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# Changes in the mitochondrial network during ectromelia virus infection of permissive L929 cells*

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**Mitochondria are extremely important organelles in the life of a cell. Recent studies indicate that mitochondria also play a fundamental role in the cellular innate immune mechanisms against viral infections. Moreover, mitochondria are able to alter their shape continuously through fusion and fission. These tightly regulated processes are activated or inhibited under physiological or pathological (e.g. viral infection) conditions to help restore homeostasis. However, many types of viruses, such as orthopoxviruses, have developed various strategies to evade the mitochondrial-mediated antiviral innate immune responses. Moreover, orthopoxviruses exploit the mitochondria for their survival. Such viral activity has been reported during vaccinia virus (VACV) infection. Our study shows that the Moscow strain of ectromelia virus (ECTV-MOS), an orthopoxvirus, alters the mitochondrial network in permissive L929 cells. Upon infection, the branching structure of the mitochondrial network collapses and becomes disorganized followed by destruction of mitochondrial tubules during the late stage of infection. Small, discrete mitochondria co-localize with progeny virions, close to the cell membrane. Furthermore, clustering of mitochondria is observed around viral factories, particularly between the nucleus and viroplasm. Our findings suggest that ECTV-MOS modulates mitochondrial cellular distribution during later stages of the replication cycle, probably enabling viral replication and/or assembly as well as transport of progeny virions inside the cell. However, this requires further investigation.**

**Key words**: mitochondrial network, ECTV-MOS, apoptosis

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## INTRODUCTION

Ectromelia virus (ECTV) is an enveloped DNA virus that belongs to the *Orthopoxvirus* genus of the *Poxviridae* family. It is closely related to variola virus (VARV), a causative agent of smallpox, an infectious disease with the highest number of fatalities ever (McFadden, 2005). Vaccinia virus (VACV), also an orthopoxvirus, has been used successfully as a vaccine to eradicate smallpox (Fenner, 2000). ECTV causes mousepox in various breeds of mice. Because symptoms of mousepox are reminiscent of smallpox in humans, the disease in mice is occasionally referred to as "smallpox of mice". However, ECTV does not cause disease in humans. For that reasons,

ECTV has repeatedly been used as a model to study pathogenesis of orthopoxviruses with the aim of elucidating mechanisms of host defense against these viruses.

Mitochondria are key organelles in eukaryotic cells. They are involved in a range of cellular metabolic processes, such as cellular energy supply, cellular differentiation, aging, calcium buffering to red-ox homeostasis, as well as cell death. In addition to their role in elimination of infected cells through apoptosis, mitochondria contribute to interferon (IFN)-signaling, leading to production of type I IFNs, thus indicating their function in the primary host defense against viral infections (Tait & Green, 2010). Numerous orthopoxviral genes modulate host immunity including mitochondrial-mediated antiviral response (Melo-Silva, 2011). For instance, they can cordon off mitochondria to prevent the release of mediators of apoptosis and/or activation of IFN production. Furthermore, poxviruses can exploit mitochondria for their survival through viral protein-mediated increase in concentration of mitochondria near the viral factories for the purpose of providing energy during viral replication and/or morphogenesis. An example of such evasive strategy is exhibited by VACV through extensive modification of the intracellular distribution of mitochondria during infection (Schepis *et al.*, 2006).

Mitochondria are highly dynamic organelles (Bereiter-Hahn & Voth, 1994). In many cell types, mitochondria have a tubular morphology; they form a branched, interconnecting network and undergo morphological and functional changes due to their frequent fusion and fission in response to local environmental conditions. The fusion/fission machinery is represented by the mitochondria shaping proteins, such as mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), optic atrophy 1 (Opa1), regulators of fusion, and dynamin related protein 1 (Drp1), fission protein 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial elongation factor 1 (MIEF1), which modulate fission (Bereiter-Hahn & Voth, 1994). During the fusion/fission processes mitochondria can shift between large, branched networks and small, circular or fragmented units. Cells undergoing mitochondrial fission have mitochondria shorter in length when compared to cells that are undergoing mitochondrial fusion. The balance between fusion and fragmentation is important to main-

tain normal mitochondrial size, shape and distribution, and to supply energy to high-demand sites. Significantly, the cytoskeleton interacts with mitochondria and is responsible for mitochondrial transport throughout the cytoplasm (Boldogh & Pon, 2006; Guzun *et al.*, 2011).

Normally, mitochondria are tubular in shape but may attain various forms under different pathological conditions (stress) (Wasilewski & Scorrano, 2009; Arnoult, 2007). There are three main types of mammalian cell deaths, which can be characterized by different mitochondrial morphology. Fragmented, round tubes or blob-shaped mitochondria are observed during apoptosis, whereas swollen mitochondria are characteristic for necrosis (Ahmad *et al.*, 2013) and in autophagy mitochondria become elongated and are spared from degradation (Gomes, 2011). Overall, mitochondria can form circular tubes with a clear center sometimes referred to as donut-shaped mitochondria (Benard *et al.*, 2007; Liu & Hajnóczky, 2011), which according to a recent study may indicate their role in the mitochondrial adaptation to hypoxia–reoxygenation-induced cellular stress (Liu & Hajnóczky, 2011).

In the present study we evaluated the mitochondrial network organization in L929 cells infected with Moscow strain of ECTV (ECTV-MOS). Our results show that upon infection, the branching structure of mitochondrial network collapses and becomes disorganized. Significantly, mitochondrial tubules are destroyed during the late stage of infection. Moreover small, discrete mitochondria co-localize with viral progeny close to the cell membrane. Furthermore, clustering of mitochondria is observed around viral factories, particularly between the nucleus and viroplasm. Results are discussed in the context of poxvirus immune evasion mechanisms.

## MATERIALS AND METHODS

**Virus**. ECTV-MOS was obtained from the American Type Culture Collection (ATCC 1374, Manassas, VA, USA), propagated and later titrated by plaque assay (PFU/ml) in Vero cell culture (kidney epithelial cells extracted from African green monkey; ATCC CCL-81) in DMEM medium containing L-glutamine (Gibco BRL, Grand Island, NY, USA), supplemented with 1% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% antibiotic/antimycotic (100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amfotericin-B) (Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% $CO_2$ in the air. The virus was purified and stored at –70°C.

**Cell culture**. L929 cells (mouse fibroblastic cell line, haplotype H-2k; ATCC CCL-1), which are permissive to ECTV infection, were used in this research. The L929 cells were cultivated in DMEM high glucose medium containing 4.0 mM L-glutamine (Gibco BRL) and supplemented with 5% FBS and 1% antibiotic/antimycotic. Twenty-four hour cell culture was infected with ECTV-MOS at the multiplicity of infection (M.O.I.) of 1 in culture medium supplemented with 1% FBS and 1% antibiotic/antimycotic. Uninfected (culture medium without ECTV-MOS) L929 cells were used as a negative control. At 4, 8, 18, and 24 h post-infection (h.p.i.), the cells were harvested for further analysis. Each experiment was performed in triplicate.

**Mitochondria labeling with MitoTracker Red.** L929 cells seeded on glass coverslips in a 24-well plate were infected with ECTV-MOS. At 4, 8, 18 or 24 h.p.i. cells were incubated for 30 min at 37°C with 100 nM MitoTracker Red CMXRos (Molecular Probes, Inc., Eugene, OR, USA), washed three times with culture medium and fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) or methanol (Sigma-Aldrich).

**Immunofluorescent staining.** Intracellular staining of viral proteins was performed as previously described (Boratynska *et al.*, 2010). Briefly, fixed L929 cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) and blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich) to prevent nonspecific binding. The cells were subsequently incubated with rabbit antibodies directed against ECTV-MOS conjugated with FITC or unconjugated mouse anti-α-tubulin monoclonal antibodies (Sigma-Aldrich). Next, secondary anti-mouse antibodies conjugated with FITC (Jackson ImmunoResearch) were used. The nuclei were stained with Hoechst 33342 (Sigma-Aldrich) and cells were mounted in ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA, USA).

**Fluorescence microscopy.** Images were recorded using Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) equipped with Color View III cooled CCD camera and processed using the Cell^F (Olympus), CellSens Dimension (Olympus) and ImageJ (NIH Image, Bethesda, MD, USA) software.

## RESULTS AND DISCUSSION

### Mitochondria display close association with ECTV-MOS factories in L929 cells

Poxviruses are unique among most DNA viruses in that their replication occurs in the cytoplasm of the infected cells. The characteristic sites of replication and assembly are large cytoplasmic perinuclear areas termed viral factories, where viral and cell components move in and out depending on the stage of the viral replication. Studies on VACV have shown that the striking feature of these areas is their close association with the nucleus and mitochondria (Tolonen *et al.*, 2001; Risco *et al.*, 2002; Schepis *et al.*, 2006).

Mitochondrial network in uninfected control L929 fibroblasts was characterized by extended wavy tubules distributed differently depending on a subcellular region of the cell (Fig. 1a). Near the nucleus (perinuclear region) mitochondria were arranged in a concentric manner, whereas radial distribution was observed closer to the cell membrane (periplasmic region). Moreover, the control cells clearly showed a microtubule organizing center (MTOC), from which mitochondrial tubules appear to radiate (Fig. 1b).

At the early stage of ECTV-MOS infection (4 h.p.i.) only a few cells had visible viral factories compared to vast majority of infected cells without viral factories and no significant alterations to the mitochondrial network structure (Fig. 2a). However, inconsiderable differences were noted in mitochondrial distribution compared to uninfected control cells (Figs. 1, 2). The noticeable accumulation of mitochondria around the nucleus at the presence of virions was observed (Fig. 2a). Interestingly, at 8 h.p.i we noticed formation of viral factories in the perinuclear region and the characteristic mitochondrial clustering around these areas of viral replication (Fig. 2b). Later, progressive changes occurred in mitochondrial distribution. Mitochondria began to accumulate between nucleus and viral factory/factories and disconnections between mitochondria within the cytoplasm occurred (Fig. 3a, 3d). At 18 and 24 h.p.i., viral factories lost their regular shape and took dispersed or expanded

forms thus intensifying mitochondrial localization around viral replication/assembly sites (Fig. 3a, 3d).

Interestingly, MTOC gradually disappeared during ECTV-MOS infection (Figs. 1c, 2b, 3a, 3d) as opposed to MTOC in uninfected control cells which was intact and distinctly marked (Fig. 1b). We initially observed these events in BALB/3T3 and Vero cells during ECTV-MOS infection (data not shown). Additionally, viral particles and viral factories accumulate near MTOC in infected cells. ECTV-MOS virions co-localize with tubulin in BALB/3T3 and Vero cells (data not shown). Another study indicated that both mitochondria and virions localize to MTOC during African swine fever virus (ASFV) infection (Heath *et al.*, 2001). Our observations confirm that mitochondria localize to MTOC in L929 cells during ECTV-MOS infection. Altogether, these findings suggest the participation of microtubules in the transport of viral particles as well as mitochondria during ECTV-MOS infection.

During VACV infection viral factories are enclosed by elements of the rough endoplasmic reticulum (RER) (Tolonen *et al.*, 2001). Since close association between vi-

ral factories and mitochondria was also indicated, it can be assumed that mitochondria interact with RER. It is well known that these structures collaborate during viral infection, because they are involved in the production of proinflammatory cytokines and type I IFNs (Ishikawa & Barber, 2008). These findings may suggest that mitochondrial recruitment to viral factories is related to the early antiviral responses during VACV infection. It is highly possible that mitochondria engage in a similar mechanism in ECTV-MOS-infected L929 cells. However, VACV encodes proteins which inhibit mitochondria-mediated antiviral response (Deng *et al.*, 2008; Chen *et al.*, 2008). Only elongated mitochondria are able to transduce signals for production of type I IFNs and cytokines. Mitochondrial fragmentation leads to the loss of connection with endoplasmic reticulum and inhibits antiviral response. Fragmentation of mitochondrial network during VACV (Schepis *et al.*, 2006) and ECTV-MOS infection indicates that these viruses may inhibit production of antiviral factors.

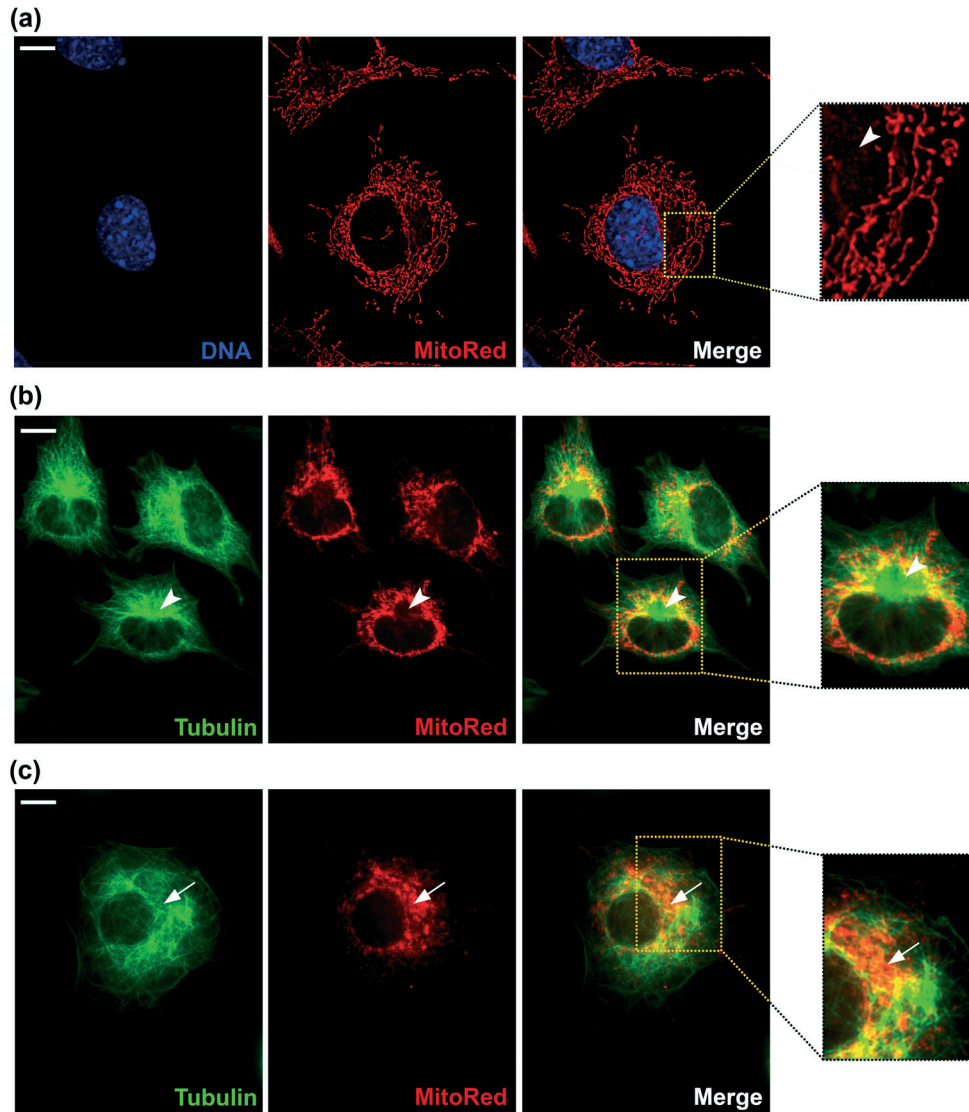Our findings suggest that infection of L929 cells with ECTV-MOS rearranges mitochondrial distribu-



**Figure 1. Mitochondrial morphology in control uninfected L929 cells (a, b) and localization of MTOC in control uninfected cells (a, b) and loss of the MTOC at 18 h.p.i. with ECTV-MOS (c).**
(a, b) arrowheads show the MTOC (area without mitochondria), (c) arrows indicate aggregation of mitochondria in place of MTOC. Nuclei (**blue**), α-tubulin (**green**), mitochondria (**red**). Scale bars: 10 μm (**a**), 25 μm (**b, c**).
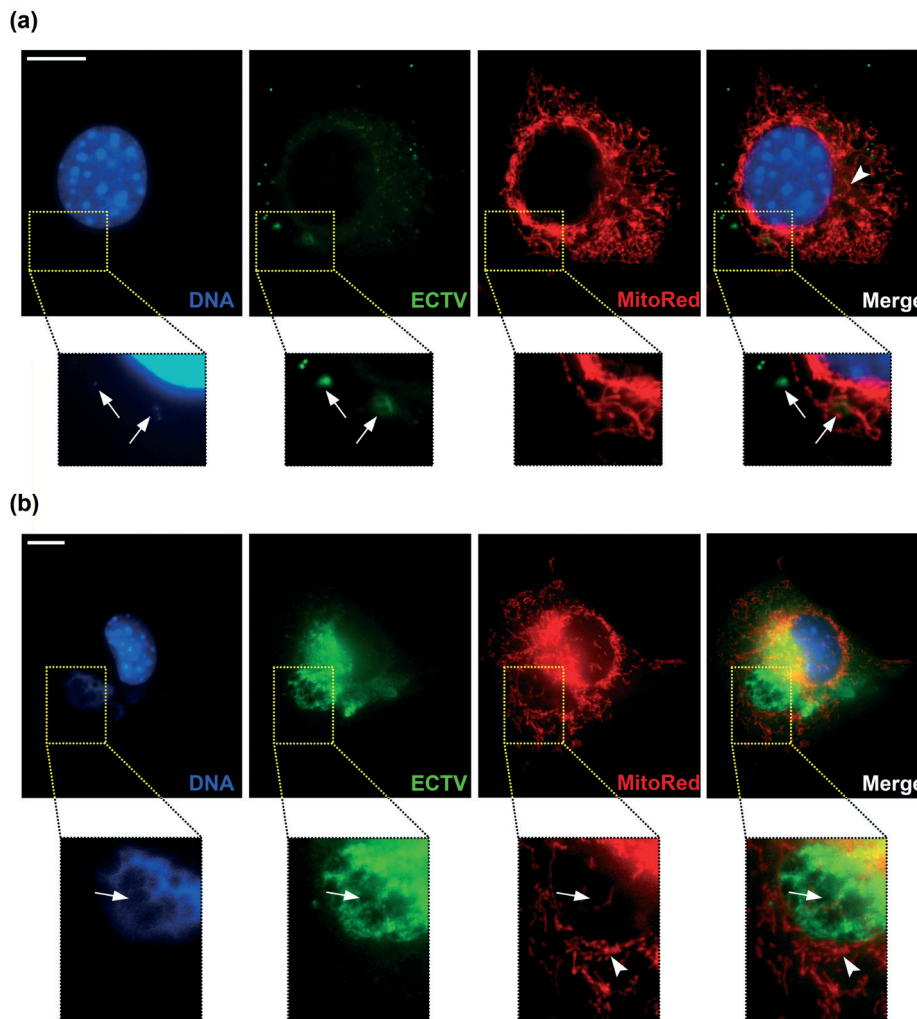
**Figure 2. Mitochondrial network organization in infected L929 cells at 4 (a) and 8 (b) h.p.i. with ECTV-MOS.**
(**a**) arrowhead shows MTOC, arrows indicate co-localization of viral DNA with antibodies directed against ECTV-MOS. (**b**) arrowheads indicate localization of mitochondria around a viral factory, arrows show viral factory formed in the cytoplasm of the infected cell. Nuclei (**blue**), viral antigens (green), mitochondria (**red**). Scale bars: 10 µm.

tion, implying that ECTV-MOS "prepares" mitochondria for participation in viral replication. It is likely that these organelles may supply energy for viral replication and maturation as suggested for ASFV infection (Rojo *et al.*, 1998). In our future research, we plan to check the level of ATP during ECTV-MOS infection in L929 cells which will be able to give us an information about ECTV-MOS-mediated changes in the level of cellular energy. These results will be essential for a better understanding of mitochondrial participation in the replication and maturation of ECTV-MOS. On the other hand, orthopoxviruses are known to inhibit programmed cell death (Everett *et al.*, 2000; Wasilenko *et al.*, 2003). For that reason, ECTV-MOS may contribute to the accumulation of mitochondria thus preventing the release of mediators of apoptosis. Such behaviour of mitochondria has been reported during hepatitis B (HBV) or C (HCV) virus infection (Nomura-Takigawa *et al.*, 2006; Kim *et al.*, 2007). Our previous studies (Krzyżowska *et al.*, 2002) show that ECTV-MOS modulates apoptosis of L929 and RK-13 cells through inhibition of caspase activity (caspase 3 and 7) before successful virus replication is completed. Within the first few hours of infection (2–8

h.p.i.) there are no significant differences in the percentage of apoptotic cells between infected and uninfected control cells. However, release of the first ECTV-MOS progeny from infected cells may signal to stop inhibition of apoptosis by ECTV-MOS-encoded proteins and consequently increase the percentage of apoptotic cells, as seen between 12 and 18 h.p.i, compared to uninfected control cells (Krzyżowska *et al.*, 2002). Nevertheless, caspase 3 and 7 are executive caspases in apoptosis and are common to both intrinsic (mitochondrial) and extrinsic pathways of this type of cell death. Therefore, in the future studies, we will focus on examining the proteins of the mitochondrial pathway of apoptosis in order to determine the importance of mitochondria in the ECTV-MOS replication cycle.

With regard to the ability to manipulate cells, VACV and ECTV-MOS may be involved in mitochondrial recruitment to the proximity of viral factories and responsible for their fragmentation to inhibit antiviral factors during infection. It is highly likely that ECTV-MOS exploits mitochondrial energy production, and has capability to inhibit antiviral innate immunity.
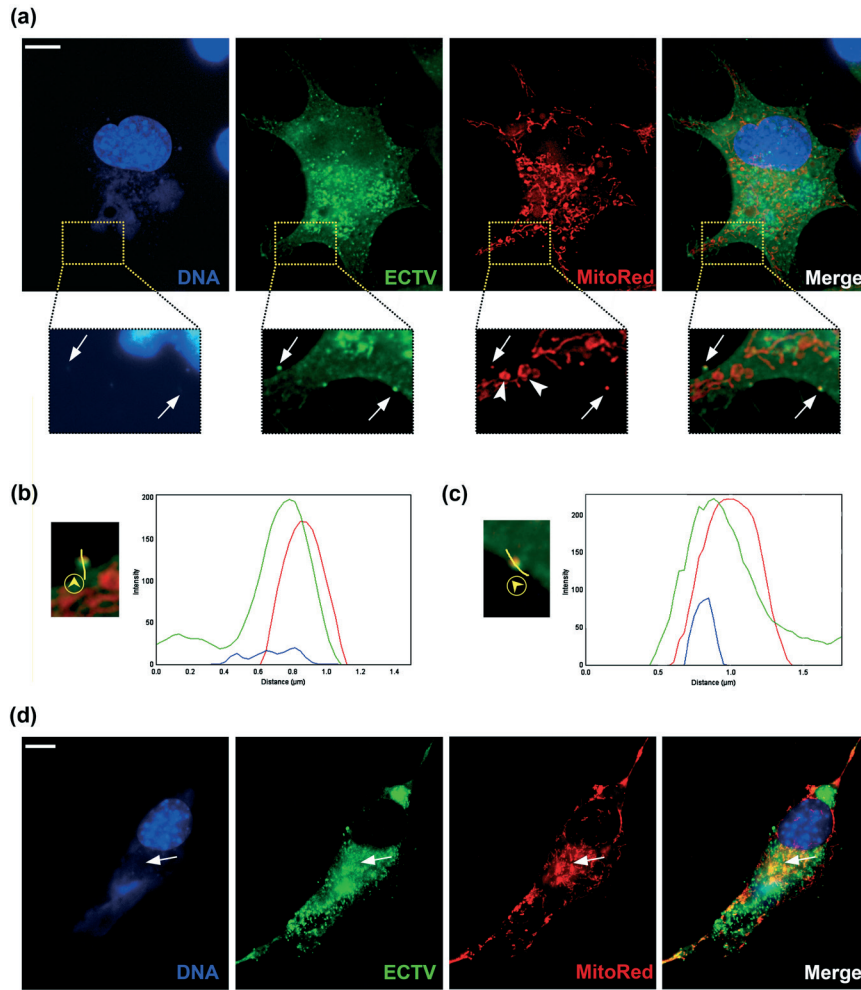
**Figure 3. Disruption of mitochondrial network in L929 cells at 18 (a) and 24 (d) h.p.i. with ECTV-MOS.**
(**a**) arrowheads show donut-like mitochondria, arrows indicate co-localization of progeny virions with discrete mitochondria. (**b, c**) histograms showing relative fluorescence intensity measured along sites of co-localization of a viral particle and mitochondria. (**d**) arrows show localization of mitochondria between nucleus and viral factory. Nuclei (**blue**), viral antigens (**green**), mitochondria (**red**). Scale bars: 10 μm.

## ECTV-MOS is responsible for destruction of mitochondrial network in permissive L929 cells during the late phase of infection

It is well known that mitochondria are highly dynamic organelles that frequently divide and fuse. Changes of mitochondrial network depend on physiological or pathological conditions of mitochondrial metabolism. Under normal conditions, mitochondrial fragmentation contributes mitochondria for the daughter cells during cell division and improves distribution of mitochondria along microtubule tracks. However, severe cellular stress leads to mitochondrial fission and consequently to the selective removal of dividing mitochondria by an autophagy process, termed mitophagy. The intensification of mitochondrial division during severe stress may lead to apoptotic cell death (Bossy-Wetzel et al., 2003).

Our findings indicate that ECTV-MOS contributes to destruction of mitochondrial network in L929 cells during late stages of infection. We detected the dispersal of mitochondria at 18 and 24 h.p.i. (Fig. 3a, 3d). Schepis and colleagues (2006) have shown that VACV leads to the collapse of mitochondria also in the early hours after infection. The discrepancy between those results and ours may be due to different rates of ECTV-MOS and VACV replication. At 4 h.p.i. we observed only single ECTV virions on/inside the cell, without viral factories, in contrast to VACV-infected cells (Schepis et al., 2006; Lynn et al., 2012) where viral factories as well as mitochondrial changes were detected at the indicated time. Recent studies by Lynn et al. (2012) have shown that replication of ECTV is approximately 1.5-fold slower than that of VACV. Probably, this accounted for the observation of intact mitochondria at early time points following infection with ECTV.

In our studies, at subsequent time points, i.e. 18 and 24 h.p.i., mitochondria displayed 4 types of morphological forms in ECTV-MOS-infected L929 cells: 1) loose mitochondrial network ("loose net"), when interconnected network is relaxed, 2) short, individual mitochondrial tubules, 3) donut-like mitochondria, and 4) single, separated, circular mitochondria (discrete mitochondria) (Fig. 3a). Each form is characteristic for particular cellular stress. Mild oxidative stress may lead to formation of donut-like mitochondria, but high oxidative stress extensively destroys the mitochondrial network leaving only discrete mitochondria (Ahmad et al., 2013). Additionally, donut-like mitochondria could result from hypoxia–re-
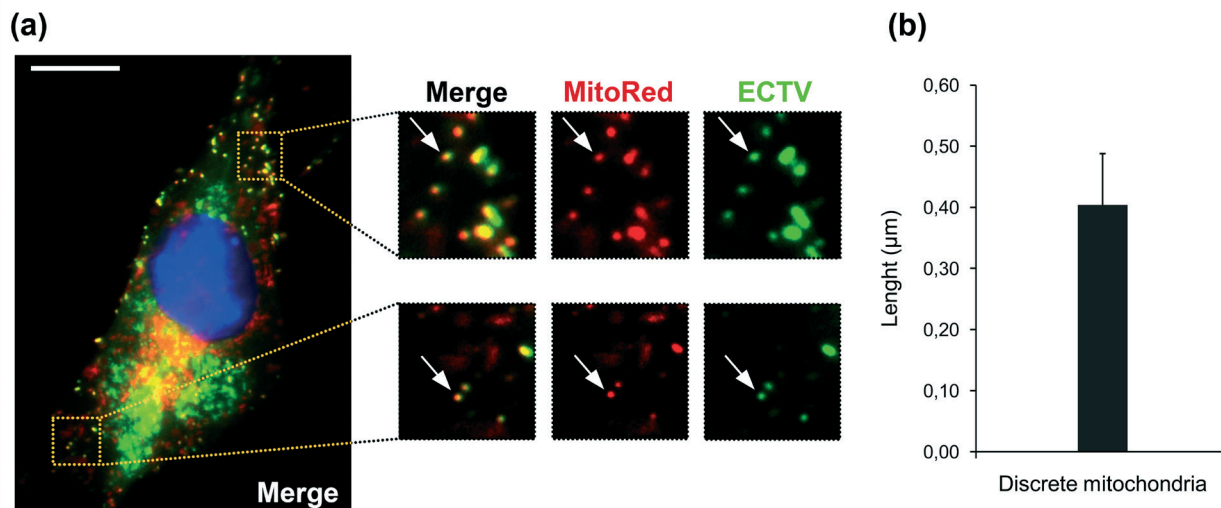
**Figure 4. Co-localization of discrete mitochondria and progeny virions in L929 cells at 18 h.p.i. with ECTV-MOS.**
(**a**) arrows indicate co-localization of progeny virions with discrete mitochondria. (**b**) bar shows length (µm) of discrete mitochondria co-localized with virions (150 samples). Nuclei (**blue**), viral antigens (**green**), mitochondria (**red**). Scale bars: 10 µm.

oxygenation stress, when anomalous fusion events are favored (fusion of 2-3 mitochondria) (Liu & Hajnóczky, 2011). Available evidence shows that fragmented mitochondria lose their membrane potential, however, donut-like mitochondria quickly regain the membrane potential and so retain competence, thus preserving the organelles under conditions of metabolic stress. This may explain the donut-like mitochondria formation during ECTV-MOS infection where apoptosis is not instantly evident and it is known that the loss of mitochondrial potential can led to apoptosis. Moreover, recent research has revealed that VACV creates hypoxic response under normal oxygen levels that imitates the situation in solid tumors (Mazzon et al., 2013). Cells predominantly produce energy by a high rate of glycolysis and reduce oxidative phosphorylation during hypoxia. This observation, described as a Warburg effect, may simply be a consequence of mitochondrial metabolism reduction, which limits reactive oxygen species (ROS) production, and then protects the cell from death (Mazzon et al., 2013). Interestingly, donut-like mitochondria, which form during hypoxia, and discrete mitochondria are the main source of ROS. As both donut-like mitochondria and small, fragmented mitochondria are observed during late stages of ECTV-MOS infection, we hypothesize that ROS may accumulate in a cell after the replication and assembly of progeny virions, but this requires further investigation. On the other hand, the mitochondrial network disintegrates into shorter fragments although not completely. The number of donut-like or discrete mitochondria is quite inconsiderable compared to short tubules and loose net in ECTV-MOS-infected L929 cells at 18 and 24 h.p.i., which may suggest that the virus prevents full mitochondrial degradation and protects the cell from ROS accumulation and finally from death.

**Progeny virions of ECTV-MOS co-localize with discrete mitochondria in L929 cells during late stages of infection**

Mitochondrial transport depends on both actin and tubulin cytoskeleton in mammalian cells (Morris & Hollenbeck, 1995; Ligon & Steward, 2000; Hollenbeck & Saxton, 2005). Physiological shift between mitochondrial network and mitochondrial particles closely relates to the need for organelle trafficking. When mitochondrial fission is blocked, the network appears to be branched and mitochondrial movement along actin cables is abolished (Varadi et al., 2004). These findings demonstrate that only fragmented mitochondria are able to be transported throughout the cell.

Destruction of mitochondrial network in ECTV-MOS-infected L929 cells leads to the formation of small discrete mitochondria (Figs. 3a–c, 4). Interestingly, these structures were observed only during the late stage of ECTV-MOS infection, when progeny viral particles localize near the cellular membrane. Our observations have led to a surprising discovery that discrete mitochondria localize in close association with ECTV-MOS virions (Figs. 3a–c, 4a). Discrete mitochondria are approximately 400 nm in length (Fig. 4b). This is consistent with our previous study, which indicated that the actin tails or filopodia with viral particle/s are formed during ECTV-MOS infection in permissive Vero and BALB/3T3 cells (Boratyńska et al., 2010). The actin tails and filopodia consist of actin filaments and are important in the spread of ECTV from cell to cell in vitro. Besides their participation in mitochondrial transport under normal conditions, a role for actin filaments is implied in viral infections. This suggests that discrete mitochondria may be important for the transportation of mature progeny virions using actin filaments.

In conclusion, our study shows that ECTV-MOS contributes to changes in the mitochondrial network in permissive L929 cells during infection. These changes are manifested by destruction of the mitochondrial network as well as loosening of the connections between individual mitochondrial tubules. Mitochondrial intracellular distribution is also rearranged upon infection with ECTV-MOS. These findings suggest a strategy for orthopoxviruses to effectively escape from mitochondria-mediated innate immunity. Furthermore, our findings point to the possibility that ECTV-MOS may use mitochondria for energy requirements of replication or viral particle transportation using the cytoskeleton.

## Acknowledgements

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