

Caveolae in Rabbit Ventricular Cardiomyocytes: Distribution and Dynamic Diminution after Cell Isolation

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Abstract

Introduction: Caveolae are signal transduction centres, yet their subcellular distribution and preservation in cardiac myocytes after cell-isolation are not well documented. Here, we quantify caveolae, located within 100 nm of the outer cell surface membrane, in rabbit single ventricular cardiomyocytes over 8h post-isolation and relate this to caveolae presence in intact tissue.

Methods: Hearts from New Zealand white rabbits were either chemically fixed by coronary perfusion, or enzymatically digested to isolate ventricular myocytes that were subsequently fixed at 0h, 3h and 8h post-isolation. In live cells, the patch-clamp technique was used to measure whole-cell plasma membrane capacitance, and in fixed cells caveolae were quantified by transmission electron microscopy (TEM). Changes in cell surface topology were assessed using scanning electron microscopy (SEM). In fixed ventricular myocardium, dual-axis EM tomography (ET) was used for three-dimensional (3D) reconstruction and analysis of caveolae *in situ*.

Results: Surface-sarcolemmal caveolae presence and distribution in freshly isolated cells matches that of intact myocardium. With time, the number of surface-sarcolemmal caveolae decreases in isolated cardiomyocytes. This is associated with a gradual increase in whole-cell membrane capacitance. Concurrently, there is a significant increase in area, diameter and circularity of sub-sarcolemmal mitochondria, indicative of swelling. In addition, ET data from intact heart illustrate the regular presence of caveolae not only at the surface sarcolemma, but also on T-tubular (T-tub) membranes in ventricular myocardium.

Conclusions: Caveolae are dynamic structures, present both at surface sarcolemmal and T-tub membranes. After cell isolation, surface-sarcolemmal caveolae numbers decrease significantly, within a time-frame relevant for single cell research. The concurrent increase in cell capacitance suggests that membrane incorporation of surface-sarcolemmal caveolae underlies this, but internalization and/or micro-vesicle loss to the extracellular space may also contribute. Given that much of the research into cardiac caveolae-dependent signalling utilises isolated cells, and since caveolae-dependent pathways matter for a wide range of other study targets, analysis of isolated cell data should take the time post-isolation into account.

Introduction

Caveolae (Latin for *little caves*), cholesterol- and sphingolipid-rich spheroid plasma membrane domains of 50-100 nm diameter, are found in close proximity to the surface sarcolemma of cells, usually linked to it *via* a “bottle-neck”-like connection [1, 2]. Caveolae are present in many cell types, including those of the cardiovascular system (cardiomyocytes, endothelial cells, fibroblasts, smooth muscle cells) [3]. A defining feature of caveolae is the presence of specialized scaffolding proteins - caveolins and cavins [4-6]. Caveolins (Cav-1 to -3) are responsible, in part, for the spheroid morphology of caveolae, through their asymmetrical membrane insertion and their tendency to form oligomers that promote local concave membrane invagination [7]. Cav-3 is a muscle-specific isoform [8, 9], whereas Cav-1 is widely expressed in many cell types including adipocytes, endothelial cells, pneumocytes, and fibroblasts [9-11]. The muscle-specific Cav-3 is essential for caveolae formation in cardiomyocytes, and Cav-3- deficient mice develop cardiomyopathies [12-16].

After their discovery in the 1950s, caveolae have been shown to play essential roles in a wide range of cellular processes including signal transduction [17-19], macromolecular complex trafficking [20, 21], and – owing to the presence of several ion channel and exchanger proteins in caveolae membranes – electrophysiology [22-26]. It is thought that caveolae thus segregate and integrate certain signalling pathways in microdomains of the plasma membrane.

Their shape and composition enable surface sarcolemmal caveolae in skeletal [27] and cardiac muscle [28] to act as “spare” plasma membrane, which can be recruited during mechanical perturbations, such as stretch. Indeed, mechanical stretch or osmotic swelling can lead to sarcolemmal membrane incorporation of surface-sarcolemmal caveolae, preventing excessive sarcolemmal membrane tension [27-33]. The dynamic recruitment of surface-sarcolemmal caveolae into the surface membrane increases membrane capacitance [29] and affects the density and distribution of sarcolemmal ion channels [18]. Depletion of surface-sarcolemmal caveolae in turn prevents the stretch-induced increase in membrane capacitance, and inhibits the slowing of conduction otherwise seen upon mechanical distension of the intact heart [29]. Surface-sarcolemmal caveolae are therefore one of the mechano-sensors/-transducers of cardiomyocytes [23, 28, 34, 35].

In cardiac and skeletal muscle, Cav-3 is distributed throughout the sarcolemma, including external surface and T-tubular (T-tub) membranes, as visualised by immunofluorescence [24, 36]. Nonetheless, caveolae are traditionally thought to be structures associated with the ‘outermost’ surface sarcolemma only, despite reports suggesting caveolae presence in T-tub based on high resolution imaging data [37-39]. However, presence and relevance of T-tub caveolae have remained controversial [37, 40, 41]. Here we describe in detail the 3D organisation of caveolae at surface sarcolemmal and T-tub membranes in rabbit ventricular myocardium.

The rabbit model was chosen, because it represents a species that mimics human cardiac physiology surprisingly well. The characteristics of the rabbit heart resemble those of the human in terms of structure [42, 43], regional contractile and diastolic properties [44], and responses to pathophysiological stimuli (such as ischemia, [43, 45]). Key electrical features

(such as action potential properties) and responses to relevant pharmacological interventions also show pronounced similarities between the two species [46-48]. Additionally, rabbit cardiomyocyte ultrastructure (for example, of the T-tub system) is closely reminiscent of that in human cells [49]. As a consequence, there is a growing appreciation for the utility of rabbit models to study basic electrophysiological concepts, human heritable diseases [50] and preclinical cardiac safety testing [51].

Isolated ventricular cardiomyocytes are an important model system in molecular and cellular cardiology, and are used in many studies of caveolar signalling. At the same time, the extent to which structural and functional *in vivo* properties of caveolae are preserved *in vitro* is unknown. What is known is that isolated myocytes, even if optimally isolated and maintained, gradually change shape, structure and function post-isolation, towards a less *in vivo*-like state [52]. This includes a progressive reduction in T-tub density, by ~40% within 24h in rabbit [53, 54] and by ~60%-100% within 72h in rat [55]. These structural changes have marked effects on cell physiology, including excitation-contraction coupling. Here, we document a dynamic reduction in the presence of near-surface caveolae in rabbit ventricular cardiomyocytes within the first 8h post-isolation – a time-course that is directly relevant for research on acutely isolated cells.

Materials and Methods

All investigations conformed to the UK Home Office guidance on the Operation of Animals (Scientific Procedures) Act of 1986.

Heart and Cell Isolation

New Zealand white rabbit hearts (n=6 for intact tissue studies) were swiftly excised after Schedule 1 killing, Langendorff-perfused with normal Krebs-Henseleit solution (containing [in mM]: NaCl 118, KCl 4.75, CaCl₂ 2.5, NaHCO₃ 24.8, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 11, insulin 10 U/L, pH 7.4) and then cardioplegically arrested using high-K⁺ (25 mM). For tissue fixation, cardioplegically arrested hearts were coronary perfusion-fixed with iso-osmotic Karnovsky's reagent [56] (2.4% sodium cacodylate, 0.75% paraformaldehyde, 0.75% glutaraldehyde).

For cell isolation, hearts isolated as above (n=3 for imaging, n=10 for patch clamp recordings) were Langendorff-perfused with normal Tyrode solution (containing [in mM]: NaCl 140, KCl 5.4, HEPES 5, CaCl₂ 1.8, MgCl₂ 1, glucose 11, pH 7.4). The myocardium was enzymatically digested with collagenase and ventricular cardiomyocytes were isolated. After isolation, cells were held in a storage solution (containing [in mM]: NaCl 140, KCl 5.4, MgCl₂ 1, HEPES 5, glucose 11, CaCl₂ 1.8, trypsin inhibitor 0.17 g/L BSA 1 g/L, pH adjusted to 7.4 with NaOH) at room temperature until fixation. This solution was refreshed every 2 h.

All solutions were controlled for iso-osmolality (295-305 mOsm).

Electron Tomography (ET) of Ventricular Myocardium

Fixed tissue was washed with 0.1 M sodium cacodylate, post-fixed in 1% OsO₄ for 1 h, dehydrated in graded acetone, and embedded in Epon-Araldite resin. Thick sections (275 nm)

were cut, transferred onto copper slot grids and post-stained with 2% aqueous aranyl acetate, followed by Reynolds' lead citrate. Colloidal gold particles (15 nm) were added to both surfaces of the sections to serve as fiducial markers for post-processing tilt series alignment.

Preparations were imaged at the Boulder Laboratory for 3D Electron Microscopy of Cells (University of Colorado at Boulder, CO) using an intermediate voltage electron microscope (Tecnai F30; FEI-Company, Eindhoven, The Netherlands) operating at 300 kV, with images captured on a charge-coupled device camera (UltraScan; Gatan, Pleasanton, CA), at a pixel size of $(1.206 \text{ nm})^2$. For dual-axis tomography, series of tilted views were collected from $+60^\circ$ to -60° at 1° increments. After the first tilt series was acquired, the specimen was rotated by 90° in the horizontal plane, and another $+60^\circ$ to -60° tilt series was taken. The images from each tilt-series were aligned by fiducial marker tracking and back-projected to generate two single full-thickness reconstructed volumes (tomograms), which were then combined to generate a high-resolution 3D reconstruction of the original partial cell volume [57-59]. All tomograms were processed and analysed using the IMOD software, which was also used to generate 3D models of the relevant structures of interest [60]. Models were smoothed and meshed to obtain the final 3D representation, in which spatial relationships of surface sarcolemma, T-tub, and caveolae were visualised.

Transmission Electron Microscopy (TEM) of Isolated Cardiomyocytes

Cells were fixed (at 0h, 3h and 8h post-isolation) using iso-osmotic Karnovsky's reagent [57], embedded in LR white (Agar Scientific, UK), and sectioned at 80 nm thickness (Reichert Ultracut). Sections were post-stained with 2% aqueous uranyl acetate, followed by Reynolds' lead citrate. Cells were imaged by TEM (JEOL 1200EX II).

Scanning Electron Microscopy (SEM) of Isolated Cardiomyocytes

Cells were fixed as above, treated with 1% osmium tetroxide for 1 h, washed in PBS, dehydrated in ethanol and subjected to HDMS (hexamethyldisilazane). The protocol involved the following steps: transfer of cells on to a coverslip, ethanol dehydration series, drying for 3 minutes with HMDS, transfer onto SEM stub, coating with gold in a sputter coater, and imaging using SEM (JEOL JSM-5510); for detail – see [61].

For analysis of changes in area, diameter and circularity (ratio of short to long axis) of sub-sarcolemmal mitochondria, SEM images were analysed using custom written software (available by request from GB). Custom software (written in the Java programming language) allowed the operator to outline pixels containing mitochondria. The sub-image within the user generated outline was auto scaled and thresholded to select pixels corresponding to the mitochondria, and dilation operations were applied to the pixels to obtain a contiguous (filled) region that corresponded to the mitochondria's shape. The area of each mitochondrion is calculated by summing pixels in the contiguous region. The operator then uses an on-screen ruler to find the long and short axis of the mitochondria, and circularity is calculated from their ratio. The software generates an intensity profile for the ruler's path by summing pixel intensity values for three pixels perpendicularly on either side of the path along its entire length. Peaks in the intensity profile are used to measure distance between features in the image [62].

Cells were randomly distributed, relative to the observation angle of SEM image acquisition. While this increases the standard deviation of measurements (due to the cosine error), it does so in equal measure for all time points.

Membrane capacitance measurements

The patch-clamp technique (Axopatch 200B, Axon Instruments) was used to record whole-cell plasma membrane capacitance in whole cell mode. Cells were allowed to rest after isolation for 2h. To match structural observations, patch clamp data were binned into 0-3h, 4-5h and 6-8h (although the first group effectively contains data collected during the third hour only; it is accordingly a smaller sample). Data were analysed using pClamp10 from a standard pulse protocol (5 mV amplitude, 10 ms duration) to determine the value of cell capacitance. Capacitance was recorded immediately after membrane rupture; recordings with access resistance over 10 M Ω were rejected.

Statistics

All values are expressed as mean \pm SEM, statistical significance was assessed by one-way ANOVA.

Results

Dynamic changes in cardiomyocyte plasma membrane surface area were monitored by whole-cell membrane capacitance measurement [63] using the patch clamp technique. Cell capacitance gradually increased over an 8h period post-isolation.

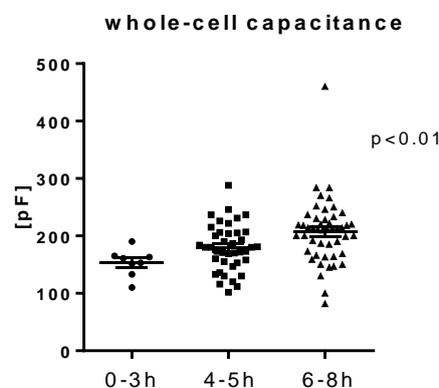


Fig 1: Whole-cell plasma membrane capacitance recordings of rabbit ventricular myocytes, showing gradual increase over time post-isolation. Data were analysed using one way ANOVA, significance indicated for overall effect of time; n=8 (0-3h), n=40 (4-5h), n=48 (6-8h) cells.

Surface-sarcolemmal caveolae numbers in ventricular cardiomyocytes post-isolation

The ultrastructure of the surface membrane, and the distribution of surface-sarcolemmal caveolae was studied over time following isolation using TEM. Surface-sarcolemmal

caveolae were defined as either visibly connected to the surface sarcolemma, or separate in a given TEM image by no more than ≤ 100 nm from the plasma membrane (measured from the surface-sarcolemmal caveolae-centre). There was a significant decrease over time post isolation (by 34% at 3h, and by 49% at 8h post-isolation, when compared to 0h) in the overall number of surface-sarcolemmal caveolae per membrane segment spanning two neighbouring z-lines (henceforth referred to as ‘per sarcomere’; Fig. 2, Table 1). Additionally, we observed an overall reduction in total cellular Cav-3 protein after 8h post-isolation (Supplementary Fig. S1).

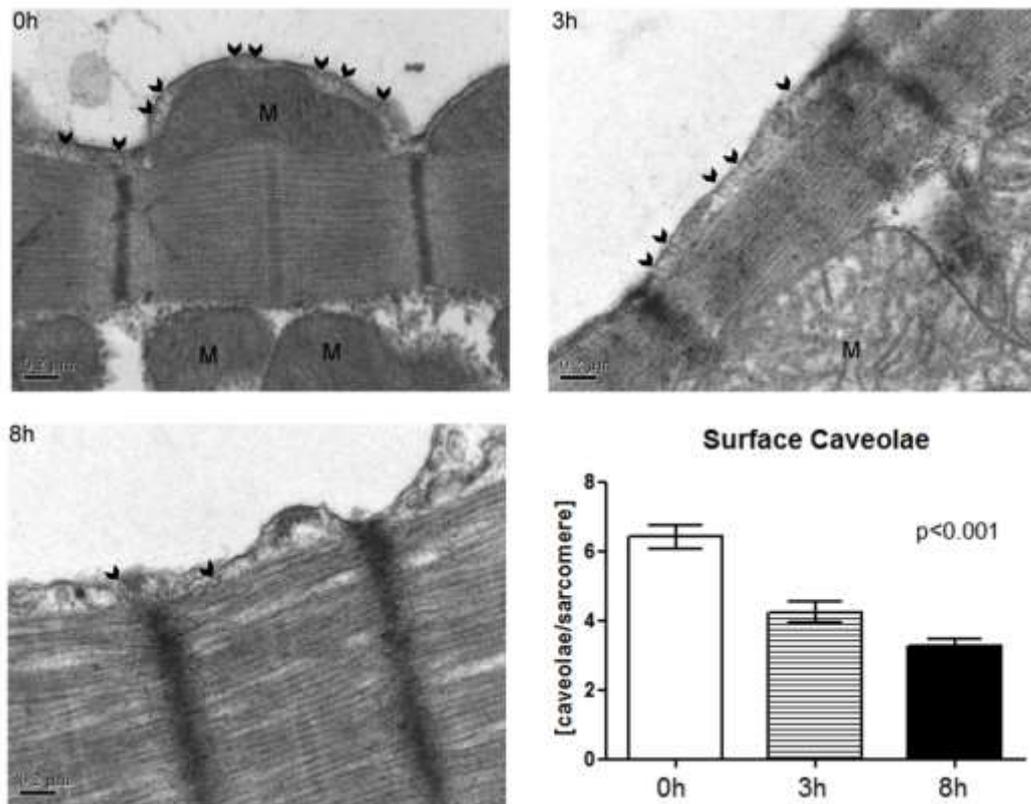


Fig 2: Transmission electron micrographs (TEM) showing changes in the distribution of sarcolemmal caveolae (surface-sarcolemmal caveolae, examples indicated by arrows) in rabbit ventricular myocytes at 0h, 3h and 8h post-isolation. Numbers of surface-sarcolemmal caveolae is scaled per membrane segment spanning two neighbouring z-lines (‘per sarcomere’). This number declined with time after cell isolation. Surface-sarcolemmal caveolae were quantified for 9-12 sarcomeres per cell, in 6 myocytes at each time point. Data were analysed using one way ANOVA, significance indicated for overall effect of time; M-mitochondria. Scale bar = 0.2 μ m.

Table 1. Surface-sarcolemmal caveolae presence in intact tissue (ET data), and in isolated cardiomyocytes (TEM data) over time post-isolation.

Time-point	Surface-sarcolemmal caveolae/sarcomere (mean±SEM)	Sarcomere/cells analysed	Fractional Change (vs. intact tissue)
Reference: Intact tissue (ET)	6.22 ± 0.64	26 sarcomeres/21 cells	1.00
0h post-isolation	6.41 ± 0.35	54 sarcomeres/6 cells	1.03
3h post-isolation	4.24 ± 0.31	59 sarcomeres/6 cells	0.68 (0.66 vs 0h)
8h post-isolation	3.27 ± 0.19	60 sarcomeres/6 cells	0.53 (0.51 vs 0h)

Note: surface-sarcolemmal caveolae/sarcomere in ET data includes an 80 nm wide sarcolemmal strip, connecting two z-lines, mimicking the section thickness of TEM.

Changes in surface topology of ventricular cardiomyocytes post-isolation

SEM revealed an increase in area, diameter and circularity of sub-sarcolemmal membrane protrusions, commonly associated with sites of mitochondria (Fig. 3). Protrusions typically correspond to a single mitochondrion (e.g. Fig. 2, 0h). Observed changes in size and shape are indicative of mitochondrial swelling.

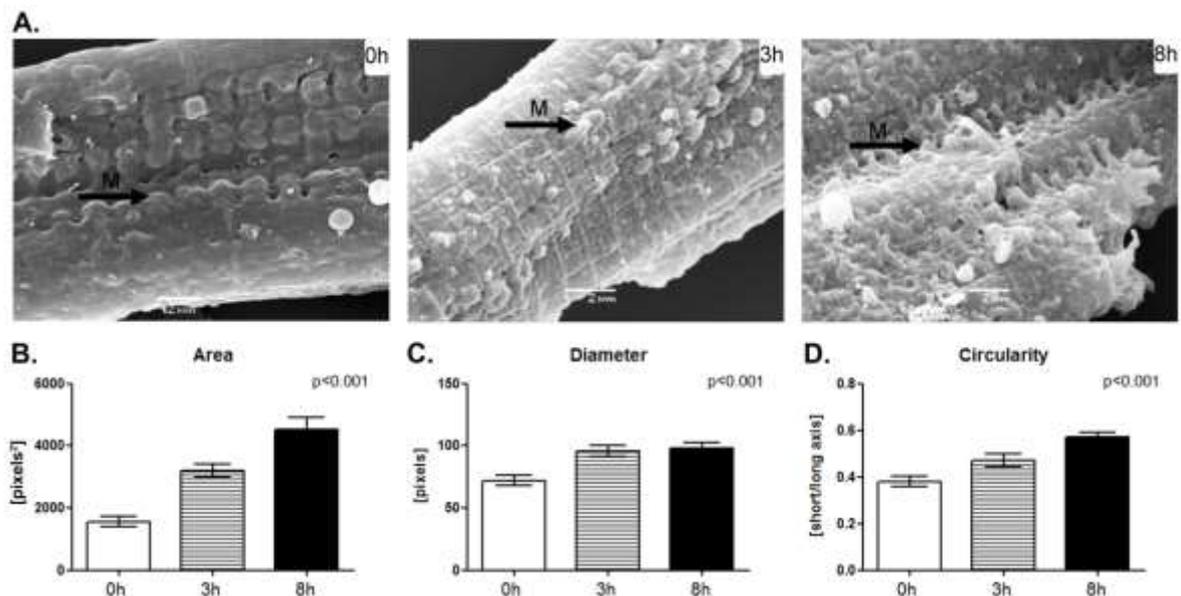


Fig 3: Scanning electron micrographs of cardiomyocyte surface topology (A) at 0h, 3h and 8h post-isolation, with protrusions of the sarcolemmal membrane (arrows labelled M) increasingly prominent. Area (B), diameter (C), and circularity (D) of membrane protrusions

increased with time. [3 cells per time point; mitochondria n=40 (0h), n=27 (3h) and n=39 (8h)]. Significance indicated for overall effect of time. Scale bar = 2 μm .

Intracellular caveolae distribution in ventricular tissue and isolated cells

Using ET we quantified the presence of caveolae in intact left ventricular rabbit tissue (Fig. 4, Supplementary video 1). Surface-sarcolemmal caveolae were found at a frequency of 6.22 ± 0.64 caveolae/sarcomere, similar to that seen in freshly isolated cardiomyocytes (Table 1).

Additionally, we observed the regular presence of T-tub caveolae in intact myocardial tissue (Fig. 4, Supplementary video 2). T-tub caveolae were seen in 133 (63%) of 209 T-tub segments analysed (average segment length 275 nm), and in every cell tested. The density of T-tub caveolae was lower than that of surface-sarcolemmal caveolae (3.23 ± 0.3 vs 12.32 ± 1.26 per $1 \mu\text{m}^2$ membrane area, respectively). **These structures were different to the T-tub folds described previously in the murine myocytes [64].**

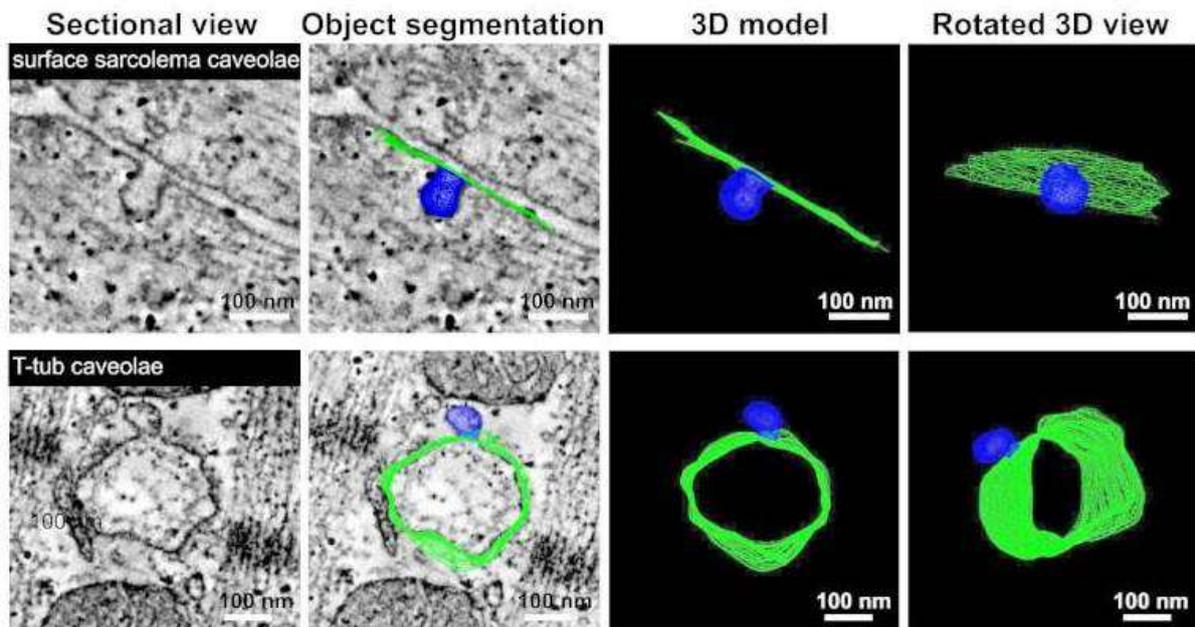


Fig 4: Representative electron tomography (ET) images of surface-sarcolemmal caveolae (top panels) and T-tub caveolae (bottom panels) in rabbit ventricular tissue. Dual-axis ET was used to image, reconstruct and model surface-sarcolemmal and T-tub caveolae and host their membranes in 3D. Isotropic voxel size $(1.206 \text{ nm})^3$, z-depth – 275 nm; green – host membrane surface, blue – caveolae.

There was no significant difference between surface area, volume and major axis diameter of caveolae from the different sub-cellular locations (Table 2).

Table 2. Surface-sarcolemmal and T-tub caveolar dimensions in intact ventricular tissue (data from ET reconstructions).

Caveolae type	Longest axis length (nm)	Volume (nm ³)	Surface (nm ²)
Surface-sarcolemmal caveolae (n=45)	77.4±2.42	(276±19) x 10 ³	(20.32±0.02) x 10 ³
T-tub caveolae (n=64)	73.9±2.38 (n.s.)	(244±19) x 10 ³ (n.s.)	(19.75±0.01) x 10 ³ (n.s.)

n.s.: no significant difference between surface-sarcolemmal and T-tub caveolae

With the knowledge of T-tub caveolae presence in rabbit ventricular cardiomyocytes, re-examination of TEM images revealed T-tub caveolae-like structures in 2D micrographs of isolated cells (Fig. 5). However, given the small radius of T-tub membrane curvature, compared to the sarcolemmal surface, nature and interrelation of probable T-tub caveolae with the T-tub surface membrane cannot be established with certainty in 2D sections (see Fig. 6).

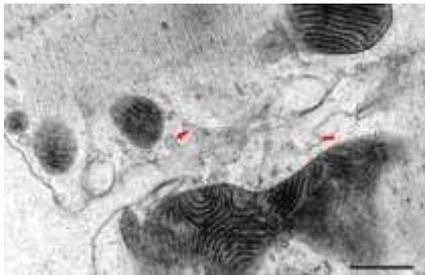


Fig 5: T-tub caveolae-compatible structures (arrows) identified in 2D (thin section) TEM of freshly isolated (0 h) rabbit ventricular cardiomyocytes. Scale bar = 500 nm.

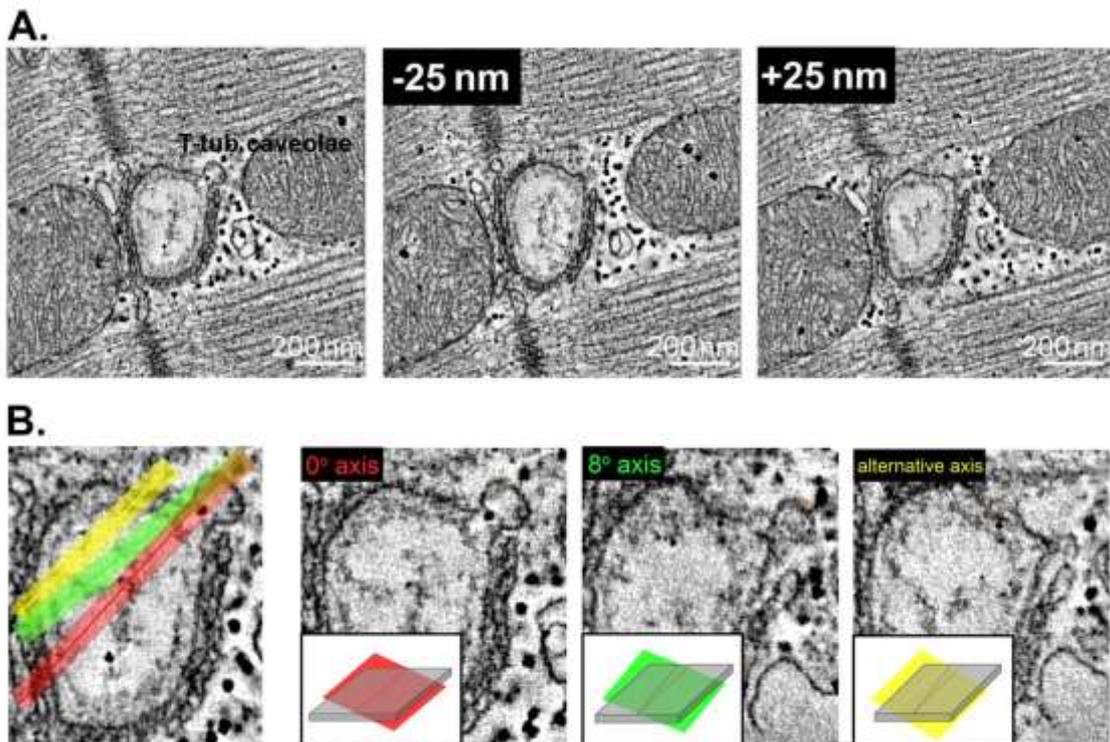


Fig 6: Illustration of technical challenges associated with identifying T-tub caveolae in 2D sections. A tomographic slice containing a representative T-tub caveolae is virtually sectioned along different planes to simulate TEM-like data with arbitrary second planes relative to structures of interest. Both the level (A) and the angle (B) of the cutting plane (shaded area illustrating thickness of a typical TEM slice) relative to the imaged structure affect the likelihood of positively identifying T-tub caveolae in non-3D data sets. Scale bar = 200 nm.

Presence of surface-sarcolemmal and T-tub caveolae in TEM images: Spatial density considerations

The relatively abundant presence of T-tub caveolae in the 3D EM data runs counter to general perception, based on decades of experience with TEM. How might this come about?

Based on experiments with cell capacitance recordings before and after de-tubulation, one can conclude that between one third and two thirds of total cell surface membrane is contained in T-tub [53]. If, for ball-park estimation, one assumes a 50:50 split, and considers the 1:4 difference in T-tub caveolae density per unit of membrane compared to surface-sarcolemmal caveolae, there should be 4 times more surface-sarcolemmal caveolae in a single cell than T-tub caveolae. This alone does not explain the hitherto rare detection of T-tub caveolae in ventricular myocyte TEM work.

Figure 7A illustrates the spatial relations between a typical TEM section (approximate thickness $\sim 0.1 \mu\text{m}$) and a cardiac myocyte (approximate dimensions $H \times W \times L = 10 \times 20 \times 150 \mu\text{m}$). The total cell surface is made up of $2A + 2B + 2C$. Given the high aspect ratio of the cell, areas $A : B : C$ are proportional to $15 : 30 : 2$, so that for the most part, the contribution from C can be ignored. Therefore, considering a TEM section through the long axis of the cell (as illustrated in Fig. 7A), the only contributions to presence of surface-sarcolemmal caveolae will come from intersections of the TEM sample with the two A -surfaces. These surfaces will contain a fraction of total surface-sarcolemmal caveolae, equivalent to $2A / (2A + 2B)$, or – using the above ratio of surface areas – $1/3$ of all surface-sarcolemmal caveolae in that cell. Any $0.1 \mu\text{m}$ TEM section cutting through this $10 \mu\text{m}$ -high cell will therefore contain $0.01 \times 1/3$ of all surface-sarcolemmal caveolae. By comparison, as the sections will cut through the entire volume of the cell, a single section will contain 0.01 of all T-tub caveolae. Combining this with the relative host-surface densities (four times higher density for surface-sarcolemmal caveolae than T-tub caveolae) the difference in the number of surface-sarcolemmal caveolae and T-tub caveolae contained in any given TEM-section is small ($\text{surface-sarcolemmal caveolae}_{\text{TEM}} = (4/3) \times \text{T-tub caveolae}_{\text{TEM}}$). This makes it even more surprising that T-tub caveolae have not been reported more regularly in prior TEM studies.

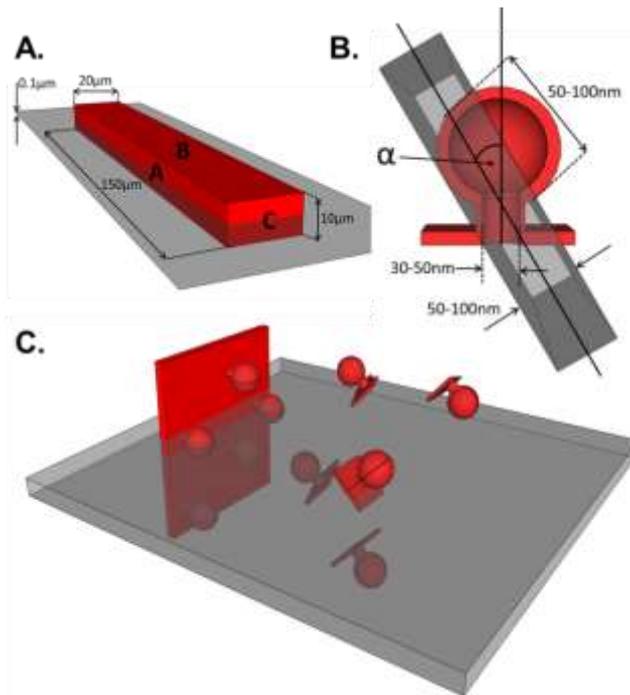


Fig 7: (A) Model cardiomyocyte showing approximate interrelations of surfaces ‘A’, ‘B’ and ‘C’ with a TEM cutting plane (grey). (B) A cut-away through a model caveolae showing approximate dimensions. The TEM section is oriented at an angle α relative to the caveolae-centric orientation that would be optimal for identification. (C) Illustration showing the different likelihood of imaging caveolae on the sarcolemma (left) at an angle amenable for identification, compared to randomly

oriented caveolae on T-tub.

Identifiability of surface-sarcolemmal and T-tub caveolae in TEM images:

If there is a similar net presence of surface-sarcolemmal and T-tub caveolae per whole-cell TEM section, the question arises as to why T-tub caveolae may still end up being under-detected. In order to identify caveolae beyond doubt, a TEM section should run through the lumen of the ‘bottle-neck’ connecting the circular caveolae structure to the host membrane. In Figure 7B, the caveolae-centric line, running parallel to the axis of the neck, illustrates the orientation of an ideally-angled and -positioned TEM section. Shown in grey and at an angle α to this line is the case for a TEM sectioning plane that runs through the lumen of the caveolae-connection at a non-ideal angle. If α exceeds a critical level (α_c), the presence of a caveolae structure will not be confirmed *even if the cut runs through the centre of the neck area*. If, in addition, the TEM sectioning plane is off-set laterally (not running through the neck), one would see a caveolae-sized circular structure whose connection to the host membrane cannot be confirmed, so one would not be able to positively identify this, for example as T-tub caveolae, based on 2D TEM imaging data alone (see Fig. 6B).

The likelihood of any TEM section plane to run through the neck region of randomly distributed surface-sarcolemmal and T-tub caveolae may be assumed to be identical. However, the likelihood of a favourable angular orientation of surface-sarcolemmal or T-tub caveolae with TEM sectioning differs. This is illustrated in Figure 7C. While surface-sarcolemmal caveolae will generally be orientated roughly orthogonal to the outer cell surface, T-tub caveolae may be aligned in any direction, depending on the orientation of the host T-tub (which, in rabbit, include spoke-like transversal and additional cell-axis parallel segments [65]). From these, T-tub caveolae can protrude radially in all surface-orthogonal directions. The question then is what fraction of caveolae, cut at an optimal level – i.e.

through the centre of the neck region – will be positively identifiable as caveolae in TEM images.

The angle, α_C , defines a cone that contains all TEM imaging planes that will yield an identifiable image of caveolae. The volume fraction of this cone, relative to a half-sphere, equals $1 - \cos \alpha_C$. An estimate of α_C has been obtained by simulating TEM sections in the 3D ET data, with estimates for the value of α_C varying between 8° and 20° , and an average value of 13° (see example in Fig. 6B) This correlates to a fraction of possible TEM sectioning planes relative to caveolae orientation that permit positive identification of about 2.5% (at $\alpha_C = 13^\circ$; ranging from 1.0% at 8° to 6.0% at 20°).

If one assumes, therefore, that T-tub caveolae can be randomly distributed and oriented in the cell (Fig. 7C), one should expect to be able to identify no more than a few % of those that are present in a TEM section and cut favorably (i.e. through the lumen of the neck region). In contrast, for surface-sarcolemmal caveolae, identifiability – if cut through the neck-region – will be close to 100% for two reasons. First, they are aligned near-perpendicular to the cell surface membrane. Second, TEM imaging of densely populated cardiac myocytes is user-biased towards alignment of sectioning planes with either the long or the short axis of the cell's contractile lattice structures – and hence more or less perpendicular to the sarcolemma – the host membrane of surface-sarcolemmal caveolae.

As an aside, reliable identification of caveolae by definition also includes recognition of the host membrane, which is usually much more straightforward for surface sarcolemma, compared to less regular and variably orientated intracellular membrane systems. If one further takes into account the 'best-case scenario' of histologically convincing identification, i.e. a cut that not only captures T-tub caveolae favourably (right level and orientation), but also runs either longitudinally through a suitably long section of T-tub to allow their identification beyond doubt (such as shown in Fig. 5), or perpendicularly through a clearly identifiable T-tub (such as for example in Fig. 5C of [65]), the fraction of identifiable T-tub caveolae is further reduced by about one order of magnitude, i.e. well below 1%.

Thus, in order to successfully identify a caveolae from a TEM image, caveolae must (i) be present (expectation here: surface-sarcolemmal and T-tub caveolae may be relatively evenly matched per whole-cell section); (ii) be cut at the right location (centre of neck region, also with the expectation that this may occur equally frequently for surface-sarcolemmal and T-tub caveolae) and (iii) be cut at an amenable angle (which is likely to occur with a two to three orders of magnitude higher probability for surface-sarcolemmal caveolae compared to T-tub caveolae). This is before taking into consideration that user-'optimization' of cardiac cell TEM data will also optimize surface-sarcolemmal caveolae-detection (given the surface-normal orientation of surface-sarcolemmal caveolae: if you see one – you see all that are contained in a section and its serially cut neighbours; something that cannot be done for T-tub caveolae - even if it was desired).

Discussion

Caveolae are centres of cardiac signal transduction, frequently studied in isolated cells. Although changes in cardiomyocyte structure (such as shape) and ultrastructure (e.g. T-tub integrity [53]) have been reported after cell isolation (though generally thought to become relevant over days, not hours) [28, 66], little is known about caveolae preservation in isolated myocytes. Here we report significant changes in surface sarcolemmal caveolae distribution within hours of cell isolation, with reductions in surface-sarcolemmal caveolae numbers by a third within 3h, and down to half by 8h. This marked change has a potentially significant impact on functional studies conducted on isolated cells over matching time periods. Among the types of studies potentially affected by changing caveolae distribution over time are electrophysiological investigations (e.g. via caveolar effects on calcium-induced calcium release [67]), studies of intracellular signaling (e.g. via G-protein coupled receptors [68, 69], adrenergic and NO-mediated pathways [70, 71], PDGF and EGFR [72]) as well as experimental research into mechano-sensing [23]. Additionally, one should keep in mind the potential impact of changes in caveolae distribution on cytoskeletal function [21, 73-75].

There are several potential functional implications of the result of our study. Caveolae can act as a membrane reserve which buffers membrane tension and protects the membrane from rupture. Loss of 75% of caveolae from the cardiac myocyte membrane by methyl-beta-cyclodextrin (MBCD) has been shown to enhance mechanotransductive processes and cell lysis in response to cell swelling [76]. In addition, the same degree of caveolar disruption enhances the contractile response to β 2-adrenergic stimulation in the cardiac cell, through the loss of normal compartmentalisation of signal transduction components concentrated within the caveolar microdomain [77]. The profound effect of a 75% reduction of caveolae on cardiac myocyte function suggests that the 50% loss of caveolae we report here will likewise have functional consequences.

Reduction in surface-sarcolemmal caveolae could be occurring by internalization, loss to the extracellular space, or *via* incorporation of caveolae into the surface sarcolemma. In support of the latter we observed an increase in cell capacitance in the first 8 hours after isolation, suggesting an increase in the total surface membrane area. The increased membrane capacitance and altered membrane surface to volume relationship could lead to changes in the charge required to depolarise the membrane, reduced upstroke velocity, and slowing of conduction [29]. This should be taken into account in research targeted at, or affected by, caveolae structure and function.

Since cardiomyocytes swell after isolation [78], even in iso-osmotic conditions (attributed predominantly to oncotic pressure gradients), it is possible that associated mechanical factors contribute to the destabilisation of caveolar structures, with membrane incorporation (“flattening”) of caveolae, as reported previously in rabbit ventricular tissue subjected to volume overload (stretching cells) or hyposmotic perfusion (swelling cells) [28, 30, 33]. This is in keeping with indications of mitochondrial swelling, observed in SEM images of cell surface topology. As sub-sarcolemmal mitochondria are constrained from centripetal movement by the contractile filament lattice [79], their swelling could enhance local stretch of the cell membrane, perhaps aiding incorporation of surface-sarcolemmal caveolae into the

sarcolemma.

In addition to surface-sarcolemmal caveolae, we document the regular presence of T-tub caveolae in T-tub membranes. Diameter and volumes of T-tub caveolae are indistinguishable from surface-sarcolemmal caveolae, though T-tub caveolae have a roughly four-fold lower density per host membrane area (~3 T-tub caveolae per $1 \mu\text{m}^2$ T-tub membrane area) compared to surface-sarcolemmal caveolae (~12 per $1 \mu\text{m}^2$ sarcolemma). In either case, membrane contained in caveolae represents an additional 6-24% membrane 'reserve' ($3-12 \times 0.02 \mu\text{m}^2$ per $1 \mu\text{m}^2$ of host membrane), based on our rabbit ventricular ET data.

Caveolae-like shapes have been observed in close proximity to T-tub in earlier TEM studies [37, 38, 80]. However, their existence has remained controversial [81]. This is, in part, due to the technical challenges associated with reliable T-tub caveolae identification in typical 80 nm thin sections, and no prior studies have assessed T-tub caveolae in 3D EM tomography data. As an illustration, Fig. 6 documents what one *bona-fide* T-tub caveolae, identified by ET, would look like in a series of 2D TEM sections at different imaging planes. This highlights that without 3D tracing it is difficult to identify the presence T-tub caveolae and their interrelation with the T-tub host membrane.

Early studies by Parton *et al.* [40] found that Cav-3 is transiently associated with T-tub during development. It has been suggested that caveolae are required for the formation of the T-tub system via repetitive generation of inwardly directed sarcolemmal membrane nano-bulges ("beaded tubules" theory) [40, 82-84]. Supporting this theory is the fact that both T-tub and caveolae share similarities in protein and lipid composition which is considerably different from that of the surface sarcolemma [41]. Furthermore, Cav-3 null mice show not only loss of caveolae, but also abnormalities in T-tub system organization (in skeletal muscle), such as dilatation and loss of transverse orientation [12] and double Cav-1/Cav-3 knockout mice show complete loss of cardiac caveolae, T-tub disorganization and severe cardiomyopathy [12, 14]. In contrast, in adult myocytes, it has been proposed that T-tub Cav-3 forms scaffolds rather than morphologically identifiable caveolae structures [70]. Since T-tub are an important determinant of cardiac cell function and changes in their structure and protein expression occur during development and heart failure (see review [85]), the presence of caveolae (and their associated proteins) on the T-tub could potentially contribute to important physiological and pathological functions. Whether T-tub caveolae serve as signaling centers, and to what extent this may involve mechano-sensitive alterations in T-tub structure during the cardiac cycle (similarly to CAVs [86, 87]), remains to be elucidated.

EM tomography has been used in this study as it is the only imaging method capable of resolving caveolar arrangements in 3 dimensions – necessary to discover and quantify these very small structures. Data from EM tomography compared favorably with those of more conventional techniques, such as TEM and SEM. To conclude, we (i) report dynamic remodeling of surface-sarcolemmal caveolae structures in rabbit ventricular cardiomyocytes post-isolation and provide functional assessment indicative of electrophysiological consequences, (ii) establish that this occurs over a 3-8h time-course which is relevant for research using acutely isolated cells, (iii) confirm the additional regular presence of caveolae on membranes of the T-tub system, and (iv) explain why the latter may have been overlooked

in previous ultrastructural research. It will be important now to investigate potential strategies to preserve caveolae integrity in isolated cells. Given the huge parameter space for possible modifications, a useful step would come from raising awareness of this as a variable parameter that should be assessed when evaluating existing cell isolation protocols by the different teams using acutely isolated cardiac cells.

Author contributions

R.A.B.B, E.A.R.-Z, G.B and P.K designed research; R.A.B.B, E.A.R.-Z, R.P.J.P, I.B., M.F and J.S performed research; A.D.C, A.H and G.B contributed analytic tools; R.A.B.B, E.A.R.-Z, R.P.J.P, I.B, A.D.C and S.C. analyzed and interpreted data; R.A.B.B, E.A.R.-Z, A.D.C, S.C and P.K wrote the paper, all authors read and approved the submitted manuscript.

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Appendix

Formulating a description of the intersection area, A

Starting with the axis of the cylinder oriented normally to the image plane ($\varphi=90^\circ$) and then gradually reducing the value of φ , it can be seen that a point is reached at which the full width of the image section is completely contained within the cylinder. At this point, the diagonal of the image section makes an angle φ_C (the critical angle) with the axis of the cylinder. The variation in A with the rotation angle φ can then be conveniently broken down into two

regimes; first for $\varphi > \varphi_C$ and then $\varphi \leq \varphi_C$. For $\varphi > \varphi_C$, the intersection area is labelled A_1 and for $\varphi \leq \varphi_{crit}$, the intersection area is labelled A_2 .

For $\varphi > \varphi_C$ the image section always extends beyond the length of the cylinder, with the intersection area reaching a minimum at $\varphi = 90^\circ$. For values smaller than 90° , the perimeter of the intersection area will describe an ellipse, with minor axis $a = r$ and major axis $b = (r/\sin(\varphi))$. A first approximation to A will therefore be given by the product of the circumference of the ellipse, C and the sectioning thickness, t . The parameter, C , can be approximated using the expression given in Eq. Equation 1 below:

$$C \approx 2\pi \cdot \left[0.5 \cdot (a^2 + b^2)\right]^{\frac{1}{2}} \quad \text{Equation 1}$$

Making substitutions for a and b we therefore have that:

$$A_1 = C \cdot t = 2\pi \cdot r \cdot t \cdot \left[0.5 \cdot \left(1 + (1/\sin(\varphi))^2\right)\right]^{\frac{1}{2}} \quad \text{Equation 2}$$

It can be seen that for $\varphi = 90^\circ$, $A = 2\pi r t$, which is as expected from the product of the cylinder circumference ($2\pi r$) and the section thickness, t . The expression in Equation 2 describes the regime for $\varphi > \varphi_C$. For $\varphi \leq \varphi_C$ the intersection area is maximised as the image section is completely contained within the walls of the cylinder. Introducing a factor of two to account for the intersection areas on both sides of the cylinder we can approximate the intersection area as:

$$A_2 = 2 \cdot W \cdot t \quad \text{Equation 3}$$

Calculating the expectation value of A

As not all orientations of the cylinder are equally likely, we need to modulate the above expressions for A by the probability, dP , of the cylinder having angle φ , to form the expectation value for A . The first task is then to form a probability distribution function that describes the likelihood of a randomly oriented cylinder being inclined at angle φ with respect to the image plane. For a purely random orientation, the end point of a vector running parallel to the cylinder axis (the axis vector) will describe a spherical shell of uniformly distributed points (Figure A1).

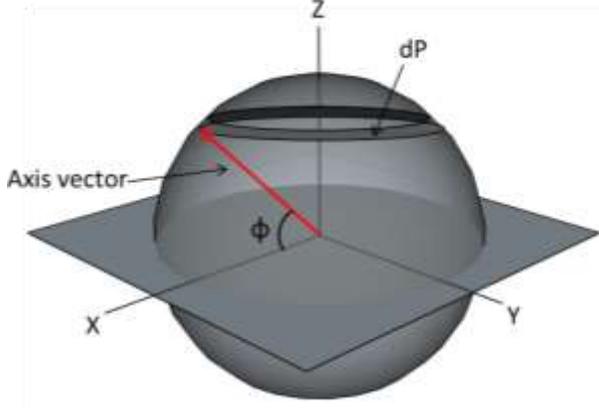


Figure A1: Showing the elemental area dP which is directly proportional to the probability of the cylinder axis having orientation angle ϕ .

The probability of a given angle, ϕ , will therefore be proportional to the area, dP , on the sphere which is given by:

$$dP = 2\pi \cdot \cos(\phi) \cdot d\phi \quad \text{Equation 4}$$

We now have sufficient information to calculate the expectation value for the intersection area:

$$\langle A \rangle = \langle A_1 \rangle + \langle A_2 \rangle = \frac{2}{4\pi} \cdot \left[\int_{\phi_c}^{\pi/2} A_1 \cdot dP + \int_0^{\phi_c} A_2 \cdot dP \right] \quad \text{Equation 5}$$

In Eq. Equation 5 we have normalized by 4π to ensure that the total probability sums to unity. The first expression for A_1 can be written as:

$$\langle A_1 \rangle = \frac{2}{4\pi} \cdot \left[\int_{\phi_c}^{\pi/2} 2\pi r t \left[0.5 \cdot \left(1 + (1/\sin(\phi))^2 \right) \right]^{\frac{1}{2}} \cdot \cos(\phi) d\phi \right] \quad \text{Equation 6}$$

It is possible to simplify this expression by setting $x = \sin(\phi)$:

$$\langle A_1 \rangle = \frac{rt}{\sqrt{2}} \cdot \int_{\sin(\varphi_c)}^1 \left(1 + (1/x)^2\right)^{\frac{1}{2}} \cdot dx \quad \text{Equation 7}$$

Eq. Equation 7 describes an elliptical integral which can be represented as:

$$\langle A_1 \rangle = \frac{2\pi \cdot rt}{\sqrt{2}} \cdot \left[\frac{x\sqrt{1+(1/x)^2}}{\sqrt{x^2+1}} \left(\sqrt{x^2+1} - \log\left(1 + \sqrt{x^2+1}\right) + \log(x) \right) \right]_{\sin(\varphi_c)}^1 \quad \text{Equation 8}$$

Evaluating this function for the experimental value of $\varphi=6.6^\circ$ we get:

$$\langle A_1 \rangle = 10.6 \cdot rt = 1.28 \cdot 10^5 \text{ nm}^2 \quad \text{Equation 9}$$

We can now calculate the expectation value of A_2 which is much more straightforward:

$$\langle A_2 \rangle = \int_0^{\varphi_c} 2Wt \cdot \cos(\varphi) d\varphi = 2Wt \cdot \sin(\varphi_c) = 4.8 \cdot 10^4 \text{ nm}^2 \quad \text{Equation 10}$$

Combining the results from Eq. Equation 9 and Eq. Equation 10 we have a total expectation value of:

$$\langle A \rangle = 1.75 \cdot 10^5 \text{ nm}^2 \quad \text{Equation 11}$$

Modelling the likelihood of detecting T-tub caveolae (abbreviated CAV_T for equations)

Using a simplistic model, one can estimate the probability of detecting a caveolae by pure chance in a cylindrical structure (e.g. a T-tub) which has a radius, r , and a length which extends beyond the field of view of the TEM image. In this model it is assumed that T-tub caveolae will be imaged if they lie within an area described by the intersection of the cylinder and a rectangular slab of width w and thickness, t , corresponding to the TEM image section (see Figure A2.A). Assuming T-tub caveolae can be randomly distributed across the surface area of the cylindrical structures, the likelihood of seeing the caveolae in close proximity to the T-tub varies in direct proportion with the size of the intersection area, A . This model determines how A varies with the relative orientation of the cylindrical structure and the image section. In principle, the orientation of either the cylinder or the image section can be changed. In this description, it is the cylinder which is reoriented, forming an angle φ ,

between the cylinder axis and the plane of the image section (Figure A2.A)

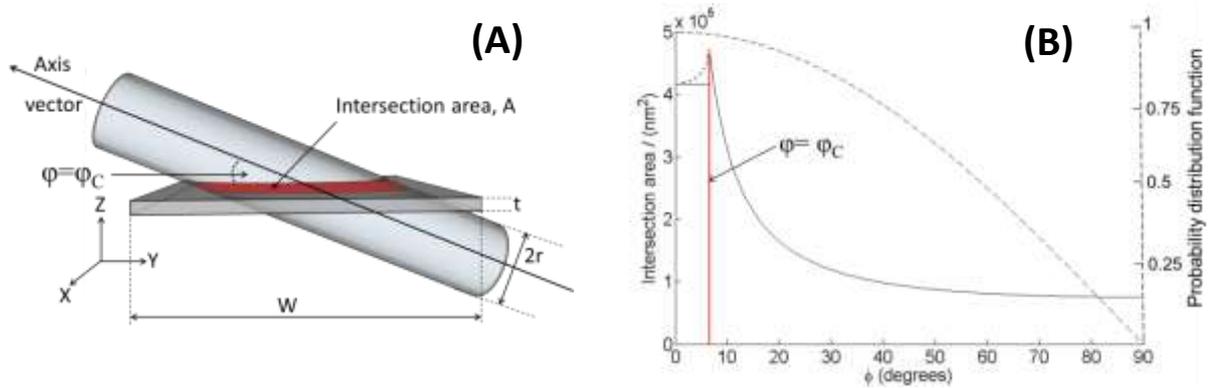


Figure A2: (A) Model of the intersection area, A , of a cylindrical structure (T-tub) of radius, r , and a slab (TEM image section) of width w and thickness t . The cylinder axis makes an angle ϕ with the plane of the slab and the cylinder is shown oriented at the critical angle ($\phi = \phi_c$). (B) Solid curve describes the variation in the interface area, A (left hand axis), as a function of the inclination angle of the cylinder, ϕ (phi) as predicted by the model. The small dashed black line predicts the variation in intersection area for $\phi < \phi_c$. The large dashed curve indicates the probability distribution function (right hand axis) for the cylinder orientation as a function of ϕ .

A plot of $A(\phi)$ can be seen in Figure A2.B. Here we have substituted experimental values for t , r and w (80 nm, 150 nm and 2600 nm respectively). It can be seen that the model of $A(\phi)$ has been split into two parts, A_1 and A_2 depending upon whether the inclination angle of the cylinder is above or below the critical angle (demonstrated in Figure A2.A) at which $\phi = \sin^{-1}(2r/W)$. The mismatch between the models for A_1 and A_2 at $\phi = \phi_c$ are a consequence of the simplicity of the model for A_2 , which underestimates A for $\phi > 0$. A more sophisticated model would appear like the small dashed line shown in Figure A2.B, although, as it is clear to see from the figure, this does not significantly change the result.

The function describing $A(\phi)$ can be multiplied by a probability distribution which determines the likelihood of a randomly oriented cylinder having an orientation of ϕ (large dashed line in Figure A2.B). Integrating this product over all possible values of ϕ delivers the expectation value of A , which is to say the statistical average value of the intersection area. For the parameter values applicable to the above experiment, the expectation value for a single T-tub was calculated to be (see appendix for full details):

$$\langle A_T \rangle = 0.175 \mu\text{m}^2 \quad \text{Equation 124}$$

From the measured surface density value of 3.23 T-tub caveolae per μm^2 of T-tub outer surface it is possible to calculate the expectation value for the number of T-tub caveolae seen in an average TEM image of a randomly oriented T-tub:

$$\langle \text{CAV}_T \rangle = 0.175 \mu\text{m}^2 \times 3.23 \text{CAV}_T / \mu\text{m}^2 = 0.57 \text{CAV}_T \quad \text{Equation 15}$$

This tells us that for a randomly oriented T-tub, we would on average expect to image around one T-tub caveolae every two TEM images. This calculation assumes only a single T-tub within the $2.6 \mu\text{m} \times 2.6 \mu\text{m}$ field of view of each TEM image, which is likely to be different in most cases, depending on the deviation of the cutting plane from a cut perpendicular to T-tub orientation. Nonetheless, this simplified analysis implies that T-tub caveolae should be a common feature in TEM images. This issue is further explored in the section “*Presence of surface-sarcolemmal caveolae and T-tub caveolae in TEM images: Spatial density considerations*”.

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Supplementary Material

Supplementary Figure S1. Whole cell Cav-3 protein levels are reduced after 8h, as shown by Western blot of isolated cell extracts (left, n = 3 cell isolations; data analysed using ANOVA, significance indicated for the overall effect of time) and measurements of fluorescence intensity in isolated cells (right, n = 18 cells from 3 isolations for each time point; data analysed using ANCOVA, # p=0.002 vs 0h; scale bar = 20 μ m).

Whether this is indicative of internalisation and protein degradation, or loss to the extracellular space, requires further elucidation.

