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Title: The structural basis for intermitochondrial communications is fundamentally different in cardiac and skeletal muscle.

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Running Title: Intermitochondrial communications in cardiac and skeletal muscle.

Abstract: This review focuses on recent discoveries in skeletal and cardiac muscles indicating that mitochondria behave as an interactive cohort with inter-organelle communication and specific reactions to stress signals. Our new finding is that inter-mitochondrial communications in cardiac and skeletal muscles rely on two distinct methods. In cardiac muscle, mitochondria are discrete entities and are fairly well immobilized in a structural context. The organelles have developed a unique method of communication, via nanotunnels, that allow temporary connection from one mitochondrion to another over distance of up to several microns, without overall movement of the individual organelles and loss of their identity. Skeletal muscle mitochondria, on the other hand, are quite dynamic. Through fusion, fission and elongation they form connections that include constrictions and connecting ducts (quite distinct from nanotunnels) and loose individual identity in the formation of extensive networks. Connecting elements in skeletal muscle are distinct from nanotunnels in

cardiac muscle.

New Findings: This review summarizes recent discoveries in mitochondria development and morphology studied with electron microscopy. Although mitochondria are generally considered as isolated from each other, this review highlights recently discovered evidence for the presence of inter-mitochondrial communication structures in cardiac and skeletal muscle, in animal models and humans. Even within striated muscles, means of inter-mitochondria exchanges and mitochondria reaction to external stimuli are uniquely dependent on the tissue, and we clearly differentiate between nanotunnels, the active protrusion of cardiac mitochondria, and the connecting ducts of skeletal muscle derived from fusion-fission and elongation events.

Dual Publication: This submission is a review. Figure 5 is an unprecedented finding and belongs to a manuscript that we are due to resubmit to the NEJM very shortly. If accepted, we respectfully ask to coordinate the publication of our review so that it appears together with our above-mentioned original article, please.

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The structural basis for intermitochondrial communications is fundamentally different in cardiac and skeletal muscle.

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Author contributions

ML provided the majority of the data; FF provided data and contributed to manuscript; CFA wrote the manuscript. All authors participated in the revision of the manuscript, approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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New Findings

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Although mitochondria are generally considered as isolated from each other, this review highlights recently discovered evidence for the presence of inter-mitochondrial communication structures in cardiac and skeletal muscle, in animal models and humans. Even within striated muscles, means of inter-mitochondria exchanges and mitochondria reaction to external stimuli are uniquely dependent on the tissue, and we clearly differentiate between nanotunnels, the active protrusion of cardiac mitochondria, and the connecting ducts of skeletal muscle derived from fusion-fission and elongation events.

Abstract

This review focuses on recent discoveries in skeletal and cardiac muscles indicating that mitochondria behave as an interactive cohort with inter-organelle communication and specific reactions to stress signals. Our new finding is that inter-mitochondrial communications in cardiac and skeletal muscles rely on two distinct methods. In cardiac muscle, mitochondria are discrete entities and are fairly well immobilized in a structural context. The organelles have developed a unique method of communication, via nanotunnels, that allow temporary connection from one mitochondrion to another over distance of up to several microns, without overall movement of the individual organelles and loss of their identity. Skeletal muscle mitochondria, on the other hand, are quite dynamic. Through fusion, fission and elongation they form connections that include constrictions and connecting ducts (quite distinct from nanotunnels) and loose individual identity in the formation of extensive networks. Connecting elements in skeletal muscle are distinct from nanotunnels in cardiac muscle.

Inter-mitochondria communication: interesting variety of means and structures

The positioning, movements and overall behavior of mitochondria are dictated by requirements of the host cell, but are also influenced by independent mitochondrial activity (Zhang *et al.*, 2016; Wang *et al.*, 2018; Strzyz, 2019). The direct cell influence on mitochondria is most obvious in skeletal and cardiac muscles, where tethering to the sarcoplasmic reticulum imposes an age-dependent stereotyped distribution of mitochondria relative to the sarcomeres (Boncompagni *et al.*, 2009; Franzini-Armstrong & Boncompagni, 2011). This disposition is of course most obviously advantageous to the muscle cells because it provides well-distributed sources of ATP production. However, recent evidence shows that mitochondria are not entirely dependent on cell commands, but assert their independence as a group by organizing means of communication between themselves that seem to by-pass the other cell organelles, serving as mitochondria-related, rather than cell-dedicated functions. This intercommunication is essential to mitochondria well-being and may be of importance to the overall cell function but perhaps only indirectly, since in general good health of mitochondria is needed as a basis for cell metabolism and other functions. Mitochondria intercommunication occurs by a variety of means and via sets of structural features that are varied in their morphology and development, and thus are functionally not equivalent. Here we present and discuss the differences between the recently described nanotunnels and a variety of other inter-mitochondrial bridging structures that form ducts or pathways with different origins. In order to emphasize the important distinction between nanotunnels and other connecting structures, nanotunnels are first described separately. Finally, we consider other proposed, more direct communications by means of fusion/fission events and at specialized “kissing junctions”. Most of the evidence presented refers to skeletal and cardiac muscle.

Mitochondrial nanotunnels: definition, origin and positioning

Mitochondrial nanotunnels were first described in cardiac myocytes and named on the basis of their structure as long thin extensions that are actively extruded from a single mitochondrion and extend to others over relatively long distances of up to several microns (Huang *et al.*, 2013). Nanotunnels are narrow (90-210 nm in diameter), with matrix and cristae included in their lumen. In general nanotunnels are larger than T tubules, have a relatively straight orientation, are in direct continuity with the mitochondria at their origin and are clearly not associated with dyads, making them a distinct anatomical structure. Nanotunnels are responsible for active intermitochondrial share of matrix content and membrane components over long distances (Huang *et al.*, 2013; Eisner *et al.*, 2017; Lavorato *et al.*, 2017). The flow of material along nanotunnels is relatively slow, requiring minutes for equilibrium, but sufficiently robust to allow distribution of mitochondrial components to all mitochondria over the whole length of cardiac myocytes, at distances of tens of millimeters within a period of time measured in hours (Huang *et al.*, 2013). Evidence for communication via nanotunnels is quite clear, direct and compelling. Live confocal imaging of communicating mitochondria, shows matrix targeted with photoactivatable green fluorescent protein (mtPAGFP) penetrating into narrow tunnels and moving along them from one mitochondrion to another (Fig.1, see also Fig. 3 in Huang *et al.* (2013) and Fig. 9B in Lavorato *et al.* (2017)). Figure 2 from thin section electron micrographs illustrates the fine structure of nanotunnels similar to those illustrated in a 3-D reconstruction from the same mouse myocardium (Lavorato *et al.*, 2017).

So far, nanotunnels have been directly imaged by electron microscopy and clearly detected by functional probes exclusively in cardiac myocytes, but not in any other muscles. Indeed, nanotunnels are quite frequent in cardiac muscle. The reason for nanotunnels' presence seems obvious in mammalian myocardium, where mitochondria are clearly discontinuous and have extremely limited mobility, being confined between the myofibrils. In 3-D scanning electron microscopy (SEM) images, cardiac mitochondria are well defined as short cylinders that extend for the length of one to a few sarcomeres between the myofibrils with no direct continuities with each other, except via the pathway provided by nanotunnels (Fig. 3). Movements of entire mitochondria have not been directly observed and matrix protein exchanges between well-separated mitochondria are very slow (Huang *et al.*, 2013; Eisner *et al.*, 2017; Lavorato *et al.*, 2017) confirming that the organelles are normally trapped in a fixed position. Essentially, in the absence of nanotunnels, cardiac mitochondria would lead a life of royal isolation with few interactions with each other. Thus, we hypothesize that nanotunnels are a feature essential to the wellbeing of cardiac mitochondria as a population and of the heart as a whole.

Nanotunnels extend over distances of several microns, so they clearly can facilitate exchanges over relatively long distances within the cell (Fig. 1). They originate as a funnel shaped extension from a donating mitochondrion and they become narrower as they get farther out, while maintaining the double external membrane and, in most cases, some cristae. They rarely have a totally clear matrix. At the distal end, they taper into a rounded shape but, despite considerable effort in this regard, we were not able to observe a direct continuity between the far end of a nanotunnels and a receiving mitochondrion. The image in Figure 2 is suggestive but not quite a proof of continuity. Since an electron microscopy (EM) image is a snapshot in time, this

indicates that direct continuity between a nanotunnel and its receiving mitochondrion is either quite rare, or of very short duration, or of very small size, or, most likely, a combination of all factors. This leaves behind a currently unresolved lack of direct ultrastructural evidence for the exact nature of the nanotunnel-to-mitochondrion continuity at the receiving end of the exchange.

Nanotunnels reach for some distances from their site of origin (Fig. 3). The frequent close proximity between nanotunnels and microtubules (Fig. 2) suggests that nanotunnels move along the microtubules as many membrane-limited cell organelles do.

One essential question about nanotunnels has not been solved: does the active transport of proteins between two separate mitochondria make use of preexisting nanotunnels or does a new tunnel develop when transport is needed, leaving behind the structural framework to be visualized by EM? In other words, how dynamic are nanotunnels and what is their life span? Static EM snapshot of myocardial structures (such as in Fig. 2 and 3) reveal the presence of numerous nanotunnels in all stages of deployment at any given time, and video recordings of active matrix transfer (Fig. 1) illustrate the movement of components along nanotunnel structure. However, it is not known whether nanotunnels may increase in density and dimensions when exchanges are required.

Mitochondrial constrictions, mitochondrial fission, intermitochondrial nanotubes, ducts or pathways.

In contrast to cardiac muscle, skeletal muscle inter-myofibrillar mitochondria do not show

any tendency towards “nanotunneling”: no long thin mitochondrial extensions have been observed to freely extend from the organelle surface, although the main shape of the individual mitochondrion is quite elongated. Additionally, skeletal muscle mitochondria in mammalian muscle differ from those in cardiac myocytes because they are part of extensive networks with branching in the transverse direction and longitudinal extensions (Amchenkova *et al.*, 1988; Picard *et al.*, 2013). The extraordinary extent of continuous mitochondria networks in mammalian muscles has been frequently noted (Franzini-Armstrong, 2007; Wei *et al.*, 2011; Patel *et al.*, 2016) and it is likely that exchanges over long distances can easily occur over the length of preexisting continuous mitochondria pathways. Additionally, although mitochondria are physically anchored to the sarcoplasmic reticulum (SR) by tethers connecting them to the SR at triads (Boncompagni *et al.*, 2009) and is constrained by the cytoskeleton, the entire network is quite variable in shape. This arrangement suggests a dynamic structure with the occurrence of fission and temporary fusion events, which allow extension of one mitochondrion domain into that of its adjacent neighbour, and provide for physiological exchanges.

Extensive mitochondrial networks are not a rule, if muscles other than mammalian muscle are considered (Franzini-Armstrong & Boncompagni, 2011). In lower vertebrates mitochondria are mostly present in small groups, where they are either separate from each other, or only partly connected. It is important to note that nanotunnels, as defined in this review, are not deployed in skeletal muscle, even where few mitochondria are present, indicating that exchanges are more likely to occur by other means, e.g. fusion events (Eisner *et al.*, 2014), or via short connecting tunnels of the type described in free bacteria (Dubey & Ben-Yehuda, 2011) as well as in higher cells (Rustom *et al.*, 2004) and detailed below.

3-D reconstructions of mitochondria in human muscles have revealed the presence of numerous connections between mitochondria via narrow ducts or pathways that directly join one mitochondrion to another (Vincent *et al.*, 2019). We propose that the origin of these connecting structures and their ultrastructural details are quite different from those of nanotunnels, so for clarity we propose to restrict the term nanotunnels to the structures specific to cardiac mitochondria and to use alternative names for other intermitochondrial connections, e.g. nanotubes, connecting ducts. Some instances of connecting ducts have been detected in skeletal muscle, often associated with evidence of severe structural alterations. The direct role of such structures in the physiology and pathology of muscle are not well defined, because they occur under a variety of pathological conditions (Vincent *et al.*, 2016; Vincent *et al.*, 2017), and may also be present as part of the normal network of mitochondria (Vincent *et al.*, 2019).

Differently from nanotunnels, the connecting ducts do not arise as active projections from the borders of mitochondria, but may be the result of slow and/or partly arrested fission. The most striking demonstration of this effect is in the work by Zhang et al (2016), who illustrated the process in a brain model of Alzheimer's disease. In this brain model, elongated mitochondria show multiple constrictions sites, the likely initial stages of multiple fission processes that would break the elongated mitochondria into many fragments. However, the process is not completed and the connections between the fragments remain *in situ* for some time and become thinner and elongated thus forming ducts.

Novel discoveries in skeletal muscle show that mitochondria in this tissue may behave

similarly to those in neuronal tissue under conditions of some stress and provide direct evidence for the derivation of intermitochondrial ducts from events involving the evolution of elongated zones that may or may not evolve into actual fission. Fatigue in fast fibers of mouse is associated with greater frequency of elongated constrictions within the body of individual mitochondria, and the subsequent development into short connecting ducts (Fig. 4) (Lavorato *et al.*, 2018). Moreover, in a novel, human genetic condition (Perrotta et al., under review) unusually thin and elongated mitochondrial tubes have been discovered (Fig. 5). Levels of the von Hippel-Lindau protein are reduced in this condition, leading to a largely hypoxic phenotype at multiple levels and exposing the mitochondria to metabolic stress. These findings suggest that the development of intermitochondrial connecting ducts may be a common response, worth investigating in other circumstances where the functioning of the hypoxia-inducible factor pathway is altered (Perrotta *et al.*, 2006; Formenti *et al.*, 2010; Formenti *et al.*, 2011; Petousi *et al.*, 2014; Thompson *et al.*, 2014; Lenglet *et al.*, 2018).

The ducts and elongated constrictions significantly differ from nanotunnels in two major details. First, they are located between two parts of an individual mitochondrion that have apparently moved apart extending a portion of the organelle into thinner elongated structures that remain associated with the two sides. Second, the connections on the two sides are always clearly patent, indicating an opening that is present over a period of time. By contrast, the nanotunnels evolve as projections from the edge of a mitochondrion and although are clearly connected on the side of the mitochondrion of origin, they are not visibly so on the side of the receiving mitochondrion.

Other mechanisms of intermitochondrial communication

In the case of the mostly immobile mitochondria in cardiac muscle, it has been proposed that a means of exchange may be present at sites where two adjacent organelles closely abut against each other, in addition to the exchange that occurs via nanotunnels. Structural specializations at these proximity sites were first described and very well illustrated by Bakeeva et al. (1983). They were later confirmed multiple times and named "kissing junctions" (Huang *et al.*, 2013; Picard *et al.*, 2015; Glancy *et al.*, 2017; Lavorato *et al.*, 2017). Despite the fact that the membranes of the two adjacent mitochondria seem to form very close punctate contacts, no direct evidence is so far available for the presence of connecting channels at these sites. These are necessary to provide a path for direct communication between adjacent mitochondria. The coordinated arrangement of cristae of two mitochondria at sites of kissing junctions (Picard *et al.*, 2015) is certainly suggestive of some exchange of information, but there is no direct evidence to indicate that intermitochondrial exchanges do take place at kissing junctions.

Finally, it has been proposed that mitochondria exchange matrix content in both skeletal and cardiac muscles by means of short-lived fusion events without loss of the organelles identity (Eisner *et al.*, 2014; Eisner *et al.*, 2017). This hypothesis is in keeping with the normal, continuous dynamic behaviour of mitochondria, as detected in cultured cells, that involves fusion and fission events and play a major role in maintenance of the organelles' integrity (Westermann, 2010; Youle & van der Bliek, 2012). However, differently from mitochondria involved in these events in cultured cells, muscle mitochondria do not move out of position during presumed fusions, offering a more physiological perspective on their development and

function. All exchange events involving mitochondria at short distances have been proposed to depend on either kissing junctions (in cardiac muscle), or fusion events (in both skeletal and cardiac muscles). However, it cannot be excluded that in cardiac muscle such exchanges at short distances may be carried out by short nanotubes that are not visualized in the fluorescent images. In skeletal muscle, exchanges may be simply a function of the network continuities, without need to assume an ad-hoc fusion.

Additionally, presumed fusion events in skeletal and cardiac muscle leave one unsolved mystery: the exchange of organelles content at presumed sites of fusion is considerably slower than it is expected from free diffusion between two compartments that are presumably in open direct connection. Thus some regulation of the exchange rate must be present, either as a physical barrier (e.g. restricted sites for diffusion) or some direct regulation of diffusion, such as binding protein(s). This challenging question has not been explored in detail yet.

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Figure legends

Figure 1. Cardiac myocyte. Previously unpublished image of a cardiac myocyte expressing photoactivatable mtPA-GFP and photobleachable mtDsRed in the mitochondrion matrix (see Lavorato et al., 2017 for details). The experiments involved activating the mtPA-GFP with a laser flash in a delimited area (within the square) and detecting its movement in time. In this image the activated green mtPA-GFP is seen to spread along narrow pathways (presumably nanotunnels, small arrows). In published work it was shown that the activated protein eventually diffuses into nearby mitochondria, again presumably via nanotunnels. Contributed by M. Lavorato.

Figure 2. Ultrathin section through a rapid frozen freeze-substituted cardiac myocyte showing nanotunnels that arise from the periphery of two donor mitochondria. Cristae and a dense matrix fill the interior of nanotunnels. Note profiles of microtubules (arrows) that probably act as guides for nanotunnel movements. (M. Lavorato, unpublished. See also Lavorato et al., 2017). M: mitochondrion; Nt: nanotunnels.

Figure 3. Novel SEM image of mitochondria in a mouse cardiac myocyte, illustrating a long nanotunnel (arrow). Cardiac myocytes are the only striated muscle in which nanotunnels have been observed. They may be the main conduits for intermitochondrial communication in myocardium. The tissue was prepared following the protocol devised by Ogata and Yamasaki, 1990. M. Lavorato, unpublished.

Figure 4. Elongated constrictions separating sections of mitochondria in fast skeletal muscle fibers of mouse. In both images (A and B) a mitochondrion shows a transition (at arrows) into a narrow region that remains associated at either end with the normal mitochondrial structure. These events were observed in fatigued mice muscle and are similar to the ones detected as a response to stress and perhaps indicative of incipient fission in Fig. 5. Note that in both cases SR elements are closely associated with the constricted section, perhaps contributing to development of the constriction. M. Lavorato, unpublished observations in collaboration with V. DeBattisti, from Jefferson University, Philadelphia, PA. (See also Lavorato et al. (2018)).

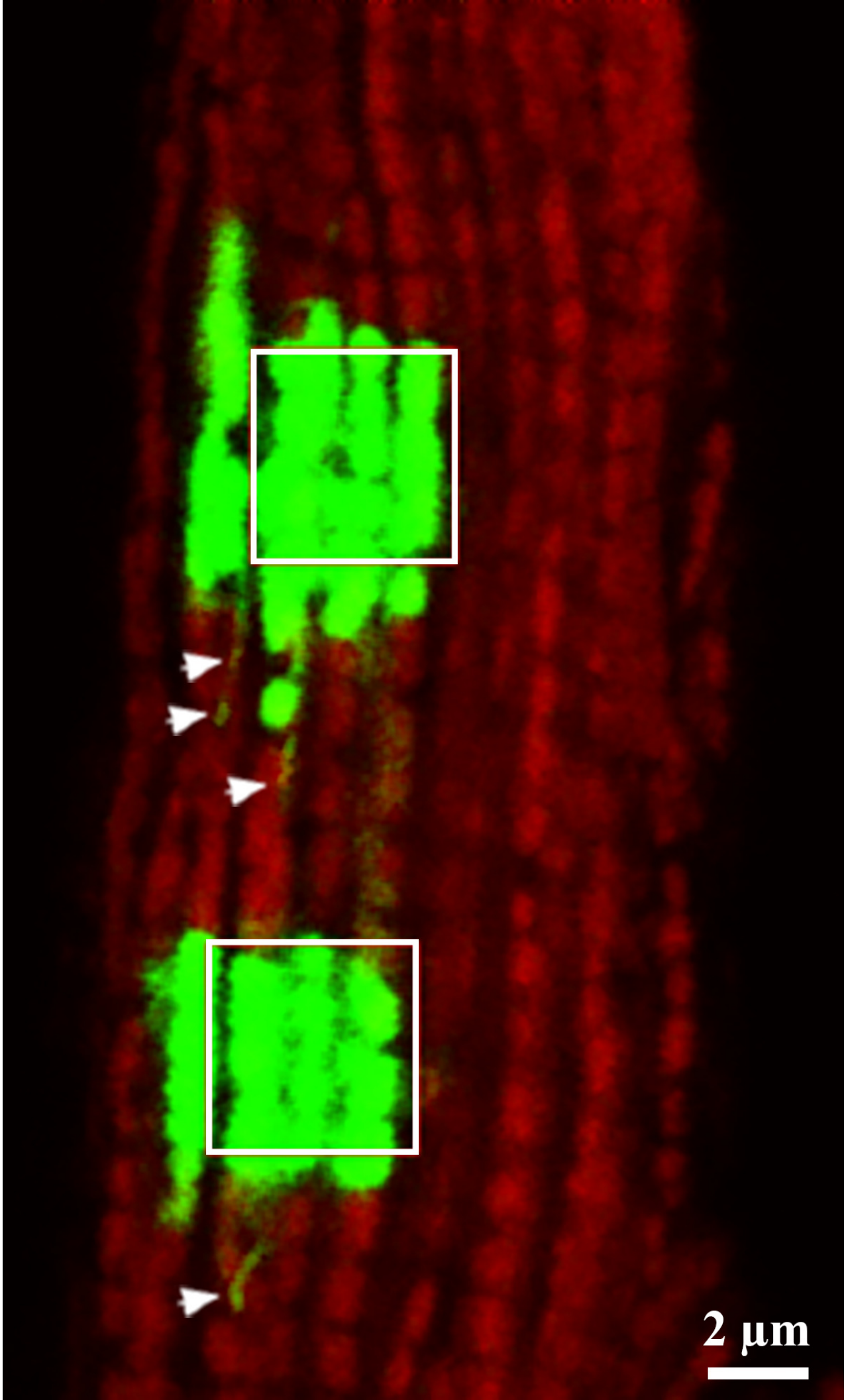
Figure 5. Intermitochondrial ducts in the muscle from a patient with a mutation leading to reduced von Hippel-Lindau protein levels.

A) Mitochondria from a *vastus lateralis* biopsy of a patient with a mutation leading to reduced von Hippel-Lindau protein levels, associated with an abnormal metabolic phenotype and mitochondrial stress (Perrotta et al., under review). The mitochondrial response in this case is an extension of the organelles (A, at arrows) so that the wider regions are connected by long extended tunnels that follow the transversely oriented path occupied by mitochondria (Perrotta et al., under review).

B) Elongated profiles are patently connected to mitochondria at either end, and so differ from the asymmetric nanotunnels illustrated in Fig. 2. The profiles are appropriately classified as "connecting ducts" rather than nanotunnels. They may have occurred as an extension in time and

448 space of the constrictions illustrated in Figure 4. These images are quite similar to those observed

449 in the formation of connecting ducts in a model of Alzheimer's disease (Zhang et al., 2016).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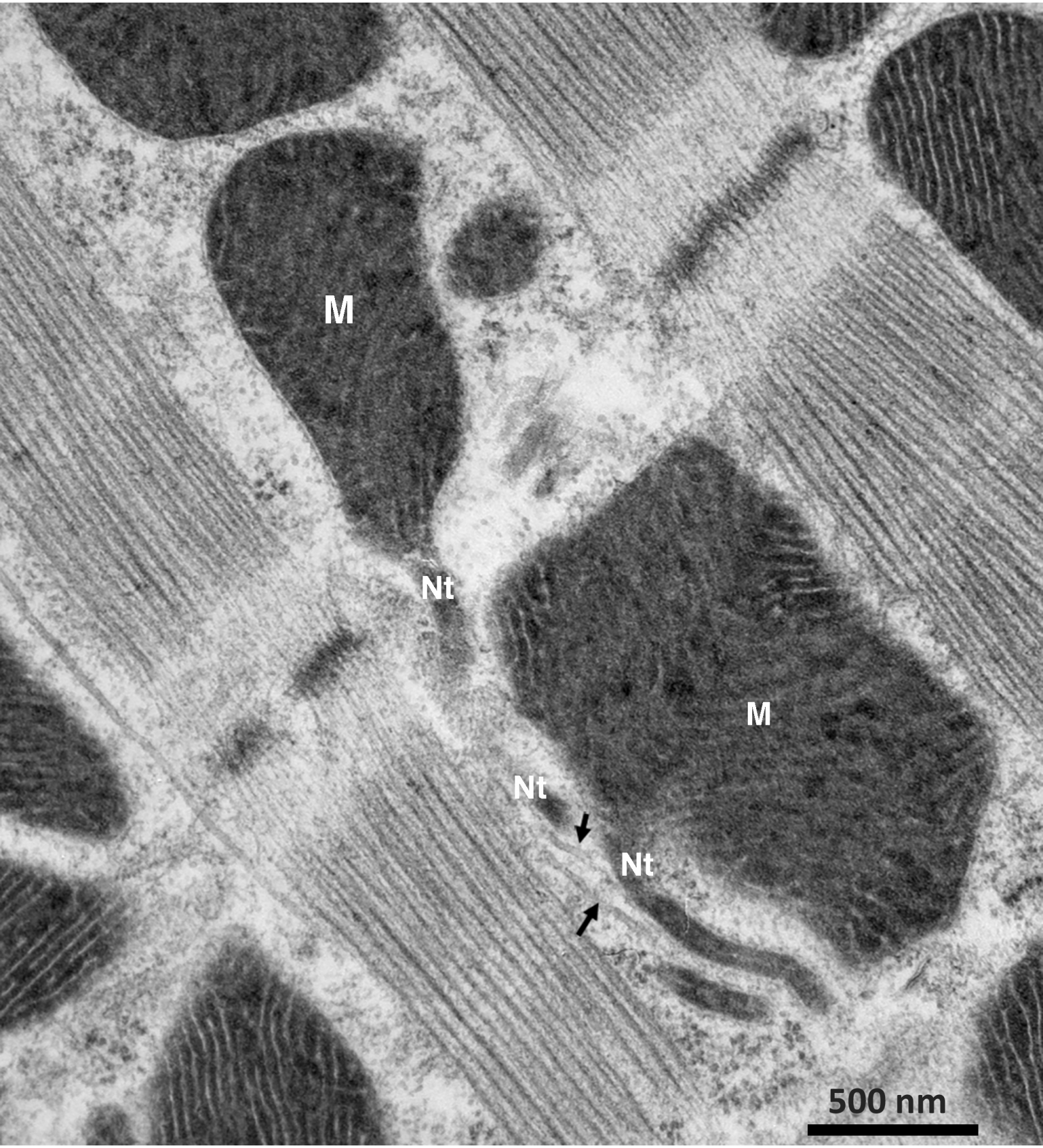
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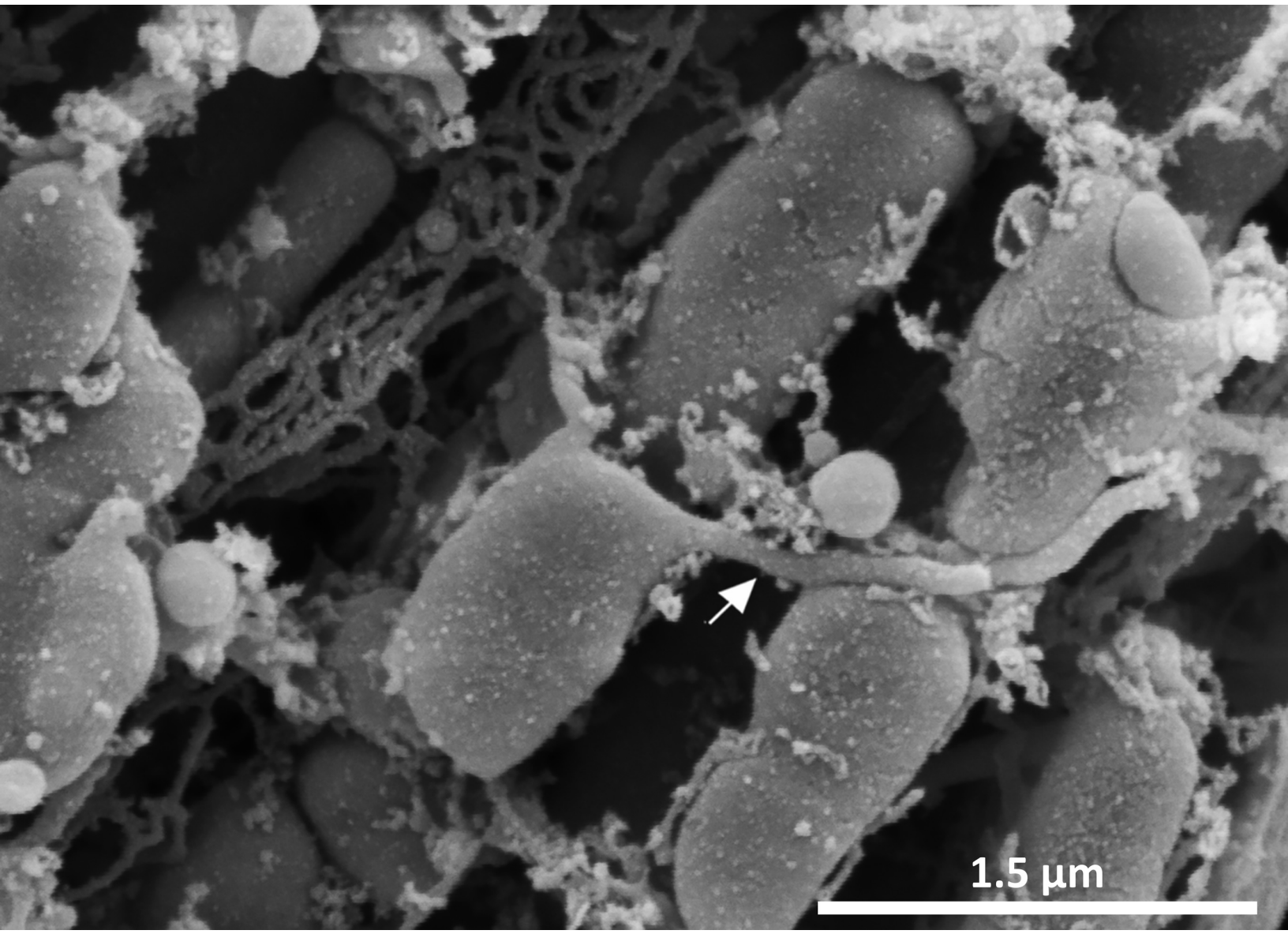
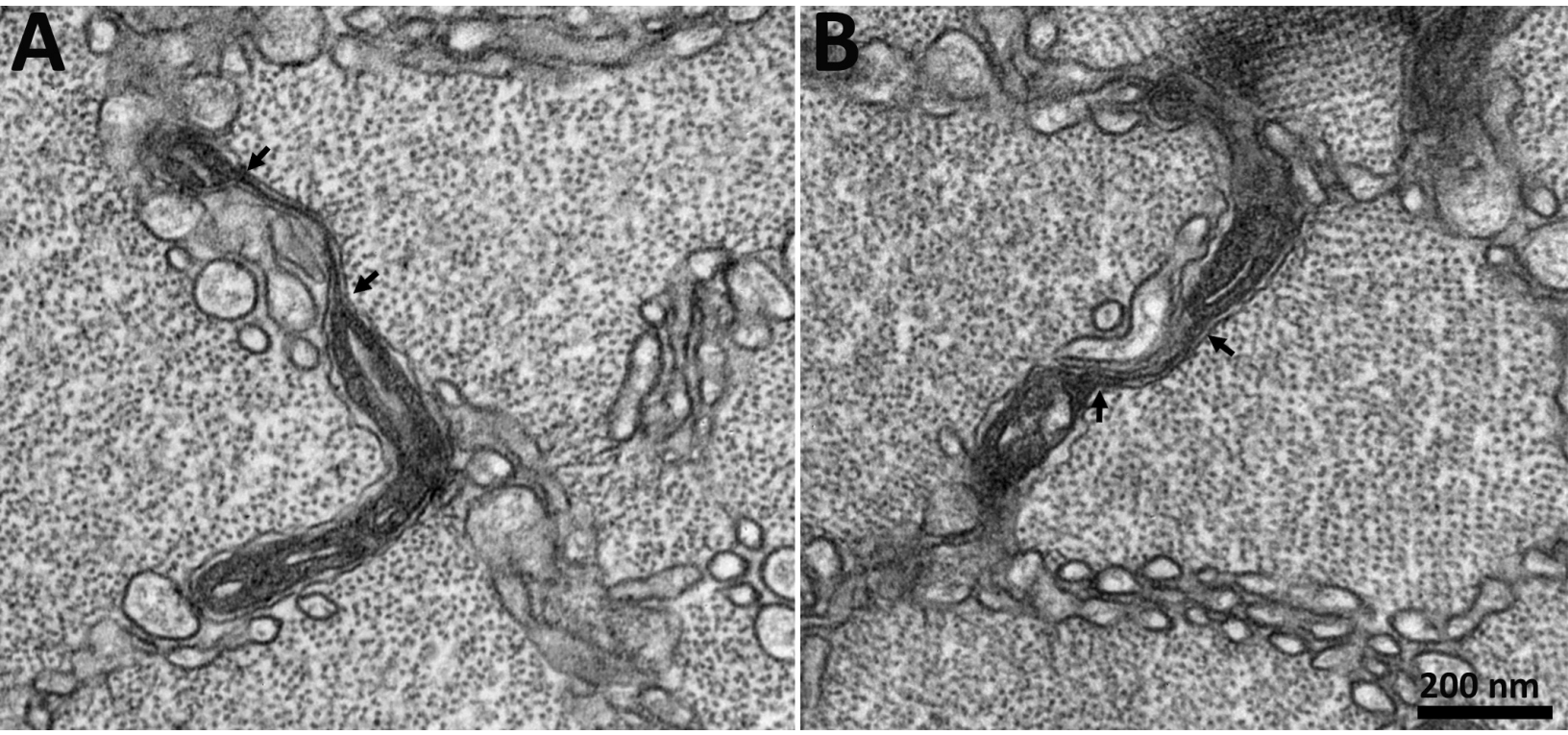
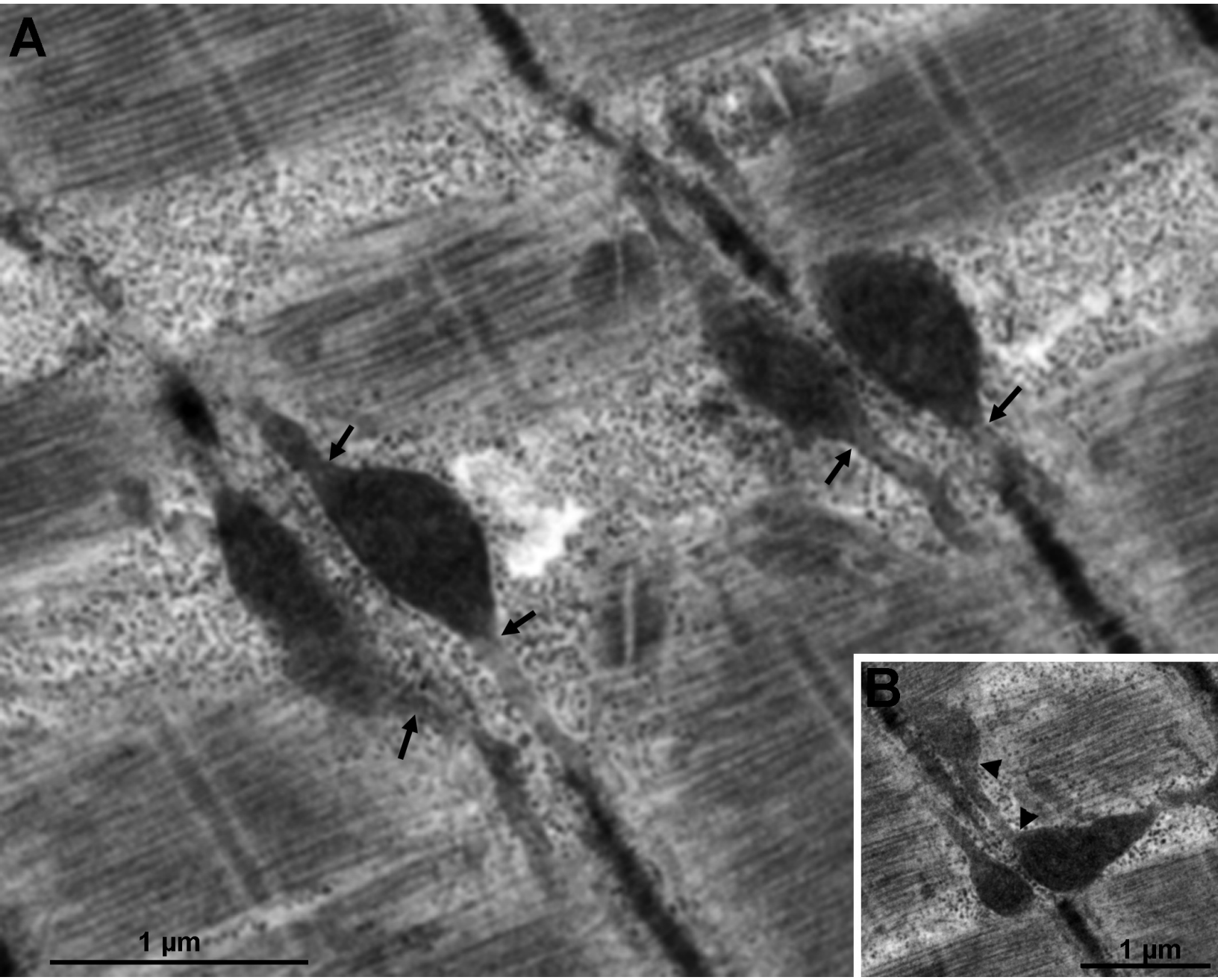
Nt
500 nm


1.5 µm


A
B
200 nm


A
1 µm
B
1 µm