; see [27]). As a result, insulin-stimulated GLUT4 translocation and glucose uptake as well as insulin-stimulated CD36 translocation and fatty acid uptake are largely inhibited. The loss of insulin-stimulated GLUT4 translocation is accompanied by a low basal glucose uptake rate, whereas the loss of insulin-stimulated CD36 translocation does not affect the already high basal rate of lipid uptake. Taken together, in the overt insulin resistant cardiomyocyte GLUT4 is mainly localized intracellularly whereas CD36 is mainly present at the cell surface. Ultimately, the combination of lipid overload and insulin resistance will lead to loss of contractile activity of cardiomyocytes [23] and to cardiac failure upon Western diet (Section 1.3) [27]. In conclusion, prolonged lipid-induced v-ATPase inhibition is a key underlying mechanism in lipid-induced cardiomyopathy. This is underscored by the observation that genetic inhibition of v-ATPase (via B2 silencing, see above) is sufficient to cause cardiomyocytic insulin resistance in the presence of low exogenous lipid concentrations that by themselves are insufficient to induce insulin resistance [32]. Furthermore, the v-ATPase inhibition-induced insulin resistance is prevented by a pharmacological blockage of the lipid transport function of CD36 [32].

2.5. Feed-forward mechanism of CD36-mediated CD36 translocation

The observation that lipid-induced CD36 translocation is dependent on entry of lipids into cardiomyocytes via CD36 already present at the cell surface is indicative of a feed-forward cycle in myocardial lipid uptake. Specifically, a rise in exogenous lipids (palmitate) leads to an
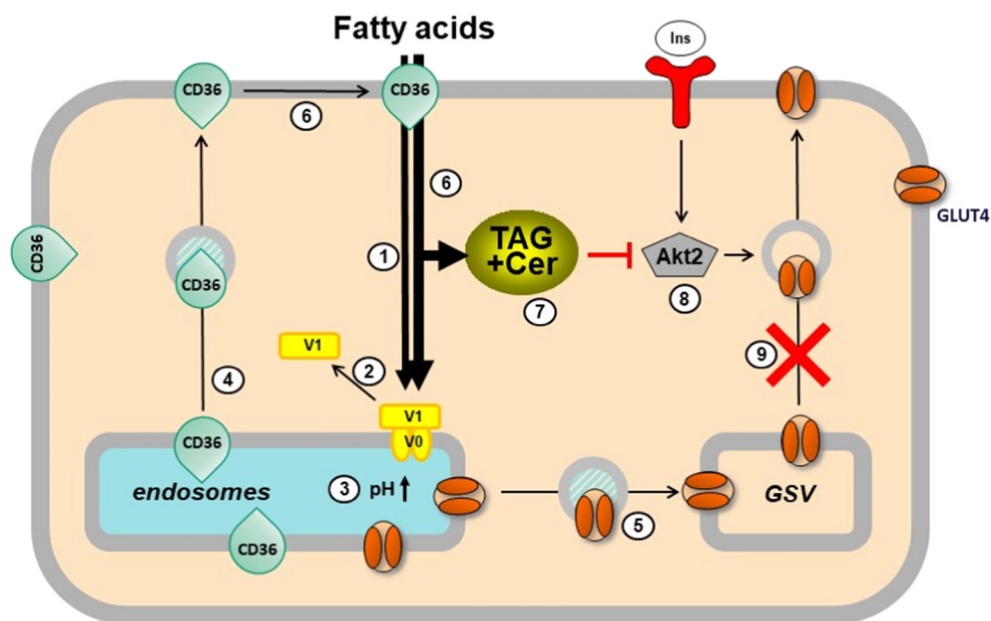
Fig. 2. Sequence of molecular changes involving CD36 and v-ATPase in lipid-induced insulin resistance. An increase in supply of fatty acids (palmitate) leads to CD36-mediated palmitate uptake into myocytes (1). Increased myocellular palmitate concentrations are sensed by v-ATPase, resulting in disassembly of subcomplex V_1 from V_0 (2) and subsequent loss of endosomal acidification (3). This endosomal dysfunction causes expulsion of CD36, which translocates to the sarcolemma (4). Simultaneously GLUT4 is expelled from the endosomes to migrate to the GSV (5). The increase in surface CD36 abundance installs a feedforward cycle of increased fatty acid uptake (6) and progressive accumulation of triacylglycerol (TAG) and bioactive lipid metabolites such as ceramides (7). Ceramides interfere with the canonical insulin signaling pathway at the level of Akt2 (8) resulting in a blockade of GLUT4 translocation (9). Eventually, in the overt insulin resistant myocyte there is a juxtaposed localization of CD36 and GLUT4. Luminal acidification (of endosomes) is indicated by a blue fill color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increased outside-to-inside gradient and a consequently increased uptake via CD36. These lipids enter the cell facilitated by CD36 and subsequently cause v-ATPase disassembly, loss of endosomal acidification, increased sarcolemmal CD36 presence and further increased lipid uptake (Fig. 2). This vicious cycle of CD36-mediated CD36 translocation is expected to be the key mechanism underlying lipid-induced cardiomyopathy. Accordingly, blocking the fatty acid-transport function of CD36 powerfully interrupts this vicious cycle. Moreover, v-ATPase could be a novel target in strategies to combat lipid-induced cardiomyopathy.

2.6. v-ATPase and GLUT4

Whereas subcellular CD36 storage has been mainly confined to endosomes [20,46], GLUT4 appears to be stored in multiple subcellular compartments [33,47,48]. Several of these compartments overlap with the endosomes, but there is strong evidence for the existence of a relatively large subcellular pool of GLUT4 outside of the endosomes. This pool is referred to as the GLUT4 storage compartment (GSV) or as the insulin-responsive vesicle compartment, and it contains about half of the intracellularly stored GLUT4, at least in adipocytes (with the endosomes containing the other half of subcellular GLUT4) [49]. Morphologically, GSV can be distinguished from the endosomes as they are formed from a collection of small (50–70 nm) vesicles [50], whereas the endosomal vesicles range from 100 to 250 nm in diameter [51]. Furthermore, GSV are not acidified, and lack v-ATPase. On the other hand, GSV are equipped with a distinct subset of proteins. They contain, among others, an insulin-regulated aminopeptidase (IRAP), which has long been recognized to translocate in a similar manner as GLUT4 in response to insulin [52]. More importantly, GSV contains TUG (tether containing a UBX domain for GLUT4). TUG is responsible for GLUT4 retention within the GSV via binding to its N-terminal region [53]. Simultaneously, the C-terminal region of TUG anchors GSV to the cis-Golgi, thereby imprisoning GLUT4. Upon insulin stimulation, TUG undergoes an endoproteolytic cleavage [53], thereby releasing the GSV from the Golgi and allowing these vesicles to rapidly translocate to the cell surface. This initial GLUT4 translocation event is known as the insulin burst [10,50]. Additionally, GLUT4 translocates from the endosomes to the cell surface in response to insulin. However, this process is quantitatively a minor player during the insulin burst but gains in importance during more prolonged insulin exposure [10]. Both the GSV and the endosomes harbor the RabGAP AS160 [54], which is involved in the intracellular retention of GLUT4 because AS160 keeps the Rabs in an inactive GTP-unloaded state. This intracellular retention mechanism appears independent of that of TUG in the GSV. The identification of yet another protein involved in GLUT4 retention, designated phosphotyrosine-interacting domain-containing protein-1 (PID1) indicates that the GSV have several different mechanisms backed up to keep GLUT4 firmly imprisoned in the absence of stimuli [55]. Similarly as the endosomes, the GSV may not only serve as storage compartment for insulin-stimulated GLUT4 translocation, but also for contraction-induced GLUT4 translocation. This novel assumption is further discussed in Section 4.2.

Studies investigating the role of endosomal acidification in GLUT4 translocation are scarce and contradictory. In adipocytes, bafilomycin-A has been reported to inhibit [56] as well as to induce GLUT4 translocation [57]. The difference between both studies may be related to the concentration of bafilomycin used, i.e., up to 50 μM in the first study, whereas bafilomycin already completely abolishes endosomal acidification at concentrations of < 100 nM. Results from our studies with primary cardiomyocytes have revealed that bafilomycin treatment leads to expulsion of GLUT4 from the endosomes [35] in agreement with the latter study in adipocytes. Subsequently, using two independent methods (sucrose gradient centrifugation and cell surface biotinylation), we observed that GLUT4 failed to arrive at the cell surface [35]. Unfortunately, by using gradient centrifugation, we could not detect the intracellular destination of GLUT4, but we assume that GLUT4 is kicked out of the endosomes to be sent away for imprisonment within the GSV. The problem of detection of GSV by biochemical methodology has been recognized as these small vesicles appear to be easily destroyed in classical fractionation procedures [10]. Accordingly, one could speculate that the GSV serves as the intracellular GLUT4 destination during endosomal de-acidification.

What happens to the endosomal pool of GLUT4 in the heart during chronic lipid overload? At present, there is no direct answer to this question. However, we must expect that this pool will undergo the same

fate as upon bafilomycin treatment of cells. The lipid-induced v-ATPase disassembly and subsequent de-acidification of the endosomes [32] therefore may lead to expulsion of both CD36 and GLUT4, after which CD36 translocates to the cell surface and GLUT4 expectedly to the GSV, where it will be anchored by TUG. This must be a true imprisonment for GLUT4 because during lipid overload conditions the insulin signaling pathway is not able to induce the endoproteolytic cleavage of TUG needed for liberation of the GSV. Although hypothetical in nature, such scenario may help explain why in the overt diabetic myocyte the sub-cellular distribution of GLUT4 becomes juxtaposed to that of CD36. However, it remains to be explained why CD36 and GLUT4 have different destinations after being expelled from the endosomes and which molecules govern the transport to the default compartment for CD36 and GLUT4 when the de-acidified endosomes release these transporters. It is not far-fetched to attribute differences in subcellular transport of CD36 and GLUT4 to accessory proteins regulating vesicle traffic, including VAMPs.

3. Role of VAMPs in CD36 and GLUT4 traffic

SNARE proteins play a key role in all mediated vesicular trafficking events by enabling membrane fusion between the transport vesicle and the target membrane. The family of SNAREs can be sub-classified into v(esicle)-SNAREs and t(arget)-SNAREs based on their presence at the transport vesicle or at the target membrane, respectively. The specificity of pairing of v-SNARE members with cognate t-SNARE members is a main determinant of the specificity of vesicular traffic. v-SNAREs and t-SNAREs have complimentary motifs that bind via 'zippering', into a trans-SNARE complex that overcomes the repelling forces of the two lipid bilayers. The v-SNAREs are made up of 7 members of the VAMP (vesicle-associated membrane protein) family while there are > 20 t-SNAREs identified, mostly originating from the protein families of the syntaxins and the synaptotagmins [58]. This unequal distribution implies that the v-SNAREs can form specific bonds with more than one t-SNARE, while this is not the case reciprocally. Evidence is accumulating that phosphorylation of t- and v-SNARE proteins is involved in regulation of their function. These phosphorylations can be either stimulatory or inhibitory for priming of SNAREs [59]. Here, we will focus mainly on the v-SNAREs (VAMPs) because they represent the different intracellular compartments from which the transport vesicles are formed. For completion, it needs to be mentioned that SNARE pairing is essential but not sufficient for membrane fusion [60]. The latter process also requires the participation of several adaptor proteins binding to the SNAREs, such as synaptosome-associated proteins (SNAPs), conferring further specificity to trafficking [61].

Since both GLUT4 translocation and CD36 translocation are mediated by transport vesicles trafficking between endosomes and the cell surface, both processes rely on SNARE proteins including VAMPs. A net translocation of each transporter to the plasma membrane, as for any other cargo protein, is determined by the relative rates of exocytosis (from endosomes to the cell surface) and endocytosis (the retrograde trafficking back to the endosomes). Because VAMPs confer specificity to vesicular trafficking, it is to be expected that different VAMP isoforms are involved in exocytotic pathways versus endocytotic pathways regulating the subcellular distribution of CD36 and GLUT4. Another level of specificity relates to the stimuli, insulin and contractions, which could utilize different VAMP isoforms to stimulate GLUT4 and CD36 translocation, as will be discussed in detail below. For GLUT4 translocation, the involvement of VAMPs has been well-investigated, especially in adipocytes, whereas studies in cardiomyocytes and on the role of VAMPs in CD36 translocation are scarce. Atrial cardiomyocytes have been studied in relation to VAMPs because of their endocrine function during stress, leading to secretion of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). Accordingly, these cells are equipped with almost the entire set of VAMP isoforms (only VAMP7 could not be detected at the transcript level; [58]). Furthermore, the atrial cardiomyocyte cell line HL-1 has been used in a systematic study of VAMPs in CD36 translocation in relation to GLUT4 via silencing each of the resident VAMP members [34]. Ventricular cardiomyocytes also have been shown to possess most of the VAMPs (only VAMP1 and VAMP8 were lacking at the protein expression level; [34]). Below, we summarize separately for each of the VAMPs the available literature information about their roles in GLUT4 and CD36 trafficking in the heart. We also include information, when available, on VAMPs in skeletal muscle and in adipocytes, in which tissues the involvements of VAMPs has been more intensively studied. At least in skeletal muscle, the translocation of GLUT4 and CD36 appears to be similarly regulated [6]. For cell lines, such as HL-1 cardiomyocytes, a word of caution seems appropriate concerning their use as model to study GLUT4 and CD36 traffic, given that these cells are mostly glycolytic.

3.1. VAMP2

VAMP2 (also known as synaptobrevin-2) has been the first VAMP identified to be involved in GLUT4 translocation, as found in adipocytes, and has been mainly associated with insulin stimulation of GLUT4 exocytosis [62,63]. Also in muscle, VAMP2 has been shown to mediate insulin-stimulated GLUT4 exocytosis [64]. Data in the heart are more limited, but VAMP2 appears to be present in the endosomes as well as in the GSV, and upon insulin stimulation translocates simultaneously with GLUT4 [65]. In skeletal muscle, VAMP2 is phosphorylated on Ser upon insulin-treatment, and there is evidence that the responsible kinase is atypical protein kinase C member PKC-ζ. Subsequently, this PKC-ζ-mediated VAMP2 phosphorylation may be essential in insulin-stimulated GLUT4 translocation [66]. However in the heart, insulin did not influence VAMP2 binding to PKC-ζ (or Akt2; [67]), pointing towards tissue-specific differences in VAMP priming. Conclusive evidence that also in the heart, VAMP2 is involved in insulin-stimulated GLUT4 translocation was derived from as siRNA-mediated silencing of VAMP2 in HL-1 cardiomyocytes [34]. In this study, CD36 trafficking was simultaneously examined: CD36 translocation was similarly stimulated by insulin as GLUT4 translocation and also blocked by VAMP2 silencing [34]. In contrast, contraction-induced GLUT4 and CD36 translocation were unaffected by VAMP2 silencing [34]. Remarkably, there was also another action of VAMP2 silencing: In basal conditions sarcolemmal CD36 levels were increased, pinpointing to a (possible) dual role of VAMP2 in CD36 traffic, namely, the involvement in insulin-stimulated CD36 exocytosis and in basal CD36 endocytosis [34].

3.2. VAMP3

VAMP3 is also known as cellubrevin, and several observations indicate that, just like VAMP2, it is involved in insulin-stimulated GLUT4 translocation. VAMP3 localizes to the insulin-sensitive GSV in adipocytes [63], and appears to be functionally involved in GLUT4 translocation [64,68]. This additional involvement of VAMP3 in insulin-stimulated GLUT4 translocation next to VAMP2 suggests redundancy of VAMPs in this process. Potent evidence for this redundancy was provided by VAMP replacement studies in adipocytes, where VAMP3 could restore insulin-stimulated GLUT4 translocation in adipocytes with combined ablation of VAMP2, VAMP3 and VAMP8 [69]. However, colocalization between GLUT4 and VAMP3 was not observed in rat and human skeletal muscle [70], and VAMP3 failed to mediate insulin-stimulated GLUT4 translocation in muscle cell lines [71], pointing towards a tissue-specific differences in the functioning of VAMPs in GLUT4 translocation. Other lines of evidence excluding a role of VAMP3 in insulin-stimulated GLUT4 translocation entailed a systematic study on all VAMPs in GLUT4 translocation using distribution analysis concluded that only VAMP2 is important for this process [72]. Moreover, VAMP3-knockout mice normally displayed insulin-stimulated GLUT4 translocation [73].

VAMP3 has also been studied in relation to exercise/contraction-induced GLUT4 translocation, and appears not to be involved in this process in skeletal muscle. Namely, VAMP3 does not translocate with GLUT4 in contracting muscle [74]. Furthermore, in skeletal muscle from VAMP3 knockout mice, contraction-induced GLUT4 translocation is not altered [73]. In contrast, in the heart VAMP3 appears to be involved in contraction-induced GLUT4 translocation and glucose uptake, given that both processes were completely abolished in HL1-cardiomyocytes, in which VAMP3 was knocked-down with siRNA methodology [34]. In these same VAMP3-silenced cells, also contraction-induced CD36 translocation was blocked [34], indicating that VAMP3 has a broader involvement in contraction-induced subcellular trafficking. Moreover, these combined data indicate again a tissue-specific involvement of VAMP3 in GLUT4 traffic. Priming of VAMP3 upon the onset of contraction in cardiomyocyte cultures may be due to phosphorylation by protein kinase-D1, a recently identified contraction-activated kinase, which increasingly binds to VAMP3 upon contraction [67]. In contrast, the canonical energy sensor AMPK failed to bind to VAMP3 either in the absence or presence of contraction [67].

3.3. VAMP4

In contrast to VAMP2 and VAMP3, much less research has been performed on the involvement of VAMP4 and the other VAMP members. In adipocytes, VAMP4 is not directly involved in GLUT4 endocytosis or exocytosis, but this isoform is needed for the biosynthetic sorting of GLUT4 from the Golgi apparatus to the recycling endosomes [47]. Studies with siRNA-mediated silencing of VAMP4 in HL-1 cardiomyocytes, indicated that this isoform is also not involved in GLUT4 trafficking to and from the cell surface in response to either insulin or contraction [34]. In contrast, in these same cardiomyocytes VAMP4 appeared to be necessary for insulin as well as contraction-induced CD36 translocation. Therefore, the involvement of VAMP4 in CD36 traffic, but not in GLUT4 traffic renders VAMP4 as a novel target to alter the balance between glucose and fatty acid uptake, which could be beneficial in cardiac metabolic diseases with altered substrate preference.

3.4. VAMP5 and VAMP7

In a systematic study, in which each of the VAMP members was silenced in adipocytes for studying their role in insulin-stimulated GLUT4 translocation, it appeared that VAMP5 and VAMP7 were not involved [47]. In skeletal muscle, VAMP5 and VAMP7 localize to the intracellular GSVs and both isoforms translocate with GLUT4 to the sarcolemma in response to electrically induced contraction [74]. However, this study unfortunately did not investigate whether both VAMPs were necessary for contraction-induced GLUT4 translocation. In other studies, VAMP5 and VAMP7 have been predominantly found at the cell surface in adipocytes, skeletal muscle and heart [75–77], suggesting that both VAMPs mediate endocytotic processes. VAMP5 and VAMP7 silencing in HL1 cardiomyocytes yielded an increase in cell surface GLUT4 under non-stimulated conditions, whereas insulin or contraction-stimulated GLUT4 translocation were unchanged [34]. Also basal, insulin or contraction-induced CD36 translocation was not affected [34]. Although definite proof is missing, these latter findings are compatible with specific roles of VAMP5 and VAMP7 in GLUT4 endocytosis in the heart. Furthermore, because of their selective involvement in glucose uptake (and not in fatty acid uptake), these VAMPs have the potential to modulate cardiac substrate preference.

3.5. VAMP8

VAMP8 (endobrevin) is distributed within the endosomal compartment, but more so during muscle differentiation than in mature muscle [78]. VAMP8 has been detected by PCR in atrial cardiomyocytes [58], but not by Western blotting in adult ventricular cardiomyocytes [34]. In adipocytes, VAMP 8 is implicated in GLUT4 traffic, and specifically to GLUT4 endocytosis back to the recycling endosomes [47]. No observations have been described linking VAMP8 to CD36 traffic.

4. Towards a roadmap of subcellular CD36 and GLUT4 traffic by integrating the roles of v-ATPase and VAMPs in trafficking of these transporters

By integrating observations regarding the influence of v-ATPase and of VAMPs on CD36 and GLUT4 translocation, we aim to create a roadmap for subcellular trafficking of both transporters, first for CD36 (Section 4.1), then GLUT4 (Section 4.2), and finally combined (Section 4.3). The basis for this roadmap in the heart is the systematic silencing of VAMPs in HL1 cells [34], as detailed in the previous sections (Sections 3.1 to 3.5).

4.1. Mapping CD36 traffic

When starting to combine CD36 traffic with the involvement of VAMPs, it immediately jumps to the attention that for CD36 translocation, either stimulated by insulin or by contraction, two VAMPs are necessary. Insulin-stimulated CD36 translocation is dependent on VAMP2 and VAMP4, and contraction-induced CD36 translocation relies on VAMP3 and VAMP4. Since each VAMP member is known to mediate a specific vesicle trafficking event from a given donor compartment to a defined acceptor compartment (see Section 3), it follows that in response to either stimulus, CD36 does not translocate to the cell surface in a direct manner, but makes a stopover at an intermediate station. Hence, the first VAMP brings CD36 from the endosomal mother compartment to this hypothetical intermediary station; the second VAMP brings CD36 from this station to the cell surface. What is the nature of the intermediary station? We only can predict that it must be part of the endosomal compartment. Namely, kinetic evidence indicates that stimulus-induced CD36 translocation shares the same mechanism as CD36 translocation induced by endosomal de-acidification [35,46]. Then it follows that if this compartment would be non-endosomal, CD36 would be trapped here upon treatment of cells with bafilomycin or monensin, resulting in an incomplete translocation (which does not match the observations). It makes most sense that the VAMP involved in bringing CD36 from the mother endosomal compartment to the intermediary endosomal station is VAMP4. This VAMP is specifically dedicated to CD36 traffic (and not involved in that of GLUT4). Subsequently, VAMP2 or VAMP3 can complete the translocation of CD36 from the intermediary station to the cell surface dependent on the type of stimulus (insulin or contraction). The reciprocal scenario that upon (insulin or contraction) stimulation, the VAMP responsible for the initial CD36 translocation step is VAMP2 or VAMP3 (dependent on the stimulus), can be rejected because in that situation there would be no subcellular segregation between CD36 and GLUT4. Namely, VAMP2 and VAMP3 do not discriminate between CD36 and GLUT4 traffic. Finally, VAMP2 additionally ensures that CD36 returns to the endosomes upon termination of the stimulation period. CD36 can then be delivered either to the mother endosomal compartment or to the intermediary endosomal station.

4.2. Mapping GLUT4 traffic

The systematic silencing of VAMPs in HL1 cells [34] has identified four VAMPs to be involved in GLUT4 traffic in the heart. VAMP2 and VAMP3 are necessary for insulin and contraction-induced translocation of GLUT4. Given that these VAMPs fulfil similar roles in CD36 translocation, VAMP2 can be assigned to as 'insulin-VAMP', and VAMP3 as 'contraction-VAMP'. The other VAMPs are VAMP5 and VAMP7, and mediate the retrograde trafficking of GLUT4. The fact two VAMPs are needed for GLUT4 internalization, is different from CD36 traffic, in
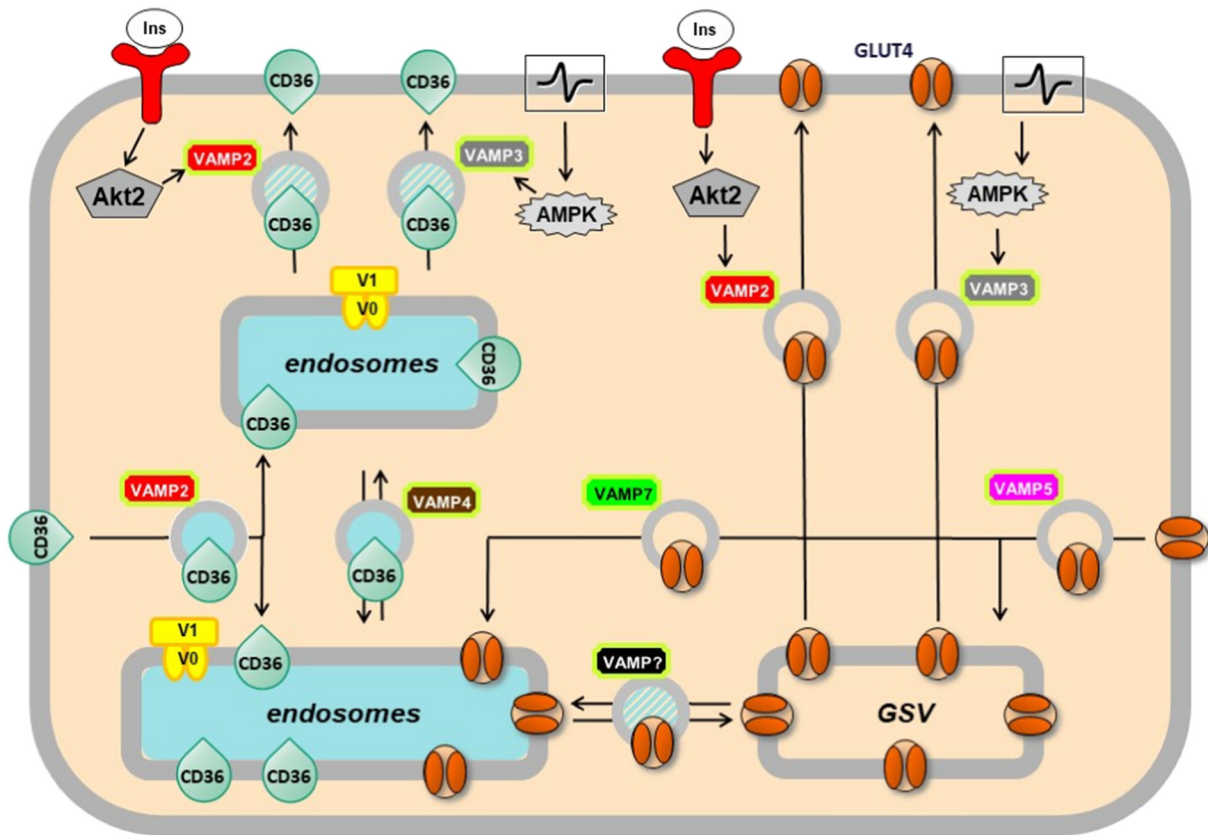
Fig. 3. Hypothetical roadmap of CD36 and GLUT4 traffic in the healthy heart. Both CD36 and GLUT4 are intracellularly stored within the (recycling) endosomes and GLUT4 additionally in non-endosomal GLUT4 storage vesicles (GSV). VAMP 4 is specifically involved in CD36 translocation and translocates CD36 between the mother endosomal compartment and a hypothetical endosomal CD36-specific intermediate compartment. A not yet identified VAMP (VAMP?, perhaps VAMP8) translocates GLUT4 between the mother endosomal compartment and the GSV. VAMP2 and/or VAMP3 bring CD36 from the endosomal CD36-specific intermediate compartment to the cell surface upon stimulation by insulin (including Akt2) and/or by contraction (including AMPK), respectively. Both VAMPs fulfil the same roles in insulin/contraction-stimulated GLUT4 translocation from the GSV to the cell surface. VAMP2 mediates CD36 return into the mother/CD36-specific endosomes. VAMP5 and VAMP7 mediate GLUT4 return to intracellular compartments. VAMP5 could be specific for internalization to GSV and VAMP7 for endosomal return, but the specific functioning of these VAMPs could also be flipped. Luminal acidification (of endosomes) is indicated by a blue fill color.

which only one VAMP isoform was found to be operative in this respect. A reason for this difference could be that in case of CD36 both involved compartments (the mother compartment and the intermediary station) are endosomal, requiring only one VAMP, whereas in case of GLUT4 the two involved compartments are endosomal as well as non-endosomal requiring different VAMPs. Still, we do not know which of the two VAMPs (VAMP5 or VAMP7) specifically brings GLUT4 from the cell surface to the GSV or which of these VAMP isoforms delivers GLUT4 back to the endosomal mother compartment.

There is another missing piece in the traffic of GLUT4. Namely, for insulin or contraction stimulation, only one VAMP has been identified to be involved in response to either stimulus (VAMP2 and VAMP3, respectively, see above), while there are clearly (at least) two GLUT4 compartments, being the endosomes and the GSV. Considering a possible symmetry with CD36 traffic, the GSV could serve as the intermediary station for GLUT4 traffic. Accordingly, VAMP2 or VAMP3 would mediate GLUT4 traffic from the GSV to the cell surface dependent on the stimulus. However, information is lacking about the VAMP responsible for traffic of GLUT4 from the endosomes to the GSV. There are a few possibilities to fill this gap. First, VAMP8 that has not been detected at the protein level in the adult heart (see Section 3.5), cannot be entirely excluded, because it may still be possible that small amounts of this VAMP that escaped detection by Western blotting would be responsible for this trafficking step. Interestingly, VAMP 8 is also implicated in GLUT4 traffic in adipocytes, but on the other hand the VAMP8-dependent GLUT4 trafficking step in this tissue appears to be

the endocytosis event [47]. Yet given the fact that VAMPs play different trafficking roles in different tissues, the GLUT4 endocytosis regulation by VAMP8 in adipose tissue is not incompatible with a possible role of this VAMP member in GLUT4 traffic from the endosomes to the GSV in the heart. Second, VAMP2 might fulfil this missing piece of traffic, because this VAMP has also been reported to shuttle GLUT4 between the endosomes and GSV in adipocytes [10]. In this scenario, VAMP2 would be also involved in contraction-stimulated GLUT4 translocation, which was not observed. Hence, this scenario can be rejected. In conclusion, in cardiomyocytes the VAMP member involved in this step awaits identification.

An additional issue of controversy is the role of the GSV in contraction-stimulated GLUT4 translocation. It has been generally accepted that the main function of the GSV is to serve as storage compartment for insulin-stimulated GLUT4 translocation [10,47,79], whereas the role of the GSV in contraction-induced GLUT4 translocation is mostly not mentioned. It is suggested (without evidence) that the endosomes are important in contraction-induced GLUT4 translocation with the GSV mediating insulin-stimulated GLUT4 translocation [80]. This scenario predicts that during lipid-induced insulin resistance, acute contraction stimulation is not able to induce GLUT4 translocation. Namely under this condition, the endosomes are devoid of GLUT4 (see Section 2.6). For gaining a better insight into this latter scenario, the key contraction-induced signaling node AMPK should be taken into focus. As depicted in Fig. 1, AMPK has been firmly implicated in contraction-induced GLUT4 translocation, and has been well-studied in the context of

insulin resistant myocytes, in which it serves as target for improvement of insulin-stimulated GLUT4 translocation. For this purpose, a great number of compounds have been tested on their ability to activate AMPK during long-term addition, including numerous compounds extracted from exotic plants (e.g., [81,82]). In contrast, the acute effect of AMPK activators on GLUT4 translocation in insulin resistant cells has been scarcely investigated. Yet, the few reports that investigated this issue, show that short-term contraction and AMPK activation lead to GLUT4 translocation in the insulin resistant setting (e.g., see [83,84]). Further evidence for a role of the GSV in contraction-induced GLUT4 translocation is that TBC1D1, the RabGAP supposedly involved in contraction-induced GLUT4 translocation, binds to IRAP in the GSV [85]. Upon AMPK-mediated phosphorylation, TBC1D1 disattaches from IRAP, thereby allowing GLUT4 translocation [85]. Yet another piece of independent evidence comes from the observation that AMPK activation can lead to IRAP translocation [86]. Taken together, the available evidence is in favor of a role of the GSV in contraction-induced GLUT4 translocation, which is mediated by VAMP3.

4.3. Integration of CD36 and GLUT4 traffic

Based on defining the roles of specific VAMP members in the distinct steps in CD36 and GLUT4 traffic in combination with the specific characteristics of endosomal and non-endosomal storage compartments (see Sections 4.1 and 4.2), a putative roadmap of CD36 and GLUT4 traffic in cardiomyocytes has been constructed, which is depicted in Fig. 3. With this roadmap we attempt to create a new starting point for discussion. It shows the effects of insulin and contraction on CD36 and GLUT4 traffic (see Legend of Fig. 3). It also provides a plausible explanation for the differential subcellular fates of CD36 and GLUT4 upon pharmacologically-induced loss of endosomal acidification (via bafilomycin-induced v-ATPase inhibition or via treatment with the H^+-ionophore monensin). Dissipation of the endosomal pH gradient will expel both CD36 and GLUT4 from the mother endosomal compartment. CD36 then travels with the aid of VAMP4 to the endosomal intermediary station, but cannot stay there since also this station is malfunctioning, and therefore has to travel completely to the cell surface. Perhaps VAMP2 and VAMP3 can substitute for each other to mediate CD36 translocation upon loss of endosomal acidification. In parallel, GLUT4 travels from the endosomes to the GSV, where it is well accommodated since this compartment is not affected by the subcellular pH gradient-lowering drugs. GLUT4 cannot travel further to the cell surface because VAMP2 and VAMP3 are likely not activated under this condition.

5. Application of the roadmap to explain altered CD36 and GLUT4 traffic in the heart during insulin resistance

A largely unresolved issue in the field of trafficking of CD36 and GLUT4 is that in the healthy heart CD36 and GLUT4 appear to travel simultaneously from intracellular compartments to the cell surface upon physiological stimuli, such as insulin and increased contractile activity, whereas at the onset of cardiac insulin resistance CD36 and GLUT4 behave quite differently: CD36 is expelled to the cell surface and GLUT4 is imprisoned intracellularly. How can the roadmap be exploited to explain these aberrant trafficking processes in the heart during excess lipid availability? This is summarized in Fig. 4A. As described in Section 1.3, the initial trigger for the aberrant CD36 and GLUT4 trafficking fates is the persistent increase in plasma lipid concentrations due to chronic overconsumption. This results in increased fatty acid (palmitate) uptake into the cardiomyocytes via the resident CD36 pool at the cell surface. As described in Section 2.4, the rise in intracellular palmitate is subsequently sensed by v-ATPase, which then disassembles, rendering the endosomes unable to serve as storage compartment for CD36 and GLUT4 (Section 2, introductory paragraph), enforcing CD36 to translocate to the cell surface (Section 1.3) and GLUT4 to the GSV

(Section 2.6). Then, the increased sarcolemmal CD36 together with the increased plasma lipid concentrations will install the feed-forward loop of CD36-mediated CD36 translocation (Section 2.5) and further massive lipid accumulation culminating into impairment of insulin signaling (Section 1.3). One of the targets of insulin signaling is VAMP2 (see Section 3.1), and the insulin resistant state would imply a lack of activation of VAMP2, rendering this VAMP unable to mediate GLUT4 translocation to the cell surface. On the other hand, contraction-induced GLUT4 translocation is not affected by lipid-induced insulin resistance [22,30] indicating that VAMP3 is normally operative. Together, this implies that during insulin resistance GLUT4 is imprisoned within the GSV. Namely, the VAMP2 inhibition precludes GLUT4 translocation to the cell surface, and the endosomal de-acidification precludes a return of GLUT4 from the GSV to the endosomes. Only VAMP3 activation would be able to release GLUT4 from the GSV during lipid overload. When assumed that expression levels of VAMP2 exceed those of VAMP3, it would be expected that VAMP3 would not be able to fully compensate for the loss of activation of VAMP2 in bringing GLUT4 to the cell surface. Additionally, VAMP2 would fail to mediate CD36 translocation during insulin resistance, but since the total CD36 pool is already at the cell surface, VAMP2 dysfunction is of no consequence for CD36 traffic. In conclusion, the roadmap may help understand the differential trafficking behavior of CD36 and GLUT4 in the heart upon the onset of lipid overload (Fig. 4A).

The roadmap may also be employed to design novel strategies to normalize CD36 and GLUT4 traffic via targeting of v-ATPase and VAMPs. One such strategy may involve overexpression of VAMP3 in cardiomyocytes, as already has been investigated [67]. In cardiomyocytes cultured under low lipid conditions, overexpression of VAMP3 did not influence insulin-stimulated glucose uptake, reconfirming that VAMP3 is not involved in insulin-stimulated glucose uptake in the healthy heart. In contrast, VAMP3 overexpression rescued insulin-stimulated GLUT4 translocation in cardiomyocytes cultured in high lipid-containing media. Additionally, cell surface accumulation of CD36 was prevented. Moreover, the build-up of excess myocellular lipid depots and the contractile dysfunction were no longer observed. Remarkably, insulin signaling was not restored within the period of measurement. From this, we deduced the following sequence of events: VAMP3 overexpression allows the formation of extra GLUT4 vesicles from the GSV, which are made available for the insulin signaling pathway during the condition of insulin resistance (Fig. 4B). These extra GLUT4 vesicles would then be sufficient to compensate for the partial inhibition of insulin signaling and subsequent insulin-induced VAMP2 activation.

These beneficial effects of VAMP3 overexpression could be mimicked by a possible drug-induced activation of VAMP3. Since VAMP3 is activated through the contraction pathway directly by PKD1 (see Section 3.2), a specific activation of PKD1 by pharmacological approaches would also normalize insulin-stimulated GLUT4 translocation during lipid overload. As proof of principle, adenoviral PKD1 overexpression led to preservation of insulin-stimulated GLUT4 translocation in lipid-overloaded cardiomyocytes [87] presumably via the PKD1 – VAMP3 axis [67]. Upon restoration of GLUT4 translocation, also glucose uptake is re-installed and intracellular glucose levels normalized. Since v-ATPase is regulated by glucose (Section 2.2), the normalization of glucose levels will lead to re-assembly of v-ATPase and re-acidification of the endosomes. This will pull CD36 back from the cell surface leading to restoration of low basal fatty acid uptake rates and breakdown of excess lipid storage (Fig. 4B). We also expect the insulin signaling pathway to recover as one of the later stage alterations, but apparently the lowering of the intracellular lipid levels is already sufficient to rescue the contractile dysfunction as seen under lipid overload.

Finally, also other VAMP members involved in GLUT4 and CD36 traffic could be targeted to protect against lipid-induced cardiac failure. The roadmap predicts that downregulation of VAMP4 and upregulation of VAMP5 and VAMP7 might be beneficial. Downregulation of VAMP4
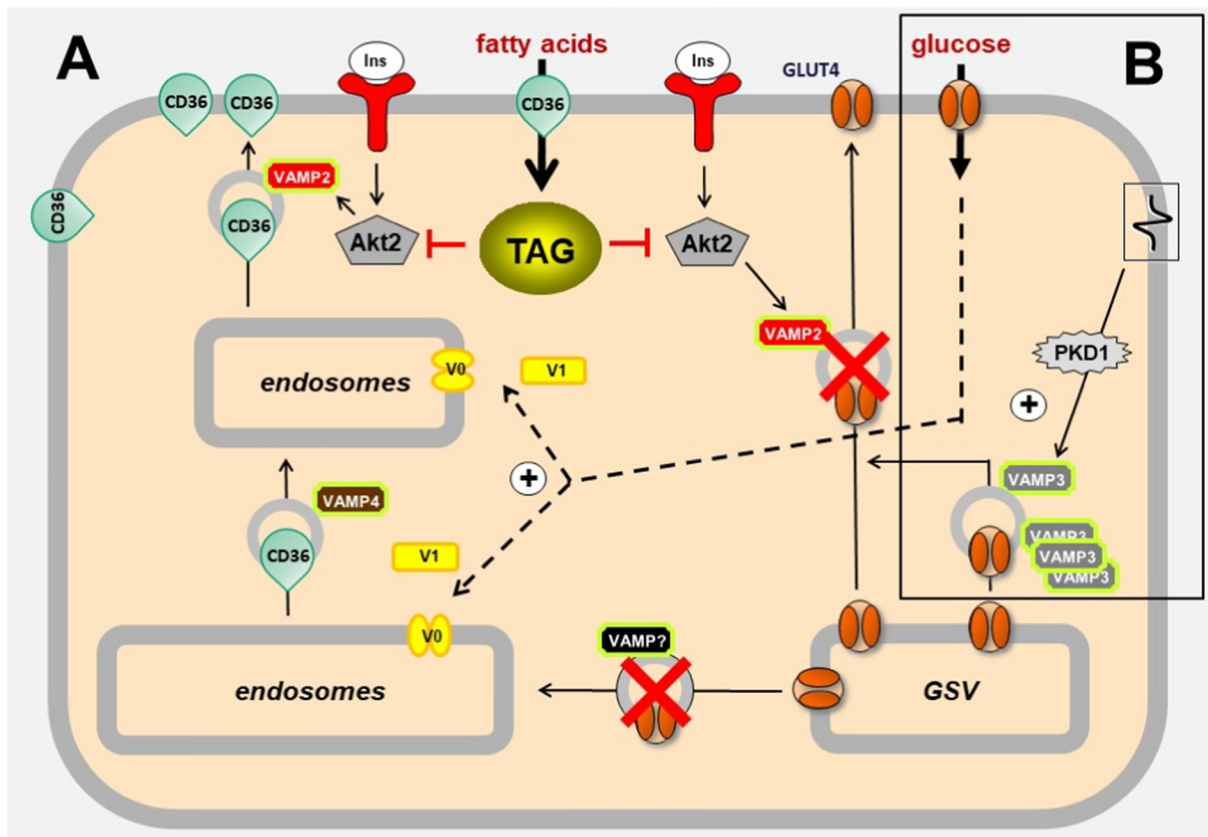
Fig. 4. Roadmap exploited to (A) visualize the aberrant reciprocal location of CD36 and GLUT4 in lipid-overloaded insulin resistant myocytes, and to (B) indicate possible novel strategies to restore normal substrate transporter localization, as exemplified by targeting VAMP3. Panel A displays a schematic presentation of a lipid-overloaded insulin resistant myocyte exposed to insulin. The chronically elevated fatty acid levels keep v-ATPase in a disassembled state, indicated by arrows, and consequently the endosomes de-acidified (endosomal lumen no longer indicated by blue fill color). The aberrantly high myocellular TAG levels and ceramides cause an inhibition of Akt2, thereby blocking insulin-induced VAMP2 activation. This has no effect on CD36 levels at the cell surface because this transporter is already there because of the loss of endosomal acidification. In contrast, VAMP2 inhibition blocks insulin-stimulated GLUT4 translocation, thereby imprisoning GLUT4 inside the GSV. Panel B (inset) displays the molecular changes by which VAMP3 targeting may rectify CD36 and GLUT4 traffic. VAMP3 overexpression or activation of VAMP3 by the contraction pathway (involving PKD1) lead to insulin-independent formation of GLUT4 vesicles, which are re-directed into the impaired insulin-stimulated GLUT4 translocation process. The next important event is the re-assembly of v-ATPase in response to increased myocellular glucose levels. The subsequent re-acidification of endosomes, CD36 internalization, decrease in myocellular lipid and restoration of insulin signaling are not displayed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

limits CD36 translocation by any stimulus and would lead to decreased cardiac fatty acid uptake. Upregulation of VAMP5 and VAMP7 would lead to decreased GLUT4 retention and stimulate glucose uptake. All these manipulations would aim to restore the substrate balance in the lipid-overloaded diabetic heart [88]. This could be tested in future research.

For the roadmap, we focussed on v-ATPase and VAMPs to regulate CD36 and GLUT4 traffic. Clearly, these proteins are not the only ones to regulate trafficking. Other proteins with an important contribution to the specificity of trafficking of cargo proteins in general and to that of GLUT4 more specifically, are the Rab proteins, their adaptors and their inhibitors (the RabGAPs). For instance, Rab10 is involved in endosomal recycling of GLUT4 whereas Rab14 mediates the GSV recycling of GLUT4 [89]. Rab11 was the first Rab member established to be involved in CD36 traffic, and associated with CD36 internalization [90]. Finally, in analogy to the VAMP members similarly involved in CD36 and GLUT4 traffic, being VAMP2 (insulin-induced translocation) and VAMP3 (contraction-induced translocation), a Rab member has been identified, being Rab8a to be similarly involved in CD36 and GLUT4 traffic (i.e., insulin-induced translocation) [91]. In conclusion, the roadmap could be expanded by including Rabs and possibly other regulatory trafficking proteins to offer a more in-depth view of regulation of CD36 and GLUT4 traffic. Then new strategies can be designed

to alter cardiac substrate preference. This could be of major importance not only in lipid-induced cardiomyopathy, but also in other cardiac diseases with metabolic fingerprints.

CRediT authorship contribution statement

Joost J.F.P. Luiken: Conceptualization, Writing - original draft, Writing - review & editing, Visualization. **Miranda Nabben:** Conceptualization, Writing - review & editing. **Dietbert Neumann:** Conceptualization, Writing - review & editing. **Jan F.C. Glatz:** Conceptualization, Writing - review & editing.

Declaration of competing interest

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

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