MITOCHONDRIAL REMODELING DURING HYPEROSMOTIC STRESS

By

Matthew Zulys

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of the Institute of Medical Science
University of Toronto
2008

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ABSTRACT

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Hyperosmotic stress represents a major threat to cellular integrity and may lead to cell death via apoptosis. Accordingly, each cell reacts to hyperosmolarity with a set of functional and structural compensatory responses. Recently it has been shown that the mitochondria remodel during hyperosmotic stress. Although changes in mitochondrial dynamics could be crucial for both adaptation and apoptosis, hyperosmolarity-induced mitochondrial remodeling has not been characterized. We found that hyperosmotic stress translocates dynamin like protein 1 (DLP-1) to the mitochondria and induces DLP-1 mediated, F-actin-modulated, Rac-dependent fragmentation of these organelles in LLC-PK1 cells. Downregulation of DLP-1 mitigates the activation of the osmotic response element and increases the susceptibility of tubular cells to hyperosmotically-induced apoptosis, suggesting that DLP-1 (or mitochondrial fragmentation) may have a protective role during osmotic stress. The hyperosmolarity-triggered remodeling of the mitochondrion represents a hitherto unrecognized response to osmotic shock, which may have significant impact on adaptation and apoptosis.
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ABBREVIATION LIST

ABC  ATP binding cassettes
ADH  antidiuretic hormone
Apaf-1  apoptosis activating factor 1
AR  aspect ratio
Arp2/3  actin related protein complex
ATM  ataxia telangiectasia-mutated
AVD  apoptotic volume decrease
BAR  bin, amphiphysin, rvs domain
CA  constitutively active
CCC  chloride coupled cotransporter
Cdk1  cyclin-dependent kinase 1
CMT  Charcot-Marie-Tooth disease
CytC  cytochrome C
Cyt D  Cytochalasin D
DISC  death inducing signaling complex
DLP-1  dynamin-like protein 1
DN  dominant negative
ECM  extracellular matrix
EGF  epidermal growth factor
EGFR  epidermal growth factor receptor
ENaC  epithelial sodium channel
ER  endoplasmic reticulum
ERM  ezrin-radixin-moesin
ETC  electron transport chain
F-actin  filamentous actin
FAK  focal adhesion kinase
fMLP  N-formyl-methionyl-leucyl-phenylalanine
G-actin  monomeric actin
GAP  GTPase-activating protein
GDAP1  ganglioside-induced differentiation-associated protein-1
GDI  guanine nucleotide dissociation inhibitor
GEF  guanine nucleotide exchange factor
GFP  green fluorescent protein
HICC  hypertonicity induced cation channel
HOG  high osmolality glycerol kinase
HR  heptad repeat
Hsp  heat shock protein
Hyp  hypertonic
IAP  inhibitor of apoptosis protein
IL-1R  interleukin-1 receptor
IM/IMM  inner mitochondrial membrane
Iso  isotonic
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>WASP</td>
<td>Wiscott Aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP family Verprolin-homologous protein</td>
</tr>
<tr>
<td>WNK</td>
<td>with-no-lysine kinase</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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GENERAL INTRODUCTION: LITERATURE OVERVIEW

Why is cell volume important?

Maintenance of normal cell volume is an essential requirement for practically all life processes. Extensive changes in cell volume (swelling or shrinkage) represent a major threat to cellular integrity. If uncompensated, these alterations unavoidably lead to cell death, either through membrane rupture and necrosis (due to hypotonic swelling), or through metabolic dysregulation and apoptosis (due to hypertonic shrinkage). Accordingly, each cell is equipped with a plethora of response systems, which either serve to restore normal volume after a perturbation, or reinforce the cell structure, enabling the cell to withstand the concomitant mechanical stress (reviewed in [1]). However, volume changes cannot be regarded only as death threats. In fact a major paradigm shift in the research of volume regulation came with the realization that cell volume is not only a regulated parameter, but it is also a regulating parameter, or in other words an important regulatory signal (reviewed in [2]). Thus, moderate volume changes not only accompany but also actively regulate a large variety of important cellular processes. These include metabolism (catabolic and anabolic states), cell migration and motility, membrane traffic (primarily endo- and exocytosis), proliferation and apoptosis. In the following, a few brief examples will be provided to underline this view.

Considering metabolism, moderate cell swelling (due to net ion and water uptake) is an important factor in the anabolic action of insulin: the volume increase per se is required for the inhibition of glycogen breakdown and intracellular proteolysis. Conversely, glucagon-induced cell shrinkage (due to opening of K⁺ and Cl⁻ channels) contributes to the catabolic effects, such as glycogenolysis and protein breakdown [3]. The major role of swelling in motility is best illustrated in neutrophils; these are the driest cells of the body that need to swell to become more...
deformable and motile. Upon activation (e.g. by the bacterial chemotactic peptide, fMLP) neutrophils take up salt (through Na+/H+ and Cl-/HCO₃⁻ exchange) and water and the hydrated cells then can perform amoeboid movement and squeeze through the endothelium [4, 5]. Interestingly, localized swelling (hydration) at the leading edge is believed to be an important general requirement for the efficient actin-based membrane protrusion of migrating cells [6]. Usually, swelling facilitates, whereas shrinkage inhibits exocytosis, effects that contribute to the degranulation of active neutrophils, and to the therapeutic inhibition of neutrophil functions by hypertonic solutions [7]. These effects are likely mediated by the corresponding depolymerization (during swelling) or polymerization (during shrinkage) of submembranous actin [7]. (Nonetheless, in neurons synaptic shrinkage has been shown to facilitate membrane fusion, indicating the complexity of the osmotic effects [8]). Shrinkage is a classic inhibitor of endocytosis in epithelia [9]. Cell proliferation is associated with cell swelling, which probably plays a permissive role in the process [2]. Prior to mitosis, the cells increase their volume due to the activation of the Na⁺/H⁺ exchanger (NHE) and/or the Na⁺/K⁺/2Cl⁻ cotransporter (NKCC), and inhibition of these transporters can halt proliferation [10, 11]. As mentioned earlier, shrinkage is intimately associated with apoptosis. However, this relationship is twofold: severe hypertonicity activates the apoptotic machinery (e.g. caspase-3 [12]), presumably by inducing shrinkage-induced clustering and activation of various death receptors (e.g. Fas, TNFR [13]). On the other hand, apoptosis induced by any other means, under iso-osmotic conditions, is accompanied with substantial cell shrinkage, due to the release of K⁺ and Cl⁻ from the cells. This process is termed Apoptotic Volume Decrease (AVD) [14]. Remarkably, inhibition of AVD (e.g. by blocking K⁺ and Cl⁻ channels) suppresses apoptosis, implying the central role of shrinkage, as a signal in the process.
Taken together, keeping cell volume within narrow boundaries is essential for life, whereas fluctuations of volume within these limits are important for the regulations of many life processes. In a way therefore cell volume is a matter of life and death.

**When does cell volume change?**

The plasma membrane is a semi-permeable barrier, which allows the diffusion of water and lipid soluble compounds in and out of the cells, but restricts the movement of polar substances. Therefore changes in intra- or extracellular osmolarity are accompanied by a change in cell volume due to the movement of water along its concentration gradient. Unicellular organisms are continuously exposed to a changing environment, and therefore it is understandable that volume regulation is a constant challenge for them. Higher organisms, on the other hand, maintain a homeostatic environment, and thus the majority of their cells are exposed to an osmotically stable milieu. Why then is there a need for cell volume regulation? There are three important answers to this question; first, because there are substantial changes in the osmotic contents of the cells even under iso-osmotic conditions (the tonicity of mammalian plasma: 290±5 mosmol). These are brought about by a) metabolic processes involving polymerization and depolymerization of substrates (e.g. glycogen vs. glucose); and b) by continuous transport of ions and metabolites across the membrane. For example, uptake of amino acids or the activation of NKCC lead to swelling, whereas release of taurine or Cl⁻ through channels leads to shrinkage [14]. Second, because certain areas in the body are exposed to severely aniso-osmotic environments, under physiologic and pathologic conditions. For example the osmolarity of the kidney medulla can be 4-times higher than that of the plasma. Medullary tubular cells constantly experience this milieu, while circulating blood cells go through it
intermittently. Similarly, intestinal epithelial cells are exposed to large changes in extracellular osmolarity, e.g. when digested food enters the small intestine and they begin to absorb nutrients. Furthermore, there are many pathologic states, which lead to substantial variations in osmolarity, either systemically or locally [2]. The systemic ones include dehydration, congestive heart failure, diabetes and other metabolic diseases. Trauma and abscesses can cause alterations in the osmotic concentration of a local environment. In both cases, cells dying by necrosis release their internal components into the surrounding area, causing local hyperosmolarity [15]. Third, the very cells that are involved in osmosensing and maintaining the constant extracellular environment should themselves undergo volume changes to initiate the compensatory responses (e.g. ADH release, thirst receptors) [16]. Finally, it is worth noting that hyperosmotic solutions are used therapeutically, in the treatment of brain edema, and also as efficient resuscitation fluids, which exert strong anti-inflammatory (neutrophil-inhibiting) properties [17]. In summary, alterations in cell volume do occur even in a controlled extracellular environment, and volume/osmoregulation represent a major task for all cells, even within a higher organism.

**OSMOTIC STRESS: SENSING, SIGNALING AND EFFECTORS**

**The Bacterial Solution**

The mechanisms by which cells detect changes in osmolarity are best understood in prokaryotes (Fig 1). A variety of channels and transporters present in the bacterial membrane are activated due to the physical and/or chemical changes caused by osmotic stress (reviewed in [18-20]). These channels and transporters serve a two-fold function, they act as both osmosensors and the effectors that will mediate adaptation to osmotic stress. The Msc family membrane channel proteins are mechanosensitive and open in response to the membrane tension created by
hypotonic swelling (reviewed in [21, 22]). During severe hypotonic shock the non-specific channel MscL opens and allows various solutes to travel out of the cell along their concentration gradient. It is the main channel responsible for volume recovery [23, 24]. During mild hypoosmotic exposure, two other channels of the Msc family, MscK [25] and MscS [26], open. These exhibit a lower threshold to membrane stretch (Fig 1).

During hyperosmotic stress OpuA, an ATP binding cassettes (ABC) family of transporters, ProP, an H\(^+\) -solute symporters of the Major Facilitator Superfamily and BetP, an Na\(^+\) -solute symporters of the Betaine-Carnitine-Choline-Transporter family are activated (reviewed in [19, 27]). These transporters bring organic osmolytes, including betaine and proline [28-30], into the cell against their concentration gradient (Fig 1). Many of these systems sense an increase in intracellular ionic strength. There is also evidence suggesting that macromolecular crowding is also involved in the activation of ProP, as an increase in the number of solute molecules at a constant ionic strength can activate the transporter [31].

Although in prokaryotes many of the major sensors are also effectors, and thus contribute to the response of the cell to osmotic stress, important signaling mechanisms have also evolved to transduce osmotic signals. The best known signaling pathways are the histidine kinase phosphorelay systems. Notably, the EnvZ-OmpR phosphorelay system can be activated in response to hyperosmotic stress, although it is not thought to be a major osmosensor (reviewed in [32]). EnvZ is an integral membrane protein that autophosphorylates on a histidine residue in response to high salinity. The phosphate group is then transferred to an aspartic acid residue on OmpR, which, when active, functions as a transcription factor and upregulates the expression of outer membrane porins.
Figure 1

Overview of major osmosensitive transport in a prokaryotic cell.

**Hypotonic Stress**

- MscL
- MscK
- MscS
- BetP: Betaine
- ProP: Betaine, Proline
- OpuA: Betaine

This represents a prototypic prokaryotic cell. These transporters do not naturally occur in the same organism.

**Hypertonic Stress**
Phosphorelay systems are very rare in eukaryotic organisms, but an osmotically activated system has been described in yeast (reviewed in [33, 34]). Under basal conditions, the membrane osmosensor Sln1 autophosphorylates and then transfers the phosphate group to another residue in its own amino acid chain. Next the phosphate group is transferred to Ypd1 (a phosphorelay protein) and finally to the transcription factor Ssk1, which is inhibited by phosphorylation. The end result is the inhibition of the transcription of genes under the regulation of Ssk1 [35]. Under hyperosmotic conditions the transfer of the phosphate group from Sln1 to Ypd1 is inhibited which relieves the inhibition of Ssk1 and allows it to bind the DNA and initiate transcription [35].

To date, no analogous phosphorelay systems have been described in mammalian cell.

The Mammalian Solution

Sensing Changes in Cell Volume and Osmolarity

Sensor and effector mechanisms are better understood in prokaryotes. Currently, little is known about the mechanisms whereby mammalian cells detect changes in their volume. The fundamental questions are: what parameters are being sensed and what components of the cell are sensitive to changes in these parameters? Several physical and chemical parameters are affected when the volume of a cell is modified and any of these could serve as an indicator of that change. These include: plasma membrane tension, osmotic concentration, ionic strength and molecular crowding. Potential cellular components that could be sensitive to changes in these parameters are membrane lipids, intramembrane proteins (e.g. receptors), the cytoskeleton, cell adhesion proteins (e.g. integrins and cadherins), and cytoplasmic proteins [14]. It is very likely that changes in cell volume are not detected by alterations in any one parameter but as a
combination of many parameters. These are probably sensed by multiple systems, which collectively contribute to the overall response.

Recently, studies on volume sensing have concentrated on examining the role of physical and chemical changes in the lipid bilayer of the plasma membrane due to osmotic stress. These changes can regulate membrane receptors, transport proteins and enzymes by mechanisms which are poorly understood [36]. Mammalian cells can compensate for stretching of the membrane primarily due to a membrane reservoir made up of invaginations and microvilli that can fold or unfold. Due to these structures, and the rigidity of the cytoskeleton, membrane stretch-induced activation of transporters is limited to a few cell types [36]. Nonetheless, there are stretch activated ion channels in the eukaryotic cell membrane; for example increased membrane tension has been shown to have a role in the hypoosmotic activation of $K^+$ and $Cl^-$ channels (reviewed in [37, 38]). Moreover, in recent years, the large family of Transient Receptor Potential (TRP) channels has emerged as key mechano- and osmosensors. Some members of this family (e.g. TRPC1, TRPC6 and TRPV1) are sensitive to membrane stretch or the concomitant changes in membrane thickness. Interestingly, they can be activated or inhibited by stretch, and accordingly they can be stimulated by hypo- or hypertonicity [39]. An important member of the latter group is TRPV1b, a hyperosmolarity activated channel involved in antidiuretic hormone release in the supraoptic nucleus and in the regulation of the thirst centre of the brain [40]. The major hypo- and hypertonic ion transport systems in mammalian cells are summarized in Fig 3.

Membrane curvature is another parameter that has been shown to be closely regulated and sensed by cells. Although no specific sensors of membrane curvature have been identified, certain proteins that contain a BAR domain (bin, amphiphysin, rvs) could be candidates. BAR domain containing proteins are usually involved in membrane dynamics [41]. The BAR domain
is a banana shaped structure that binds preferentially to curved membrane surfaces; it lies along
the cytosolic side of the plasma membrane, and thus can sense changes in membrane curvature
[42, 43]. Some TRP channels, e.g. TRPC6, seem to be genuinely responsive to curvature
changes [44]. The plasma membrane is not homogeneous and there has been a growing interest
in its microdomains, caveolae and lipid rafts, enriched in certain lipids and proteins. These
structures have roles in signal transduction, ion transport and membrane trafficking [45]. It has
been reported that both caveolae and lipid rafts are affected by changes in cell volume [46, 47]
and have been identified as possible regulators of phospholipase A₂, which generates
lysophospholipids [48], lipid derivatives that are able to affect local membrane curvature and
induce signaling pathways [49].

Protein concentration or molecular crowding has also been implicated as a potential
sensor of volume change (reviewed in [1, 50, 51]). The latter refers to “the influence of mutual
volume exclusion upon the energetics and transport properties of macromolecules [proteins,
nucleic acids and polysaccharides] within a crowded, or highly volume-occupied, medium” [52].
Under crowded conditions, the laws of dilute solutions do not apply, due to limited diffusion and
frequent intermolecular interactions. This crowded state alters protein folding, assembly and
aggregation, thereby impacting the activity of enzymes and transporters as well as the
polymerization and activity of proteins. An example for the significance of molecular crowding
in volume regulation is that shifting the internal concentration of hemoglobin in canine red blood
cells changes the set-point for activation of volume regulated transporters, thereby altering their
sensitivity to cell volume change [53].

There is also evidence that changes in ionic strength due to shifts in cell volume can be
detected by cellular components. This shift can act as the signal to stimulate transporters, such
as VRAC (a swelling activated Cl\(^{-}\) channel [54]) (Fig 2). Ionic strength has also been implicated as a signal for the induction of signaling cascades, such as the activation of Rho (of the Rho family small GTPases, which will be discussed in further detail later [55]) and the modulation of phosphoinositides (cellular lipids involved in the phosphoinositide kinase signaling pathway [56]). In addition, the intracellular concentration of specific ions may also serve as a parameter that can be sensed by the cell. For example, two shrinkage-induced transporters, NKCC and NHE are both influenced by intracellular Cl\(^{-}\) concentration, but interestingly in an opposite manner: NKCC1 is allosterically inhibited by a rise in Cl\(^{-}\), whereas NHE1 requires Cl\(^{-}\) for optimal activity. These modulatory effects are separate from the effects of volume on these transporters (reviewed in [57]).

*Integrins and cadherins* are excellent candidates as volume sensors because of their inherent properties [58, 59]; they are embedded in the plasma membrane, and connect a cell to the extracellular matrix (ECM) or its neighbours; they form a bridge between the ECM (integrins) or cell contact (cadherins) and the cytoskeleton; and are known to initiate a multitude of signaling pathways (reviewed in [60]). For example, the activation of the tonicity enhancer binding protein/osmotic response element binding protein (TonEBP/OREBP) due to hyperosmotic treatment was impaired in the kidney medulla of integrin \(\alpha_1\) (a subunit of the integrin family) null mice [61]. There is also evidence the integrins play a role in sensing volume changes in rat hepatocytes [62]; cell swelling induced the active conformation of \(\beta_1\) integrins and an integrin-antagonistic hexapeptide prevented the hypotonicity-induced stimulation of proteolysis. There is currently little evidence for cadherins as sensors of cell volume change, but they have been shown experimentally to be sensitive to mechanical stresses and are capable of transmitting signaling events [63], and their intracellular binding partners, the
catenins, become phosphorylated in hypertonically challenged cells [64]. Classical membrane receptors (e.g. growth factor receptors) may also be involved in osmosensing, but because they generate further chemical signals, they will be discussed in the next section.

The cytoskeleton could also participate in the detection of osmotic changes, either directly or by transmitting osmotic signals. Osmotically induced dehydration of G-actin facilitates actin polymerization [65] without the involvement of any other regulators. However, this reaction requires high osmotic forces and therefore is unlikely to participate in physiologic volume sensing. On the other hand, the actin cytoskeleton also undergoes mechanical stress during cell swelling and shrinkage, which may serve as a signal. Nonetheless, firm evidence for the cytoskeleton as an authentic osmosensor is lacking, and the issue remains controversial. There are also many signaling components and membrane channels / transporters that are linked either structurally or functionally to the cytoskeleton (reviewed in [66, 67]).

In summary, there have been exciting developments in the field of osmosensing, and although many osmosensing mechanisms have been defined, their relative importance is not well understood. There are also other osmosensing mechanisms that have yet to be identified.

**Signaling Mechanisms in Hyperosmotic Stress**

Although hypoosmotic signaling is equally important and extensive, the focus of this thesis is hyperosmotic stress and the discussion will be limited to some of the major hyperosmotic signaling pathways. Osmotic signaling appears to be more complex in eukaryotic cells than in prokaryotes, with sophisticated signaling pathways linking the putative sensors to better known effectors. These more refined pathways allow for the integration of various signals and amplification of the effector responses. A number of osmotic signaling pathways have been
identified and the ones that will be discussed fall into the following categories: receptor tyrosine kinases, non-receptor tyrosine kinases, MAP kinases, serine/threonine kinases, lipid kinases and the Rho family small GTPases (summarized in Table 1).

**Receptor tyrosine kinases**

Receptor tyrosine kinases are intramembrane receptors that could mediate osmotic sensing and signaling in the cell. They have been suggested to act as osmotic sensors, as epidermal growth factor receptor (EGFR), interleukin-1 receptor (IL-1R) and tumor necrosis factor receptor (TNFR) have all been reported to cluster in the plasma membrane upon hypertonic stress [13]. This clustering is ligand-independent but results in the activation of the receptor [13]. Interestingly, in rat hepatocytes, activation and phosphorylation of EGFR by cell shrinkage was also shown to occur in a ROS-dependent (reactive oxygen species) manner [68], suggesting that hypertonicity-induced oxidative stress might also be an important input. The functional role of these receptors in downstream effects was verified by showing that the osmotically induced activation of c-Jun NH2-terminal kinase (Jnk) was blocked by desensitization of receptors with their respective ligands or incubation at low temperature (which prevents receptor clustering) [13]. The physical strain acting on the membrane during hypertonic cell shrinkage could force the receptors into close proximity, thereby facilitating their clustering and their activation, leading to downstream signaling [13]. A role for the EGFR in hyperosmotic-induced apoptosis has also been shown in hepatoma cells. When EGFR is activated by hyperosmotic stress it recruits and phosphorylates CD95 [69], which then facilitates the formation of the death inducing signaling complex (DISC) [70] and the induction of apoptosis. Since EGF signaling usually supports survival, this example shows that the use of
Table 1

Summary of mammalian osmotic signaling.

<table>
<thead>
<tr>
<th>Signaling Pathway</th>
<th>Associated Proteins</th>
<th>Cellular Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor Tyrosine Kinases</td>
<td>TNFR, EGFR, IL-1R</td>
<td>Induction of cell death pathways</td>
</tr>
<tr>
<td>Non-receptor Tyrosine Kinases</td>
<td>Src family kinases, Jak, FAK</td>
<td>ORE activation, ion transport regulation</td>
</tr>
<tr>
<td>MAP kinases</td>
<td>p38, ERK, Jnk</td>
<td>ORE activation, ion transport, apoptosis</td>
</tr>
<tr>
<td>Other Serine/Threonine Kinases</td>
<td>Ste20 related kinases, WNKs</td>
<td>Cytoskeletal changes, ion transport</td>
</tr>
<tr>
<td>Lipid Kinases</td>
<td>Phosphoinositol phosphate kinase</td>
<td>Ion transport, cytoskeletal changes, Rho activation</td>
</tr>
<tr>
<td>Rho family Small GTPases</td>
<td>Rho, Rac, Cdc42</td>
<td>Cytoskeletal reorganization, MLC phosphorylation</td>
</tr>
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</table>
EGFR for osmosignaling might change the classical interaction of the receptor and reprogram it for different functions.

Non-receptor tyrosine kinases

Several non-receptor tyrosine kinases have been found to be activated by hyperosmotic stress. The discussion of these kinases will be limited to Src family kinases, Jak and FAK, enzymes which have been shown to participate in some effector responses.

The Src family kinases are cytosolic tyrosine kinases that belong to nine subfamilies including: Src, Yes, Fyn, Fgr, Hck and Lck, and are known to have roles in volume activated signaling [71]. Interestingly, Src itself is actually inhibited under hypertonic conditions [72]. The roles of some of the Src family kinases have been elucidated. For example, the association of Yes with EGFR is induced by hyperosmolarity, and this association appears necessary for the activation CD95 by EGFR [68]. This suggests that Yes plays a role in EGFR mediated apoptosis [68]. Fyn is known to be a player in both the hypertonicity-induced rearrangement of the cytoskeleton [64] and in the hyperosmotic activation of the osmotic response element (ORE), a DNA enhancer sequence for osmoprotective genes, activated by the transcription factor, TonEBP/OREBP [73]. This suggests a role for Fyn in both the short term and long term responses to osmotic stress. Previous studies in our lab have shown that the Fyn-Fer kinase pathway mediates the hyperosmotic phosphorylation of cortactin, an F-actin binding and nucleating protein, which will be discussed in more detail in the effector response section [64]. Fgr, Hck and Lck are also phosphorylated and activated by hyperosmotic stress in hematopoietic cells but little is known about the functional consequences of this reaction [68, 74].
The Janus kinase (Jak) family of non-receptor tyrosine kinases are also activated by hyperosmotic conditions [75, 76]. The main phosphorylation targets of Jak kinases are Signal Transducers and Activators of Transcription or STATs, which upon phosphorylation translocate to the nucleus and initiate the transcription of specific genes [77]. Several members of the Jak family, including Jak1, Jak2 and Tyk2 have been shown to be phosphorylated by hyperosmotic stress in different cell types [75, 76, 78]. However, the hyperosmotic phosphorylation of STATs may not completely depend on Jaks. In 2fTGH cells, STAT1 is phosphorylated by Jak1 [76], while in COS-7 cells, STAT1 phosphorylation is mediated by the p38 signaling pathway [78]. While the Jak-STAT pathway may have a role in osmotic gene regulation, further work is needed to prove this hypothesis. In addition to its effect on gene expression, Jak2 has been implicated in the osmotic activation of NHE: Garnovskaya et al. [75] proposed that osmotic stress induces Jak2-dependent tyrosine phosphorylation of calmodulin, which then binds to and activates NHE1. This intriguing mechanism awaits confirmation.

Focal adhesion kinase (FAK) localizes to the focal adhesion complex where it serves two important functions: it acts as a scaffold to recruit signaling molecules to the focal adhesion and as a tyrosine kinase that phosphorylates itself and other proteins (reviewed in [79]). FAK can be activated both by mechanical shrinkage of the cell, where its aggregation and dimerization is thought to cause autophosphorylation, and by chemical stimuli via Src [80]. FAK has been proposed to exert a protective role against hyperosmotic-induced apoptosis [80, 81]. It remains to be determined if FAK also has a role in the reduced cell motility that is associated with hyperosmotic cell shrinkage.
MAPKs

The mitogen activated protein kinases (MAPKs) were the first identified stress-stimulated kinases. Indeed, their prototype, High Osmolarity Glycerol (HOG) kinase, the yeast homolog of the mammalian p38 MAPK kinase, is a central mediator in osmotic signaling, responsible for the induction of glycerol synthesis. Glycerol is one of the most important non-perturbing osmolytes in yeast [34]. MAPK activation follows a three tiered phosphorelay system: The first proteins to be activated are MAPK kinase kinases (MKKK or MEKK), which in turn phosphorylate a MAPK kinase (MKK or MEK) which then induces dual phosphorylation (on tyrosine and threonine residues) and activation of a MAPK [82]. MAPK kinase kinases can be activated by several upstream pathways, including Ras and Rho family GTPases, and Ste20-related protein kinases [83, 84]. For the purposes of this thesis, the discussion will be limited to the three major MAPK families: p38 MAPKs, extracellular regulated kinase 1/2 (ERK1/2) and the c-Jun N-Terminal kinases (Jnk1/2) and their roles in signaling during hyperosmotic stress [85].

While the upstream mechanism of the osmotically induced activation of p38 MAPK varies in different cell types, Rac and Cdc42 have been implicated, which presumably act through the MEKK3/MK3/p38 pathway [86]. The interaction of these kinases is brought about by the adapter protein OSM [86]. So far two potentially significant roles of p38 MAPK have been identified in mammalian osmoregulation: it phosphorylates heat shock protein 27 (HSP27), thereby reducing the actin-capping properties of this molecule. This might contribute to the hypertonicity-provoked F-actin polymerization [87]. The other effect is that p38 MAPK contributes to osmotically triggered gene transcription by enhancing the activity of TonEBP/OREBP, which drives the expression of osmolyte-generating enzymes and transporters [73]. Regarding the ERK pathway, the osmotic activation of ERK1/2 seems to be dependent on
both Raf and MEKK1 [88]. Recent evidence suggests that the ERK scaffolding protein, KSR, is also involved in the hypertonic activation of ERK1/2 via direct interaction with MEKK1 [87]. The ERK signaling pathway may also serve as a link between osmotic stress and the activation of TonEBP/OREBP, as it was necessary for the TonEBP/OREBP-dependent induction of heat shock protein 70 (Hsp70) and TauT, a transporter of the osmolyte taurine [89].

In mouse embryonic fibroblasts the osmotic shrinkage induced activation of Jnk was strongly reliant on the activation of the MKKK TAK1 [90]. However, MEKK1 activity is also important for the activation of Jnk and therefore it is likely that Jnk activation requires both pathways or is cell type specific [88]. It has also been reported that in some cell types, Jnk can be activated by NHE1 [91] and that Jnk activation in general is dependent on the integrity of the cytoskeleton [88]. Jnk and the signaling mechanisms associated with it have been shown to be an inducer of apoptosis. For example, MEKK1, which regulates MKK4, an activator of Jnk was shown to ubiquitinate the Jnk target transcription factor c-Jun, which leads to its degradation and downregulation after hyperosmotic shrinkage. This sequence of events promoted hyperosmotic-induced programmed cell death in the cells [92].

Other serine / threonine kinases

In the last few years a new family of serine/threonine kinases, that may be crucial in connecting osmotic insult to cytoskeletal changes and volume regulatory ion transport, has emerged. For the purposes of this thesis, the discussion will be limited to the Ste20 related kinases and the with-no-lysine kinases (WNKs).

The Ste20 related kinases that are activated by osmotic stress include: p21 activated kinase (PAK), Ste20 related proline-alanine rich kinase (SPAk) and oxidative stress response
kinase-1 (OSR1) [93]. Both SPAK and OSR1 are known to physically associate with NKCC1 and NKCC2 and SPAK plays a role in the activation of NKCC1 by cell shrinkage [94, 95]. SPAK alone, though, is not capable of activating NKCC1, as truncated NCKK1 mutants that are not able to bind SPAK can still be activated by osmotic stress [96].

PAK2 can be found in one of two pools in fibroblasts: a soluble pool or a particulate pool. Hyperosmolarity has been shown to activate PAK2 and also cause its redistribution from the low kinase activity soluble fraction to the high kinase activity particulate fraction [97]. PAK2 activation could be dependent on Cdc42 activation, which is also redistributed to the particulate fraction under hyperosmotic conditions. These results suggest that PAK2 may have a role in the mediation of cytoskeletal changes downstream of Rac and Cdc42 [97]. PAK1 has also been shown to be activated by osmotic stress but little is known about the upstream mechanism or the functional consequences of this activation [98].

The with–no-lysine kinases (WNKs) derive their name from the substitution of a cysteine residue for a lysine in the kinase active domain of the protein. This lysine residue is known to be conserved and essential for activity in almost all other kinase proteins [99]. The WNKs are a serine/threonine kinase family and have four mammalian isoforms (WNK1-4) [99-101]. WNK1 has been shown to be activated by hyperosmolarity. This activation is dependent on the phosphorylation of a highly conserved serine residue common to all WNK family members [102]. However, the mechanisms by which WNKs are activated by changes in cell volume are not fully understood. Irrespective of the mechanism, WNKs appear to play a major role in the hyperosmotic activation of NKCC1. WNK1 has been shown to phosphorylate the Serum-and-Glucocorticoid-dependent kinase (SGK1), which in turn phosphorylates WNK4 [100]. WNK1 and WNK4 are also known to bind and activate SPAK and OSR1 by phosphorylation [103, 104],
which, as mentioned, can then phosphorylate and activate NKCC1 in response to cell shrinkage [105-107]. It is worth noting that WNK and Ste-20 related kinases are emerging as central regulators of both shrinkage- and swelling-sensitive ion transporters, and these enzymes may represent the long sought after link between cell volume changes and solute transporters.

**Lipid kinases**

Although relatively few studies have been performed regarding the osmotic stimulation of lipid kinases, these enzymes are important, and likely participate in a variety of osmotic responses. The cellular levels of phosphoinositide(4,5)P$_2$ (PIP$_2$), the cell’s most abundant phosphoinositide, have been shown to increase during hyperosmotic stress [56]. This phenomenon could be due, in part, to the activity of the phosphoinositol phosphate kinase PIP5KI [108, 109]. PIP$_2$ may be a central mediator of osmotic effects since: a) it can bind and activate NHE1[110]; b) it regulates Rho family GTPases [109]; c) it has a direct impact on F-actin assembly [111] and d) it controls the ezrin-radixin-moesin (ERM) family of proteins, which are volume-sensitive adaptors linking the cytoskeleton, ion transporters and Rho GTPases [112]. Further work is warranted to clarify the exact roles of PIP$_2$ and its regulators during osmotic stress.

**Rho family small GTPases**

The Rho family GTPases can be classified into 8 groups with 22 known members in mammals (reviewed in [113]). These proteins alternate between an active (GTP bound) and an inactive (GDP bound) form and can thereby act as molecular switches. The most extensively
studied members of the Rho subfamily are Rho, Rac and Cdc42, to which the following discussion will be limited.

Our group has shown that Rho is activated both by increased ionic strength and decreased cell volume in kidney epithelial cells [55]. It is also activated in response to hyperosmotic shrinkage in ELA cells [114] and hepatocytes [112]. Similarly Rac1 and Cdc42 are known to be activated by cell shrinkage in a variety of cell types [86, 115, 116]. Rho GTPases are regulated by the state of their GTP loading and their localization in the cell. These two factors are controlled by three separate families of proteins: 1) guanine nucleotide exchange factors (GEFs), which enhance replacement of GDP with GTP in the GTPase thereby activating it; 2) GTPase-activating proteins (GAPs), which enhance the GTPase activity thereby promoting inactivation of the GTPase, and 3) guanine nucleotide dissociation inhibitors (GDIs), whose main function is to sequester inactive GTPases, but also may have a role in the translocation of Rho GTPases to the plasma membrane, contributing to their localized activity (reviewed in [117]). The upstream regulators of GEFs, GAPs and GDIs remain largely unexplored but integrins and growth factor receptors and their associated signaling pathways are likely candidates, as they have been implicated in volume sensing and are known activators of Rho family GTPases. Recent studies have shown that ERM proteins, which are activated by hypertonicity [112], regulate both Rho-GDI [118] and the GEF Dbl. This hyperosmotic regulation leads to the activation of Rho by Rho-GDI and Cdc42 by Dbl [119].

The activity of Rho family GTPases has been coupled to cytoskeletal reorganization and ion transport through various downstream effectors. Rho is known to activate Rho kinase (ROK) which plays a role in non-muscle myosin II light chain (MLC) phosphorylation and thus contractility, while Rac and Cdc42 are known to activate PAK and WASP/WAVE (Wiscott
Aldrich syndrome protein / WASP family Verprolin-homologous protein) which play roles in decreased actin severing and peripheral actin nucleation, respectively [117]. These changes in the cytoskeleton will be discussed in a separate section. Rac activation is also involved in the hyperosmotic induction of p38 MAPK in certain cells [86, 120].

**Effectors During Hyperosmotic Stress**

There are three major cellular responses to hyperosmotic stress that will be discussed in this thesis: ion transport, cytoskeletal reorganization and gene regulation by the induction of the osmotic response element (ORE) (Fig 2).

**Ion transport**

Membrane ion transporters contribute to regulatory volume increase (RVI) by the net uptake of ions and osmotically obliged water. The most extensively studied transporters that have been linked to hyperosmotic stress are the sodium-proton exchangers (NHEs), the sodium-potassium-chloride cotransporters (NKCCs) and the hypertonicity induced cation channels (HICCs) (Fig 3). These will be discussed briefly below. Although the hypotonicity activated ion transport systems are not discussed in this thesis, for the sake of completeness, some of the major ones are represented on Fig 3.

The NHE family of transporters are antiporters, which facilitate the exchange of H\(^+\) and Na\(^+\) in an electroneutral manner [121]. NHE is a secondary active transporter, which uses the Na\(^+\) gradient generated by the Na\(^+\)-K\(^+\) ATPase to transport protons out of the cell and Na\(^+\) into the cell. The NHE family consists of at least 10 identified isoforms (NHE1-10). Structurally, each member has two distinct parts. The first is a highly homologous region at the N-terminus...
Figure 2

Key effector responses in hyperosmotic stress.

Hyperosmotic Stress

Sensors?

Signaling Mechanisms

Gene Transcription

Cytoskeletal Reorganization

Organellar Remodeling

NHE

Transport

Adaptive Responses
that contains twelve transmembrane domains and the second is a C-terminal cytoplasmic tail region that allows for the regulation of the transporter. The C-terminal domain contains residues that can be phosphorylated by different kinases and motifs for interactions with binding partners [121]. Several isoforms are sensitive to cell volume, but the main osmotically responsive isoform is NHE1 [122]. The exact mechanism by which NHE1 is activated by hyperosmotic stress is not clear. The osmotically responsive region has been localized between the N-terminus and residue 566, and the activation process is thought to relieve an autoinhibitory effect of the tail on the transmembrane domain [123]. As mentioned, a recent study suggested that the Jak2-dependent phosphorylation of calmodulin is an important requirement for the hyperosmotic activation of NHE1 [75].

The NKCC transporters NKCC1 and NKCC2 belong to the chloride coupled cotransporter (CCC) family. NKCC1 is ubiquitously expressed while NKCC2 is found only in the thick ascending loop of Henle in the kidney [124]. These symporters use the ionic gradients generated by primary active transporters (e.g. Na⁺-K⁺ ATPase) to cotransport Na⁺ and K⁺ with Cl⁻ into the cell in an electroneutral fashion [57]. In mammalian cells, the stoichiometry of the transport reaction has been experimentally determined as 1Na⁺ : 1K⁺ : 2Cl⁻ [125, 126]. The net result is the electroneutral transport of Cl⁻ into the cell as the Na⁺-K⁺ ATPase and K⁺ channels rapidly restore the Na⁺ and K⁺ gradients [57]. The NKCCs consist of twelve central transmembrane domains and cytosolic N-terminal and C-terminal domains that contain regulatory sites [57]. It has been well established that hyperosmolarity and a reduction in intracellular Cl⁻ levels can cause the phosphorylation and activation of NKCC [127-129]. Gagnon et al. showed that two serine/threonine kinases Wnk4 and SPAK (previously discussed) work together to phosphorylate and activate NKCC1 [106]. It has also been suggested that MLC
Figure 3

Key osmosensitive transport proteins in a mammalian cell.

Hypotonic Stress

Hypertonic Stress
kinase (MLCK) and MLC phosphorylation may have a role in the hyperosmotic activation of NKCC. This hypothesis is based on the finding that MLCK inhibition, by treatment with the MLCK inhibitor ML-7, reduced or prevented this hyperosmotic activation [130, 131]. However, there is controversy surrounding this hypothesis. In kidney tubular cells, the effects of hyperosmolarity on NKCC activation and MLC phosphorylation can be uncoupled [55, 132]. This does not rule out the possibility that basal contractility could be a permissive factor in the hypertonic stimulation of NKCC. The inhibition of basal myosin ATPase activity with blebbistatin significantly reduced the activation of NKCC in response to hyperosmotic treatment [132].

Hypertonicity-induced cation channels (HICC) are major contributors to RVI in the liver and have been shown to be activated by cell shrinkage in numerous cell types (reviewed in [133]). The molecular identity of these channels is still unknown and they are classified according to their sensitivities to amiloride, Gd$^{3+}$ and flufenamate [133, 134]. Despite the uncertainty surrounding their identity, they are important in the process of RVI as the rates of ion transport by HICCs are 4-5 magnitudes greater than that of other transporter systems [135]. It has been suggested that some amiloride sensitive HICCs may be related to the epithelial Na$^+$ channel (ENaC) in rat hepatocytes [136].

**Cytoskeletal reorganization**

In most cells hypertonic shock causes a remodeling of the actin cytoskeleton. This manifests in three major changes: 1) an increase in the total amount of F-actin; 2) a rearrangement of F-actin, which in certain cells involves an increase in the amount of actin in the sub-membranous ring combined with central stress fibre dissolution, while in other cells it is an
increase in both central and peripheral F-actin and 3) the phosphorylation and activation of non-muscle myosin II light chain (referred to as MLC) [117]. The reorganization of the actin cytoskeleton and myosin phosphorylation serve to reinforce cell structure, and both changes were proven to be crucial for cell survival in *Dictyostelium* [137].

There are three crucial mechanisms that lead to F-actin polymerization: i) *de novo* actin nucleation; ii) modification of actin filament severing and iii) changes in the capping of actin filaments. Each of these processes can be (and in some cases have been demonstrated to be) affected by hyperosmotic stress [117].

Actin nucleation is primarily mediated by the Arp2/3 complex downstream of Rac and Cdc42, resulting in dendritic nucleation, which generates branches on existing filaments [138]. Nucleation can also be mediated by mDia, downstream of Rho, which promotes linear F-actin assembly [139]. The severing of actin filaments is mediated by several proteins, the major ones being cofilin and gelsolin [140, 141]. Severing can generate new barbed ends, but if excessive destroys actin filaments [117]. Capping regulates G-actin addition or dissociation to and from filament ends. There are many proteins that cap the barbed ends of actin filaments, such as gelsolin and CapZ. The importance of changes in capping during hyperosmotic stress has not been explored [142, 143].

Increased phosphorylation of MLC can be mediated by MLCK, or by Rho kinase (ROK). ROK promotes the process by two mechanisms: it inhibits myosin phosphatase and causes the direct phosphorylation of MLC [55, 130].

Most of these mechanisms are under the control of the Rho family small GTPases [117] (Fig 4). As previously discussed, hyperosmotic stress was shown to activate Rho, Rac and Cdc42. Rac and Cdc42 participate in hyperosmotically-induced peripheral *de novo* actin
Rho family small GTPases and hyperosmotic cytoskeletal remodeling.

Hyperosmotic Stress

- Rho
  - ROK
    - MLC $\leftrightarrow$ MLC-P
      - $\uparrow$ contractility

- Rac / Cdc42
  - PAK
    - cortactin
      - WASP/WAVE
        - $\uparrow$ F-actin nucleation
  - Arp2/3
    - stress fibre disassembly
nucleation through the activation and peripheralization of Arp2/3 [115]. Another regulator of this process is cortactin, an additional activator of Arp2/3, which is also phosphorylated and translocated to the periphery by hyperosmotic stress [116]. It has been suggested that the Rac/Cdc42-PAK pathway mediates central stress fibre disassembly, which can be observed in certain cell types [117]. Rho activation was shown to underlie the Rho/ROK-mediated phosphorylation of MLC, and thus the increase in contractility [55]. Ongoing studies suggest that Rho/ROK is a key pathway in cofilin phosphorylation, which inhibits the severing activity of this protein [144]. This then, together with cortactin, stabilizes the newly generated actin filaments in the cell.

In summary, these major cytoskeletal changes, mostly mediated by Rho family GTPases, lead to increased actin polymerization and enhanced acto-myosin contractility; i.e. a more rigid, sturdy cytoskeleton. This provides mechanical protection against cell shrinkage but interferes with cell movement [117]. Cytoskeletal changes may also play a role in the regulation of ion transport [67] and conceivably the remodeling of organelles as well.

**Gene regulation**

Cytoskeletal reorganization and ion transport are acute responses to changes in osmolarity and cell volume. There are also mechanisms that allow a cell to adapt to chronic hyperosmotic exposure. These mechanisms are regulated at the level of gene transcription by the presence of a DNA enhancer sequence known as the osmotic response element (ORE) or the tonicity enhancer (TonE) sequence, located in front of specific osmoprotective genes [145, 146]. The transcription factor that binds to the ORE enhancer sequence was identified by several groups at the same time and is known as ORE binding protein (OREBP), TonE binding protein
(TonEBP) or NFAT5 [147-149]. TonEBP/OREBP is a 200 kDa transcription factor that binds to the ORE as a homodimer and encircles the DNA [150]. Hyperosmolarity induces the translocation of TonEBP/OREBP from the cytosol to the nucleus and it also elevates TonEBP/OREBP levels by stabilizing TonEBP/OREBP mRNA [151]. TonEBP/OREBP is phosphorylated on tyrosine and serine residues in response to hyperosmotic treatment, although the role of this reaction is not fully clear; the current paradigm is that phosphorylation is important for the hyperosmolarity-induced nuclear translocation of TonEBP/OREBP but not for its transactivating capacity[152]. However, multimerization of TonEBP/OREBP may be sufficient for translocation in the absence of phosphorylation [152]. TonEBP/OREBP also contains three transactivation domains, one of which has been shown to be osmotically sensitive [153]. The transactivation domain interacts with a specific group of proteins to form an enhanceosome, which along with the basal transcription complex facilitates the activation of genes under ORE regulation [14]. The transactivation of ORE containing genes has been shown to vary with extracellular NaCl concentration [153]. Known regulators of the transactivation of TonEBP/OREBP in various cell types are cAMP dependent kinase (PKA) [154], p38 MAPK [73], the Src family kinase, Fyn [73] and ataxia telangiectasia-mutated kinase (ATM) [155]. These regulators are also known to be activated by hyperosmotic stress [72, 154-156], further solidifying the link between hyperosmotic stress and TonEBP/OREBP transactivation. Recently, it was shown that increased reactive oxygen species (ROS) production, specifically mitochondrial ROS, contributes to the activation of TonEBP/OREBP under high NaCl conditions [157, 158].

The ORE/OREBP pathway controls the expression of a wide variety of genes. The best studied are the genes for transporters that move organic osmolytes, such as betaine, glycine,
taurine and inositol. When upregulated, these transporters bring osmolytes into the cell to try and restore normal cell volume during extended periods of hyperosmotic stress [14].

ORE/OREBP also induces the gene for aldose reductase, a key enzyme in sorbitol synthesis [159]. Other ORE-dependent genes include heat shock protein 70 (Hsp70) a chaperone protein that protects against high NaCl [160] and aquaporins (or water channels), which regulate the water permeability of cell membranes [161]. The crucial importance of the TonEBP/OREBP system is underlined by the fact that TonEBP/OREBP knockout mice exhibit severe atrophy of the renal medulla due to the apoptosis of tubular cells and lose their capacity to form concentrated urine [162, 163].

A “Novel” Osmotic Effect

Importantly, emerging research indicates the existence of another, hitherto grossly under-recognized structural change in response to hypertonicity: the remodeling of membrane-encased organelles. Specifically, both hypo- and hyperosmotic stress have been shown to inhibit endoplasmic reticulum (ER)-to-Golgi transport, while leaving retrograde transport intact. This resulted in the collapse of the Golgi apparatus into the ER [164]. While these changes were due to altered vesicular traffic, hyperosmotic stress has been shown to remodel intracellular organelles by a different means as well, namely by shifting the balance of fission and fusion. A recent single paper has reported that hypertonicity promoted the fragmentation of mammalian mitochondria in Vero cells [165]. The mechanism and significance of this intriguing phenomenon remains undefined. Cognizant of the possibility that the mitochondrion may be a critical determinant in osmoregulation (as an energy provider, signal integrator and apoptosis regulator), this recent observation directed our attention to the mitochondrion and its osmotically
induced shape changes. Because this problem is the focus of my thesis, the next sections will describe some relevant aspects of mitochondrial biology.

**THE MITOCHONDRION: MORE THEN JUST A POWERPLANT**

The best-known function of the mitochondrion, an ancient endosymbiont within the eukaryotic cell, is the aerobic generation of ATP. In addition, this organelle plays central roles in cellular Ca\(^{2+}\) homeostasis, biosynthetic pathways (steroids, heme), superoxide generation and apoptosis regulation. For the purpose of this thesis, a brief summary of its central bioenergetic function will be given, and then emphasis placed on mitochondrial dynamics and shape regulation.

**Short Overview of Mitochondrial Bioenergetics**

The mitochondrion is surrounded by an outer membrane (OM), which is permeable to solutes of <5 kDa, and an inner membrane (IM), with very restricted permeability. The IM forms large invaginations (cristae) and encloses the matrix space. The IM and the OM are separated by the intermembrane space. Enzymes of the citric acid cycle, located in the matrix, produce reducing equivalents, NADH or FADH\(_2\), the electrons of which are forwarded to molecular oxygen through the various components of the electron transport chain (ETC), which reside in, or are associated with, the IM. The energy of the electron flow is used to move protons from the mitochondrial matrix to the intermembrane space, thereby building up a proton electrochemical potential (\(\Delta\mu\text{H}^+\)). The \(\Delta\mu\text{H}^+\) (~ -220 mV) is composed of a pH gradient (~ 1 unit = -60 mV, inside alkaline) and a membrane potential (~ -160 mV, negative inside). The \(\Delta\mu\text{H}^+\) is the driving force for H\(^+\) back-flux into the matrix, which occurs through the central H\(^+\) channel of the F\(_0\)F\(_1\)
ATPase. This transmembrane and transmolecular proton movement induces a conformational change in the $F_0F_1$ ATPase (rotational theory), enabling it to phosphorylate ADP to ATP. The protons unite with reduced oxygen, the final electron receptor of the ETC, forming water. [166].

There are two additional aspects of this process, which are highly relevant to this thesis. The first is that $\approx 1\%$ of the consumed oxygen is used to produce superoxide anion by the ETC. Mitochondrial-derived ROS are thought to have important regulatory functions. The other aspect is that the negative membrane potential is a major driving force for cation (particularly $\text{Ca}^{2+}$) uptake and it is also necessary for the normal import of mitochondrial proteins [167].

**Is mitochondrial shape regulation important?**

Mitochondria are dynamic organelles that undergo continuous cycles of fusion and fission. During fusion both of the corresponding outer and inner mitochondrial membranes merge. Because of this the contents of the mitochondria are mixed and the identity of any one mitochondrion is transient. Mitochondrial dynamics is an intensely studied field, but until recently the physiological and pathological relevance of fusion and fission remained elusive. Recent research has revealed that deficiencies in the main fusion mediators ($\text{OPA1}$, mitofusin 1/2), fission mediators (DLP-1, Fis1) and associated proteins (GDAP1, Pink/Parkin) (see below) can lead to deficiencies in mitochondrial dynamics and manifest as human diseases [168].

Autosomal dominant optic atrophy is characterized by the degeneration of retinal ganglion cells [169]. The search for the cause of this disease led to the discovery of $\text{OPA1}$ the gene that encodes the protein optic atrophy 1, which is involved in the fusion of the inner mitochondrial membrane (IMM) [170, 171]. However it is still unknown how mutations in the gene and the non-functional protein that is made are linked to the actual disease. Charcot-Marie-Tooth
disease type 2A (CMT2A) is a neuropathy caused by a mutation in mitofusin 2 (Mfn2), a protein that plays a role in the fusion of the outer mitochondrial membrane (OMM) [172]. Another type of CMT, CMT4A is caused by mutations in the gene encoding ganglioside-induced differentiation-associated protein-1 (GDAP1). GDAP1 is an integral OMM protein that may be a player in mitochondrial fission [173]. A recently described dominant negative mutation in the gene that encodes a key fission protein, dynamin-like protein 1 (DLP-1) causes microcephaly, abnormal brain development, optic atrophy and hypoplasia, and is ultimately lethal [174]. Recent studies have shown that defects in Pink1, a mitochondrial fission mediator, can induce dopaminergic neuron degeneration and the onset of Parkinson’s Disease [175].

Mitochondrial Shape Change

Mitochondrial dynamics has been best studied in yeast. In this organism the fusion and fission machinery are relatively well characterized and certain details of the regulation have been elucidated. Mammalian homologs have been described for many of the major players, such as DLP-1, OPA1 and Mfn1/2, although there are some proteins which are either yeast-specific or for which the mammalian homologs have not yet been identified (reviewed in [176]). The following discussion will focus mainly on the processes of mitochondrial fusion and fission in mammalian cells.

Mitochondrial fusion

The fusion of two mitochondria is a complicated process that involves the bringing together and joining of the organelles. An added layer of complexity is that both the outer and inner membranes should fuse in a coordinated manner to ensure proper function of the “new”
mitochondrion. Three major proteins have been implicated in mitochondrial fusion: the mitofusins (Mfn1 and Mfn2) and optic atrophy 1 (OPA1) [177] (Fig 5A).

i) The Players

The mitofusins are large GTPases that localize to the OMM [178, 179]. The N-terminus of the mitofusins contains the GTPase domain as well as a hydrophobic heptad repeat region called HR1. The C-terminus contains the transmembrane domain and a second hydrophobic heptad repeat, HR2. The transmembrane domain is very long and contains a series of charged amino acids in the middle suggesting that it is U-shaped and both the N- and C-terminal regions protrude into the cytosol [179]. Mfn1 and Mfn2 differ very little in their structure, contain the same functional domains and can functionally replace each other. Cells lacking Mfn1 can be rescued by the overexpression of Mfn2 and vice versa [180]. Despite the similarities of the two isoforms, one important difference has been reported. Cipolat et al. showed that in mouse embryonic fibroblasts, OPA1 function is dependent on Mfn1 but not Mfn2 [181]. Mitofusins play an essential role in mitochondrial fusion. Cells that do not express Mfn1 or Mfn2 have greatly reduced levels of fusion [180]. Further, cells that are null for both Mfn1 and Mfn2 exhibit no mitochondrial fusion whatsoever [182].

OPA1 is a dynamin family GTPase that localizes to the intermembrane space and associates with the inner membrane [183, 184]. OPA1 contains an N-terminal mitochondrial targeting sequence (MTS), a central GTPase domain and two HR domains, one close to the GTPase domain and the other at the C-terminus [185, 186]. OPA1 is essential for mitochondrial fusion. Using short interfering RNA (siRNA) to knockdown OPA1 expression inhibited mitochondrial fusion, resulting in fragmented mitochondria [181, 182, 184]. Interestingly, in various cell types, OPA1 overexpression can lead to both mitochondrial fission and elongation
Figure 5
Mitochondrial Fusion.

A. Players
OMM
IMM
Mfn 1/2
OPA-1

B. Mechanism
Since OPA1 is the homolog of the yeast protein Mgm1p, which regulates inner membrane fusion [187], it is thought that OPA1 has the same function [176]. At least eight different isoforms of OPA1 are produced due to alternative splicing [188]. There is additional post-translational modification that is well described in yeast and thought to occur in mammals. The yeast OPA1 homolog Mgm1p has a mitochondrial targeting sequence that targets it to the inner membrane, where it is cleaved by the mitochondrial processing peptide, forming the long version of Mgm1p (l-Mgm1p). Exit from the inner membrane at this point prevents further modification. If it does not exit, a rhomboid protease, Rbd1p/Pcp1p, cleaves Mgm1p again resulting in the short form of the protein (s-Mgm1p) [185, 186, 189]. Somehow, the cellular ratio of l-Mgm1p to s-Mgm1p affects mitochondrial dynamics but the exact mechanism is poorly understood [186]. A mammalian homolog of Rbd1p/Pcp1p, Presenilin-associated rhomboid like protein (PARL), has been identified and it may be important for the processing of OPA1 [186]. Phosphorylation of PARL prevents its cleavage and activation. When PARL is inactive, mitochondrial fusion is reduced likely due to the absence of OPA1 processing [190]. This would decrease the rate of fusion, allowing fission to be the predominant mechanism and the mitochondria would fragment [191].

ii) The Fusion Mechanism

Very little is known about the regulation of fusion and the signal that initiates the process remains unknown. Mfn1 and Mfn2 on adjacent mitochondria interact with one another to begin the process [192]. The homotypic interaction of Mfn1 and Mfn2 or heterotypic interaction between Mfn1 and Mfn2 on neighbouring mitochondria is sufficient for fusion [182]. Specifically, it is the interaction of the HR2 domains of Mfn1 and Mfn2 on adjacent mitochondria that draw the outer membranes together [192], possibly by a conformational
change induced by the GTPase activity of the mitofusins [193] (Fig 5B). How the outer membranes are actually joined is not yet known. A portion of the inner membrane lies very close to the outer membrane. This area is called the inner boundary membrane, and it is likely that inner membrane fusion occurs here. Exactly how the inner membrane is fused together is not known, but OPA1 is thought to play a role [181, 182]. One interesting finding shows that disruption of the mitochondrial membrane potential can uncouple OMM and IMM fusion, allowing the outer membranes to fuse but inhibiting inner membrane fusion [194].

**Mitochondrial Fission**

The process of dividing one mitochondrion into two or more “daughter” mitochondria is also very complex. Again, there is an added layer of complexity since both the outer and inner membrane must be pinched off in order for this to occur. In mammalian systems the two major players involved are dynamin-like protein 1 (DLP-1) and Fis1 [193] (Fig 6A).

i) The Players

DLP-1 (a.k.a. dynamin related protein 1 or Drp1) is a dynamin family GTPase. It has a C-terminal GTPase domain, a central domain, and an N-terminal GTPase effector domain. This structure is typical for dynamin family GTPases [193]. Under normal conditions the majority of DLP-1 is found in the cytoplasm. There is a small subpool that localizes to small puncta on the mitochondria. These DLP-1 puncta mark sites where fission of the mitochondria may occur [195]. DLP-1, through its GTPase activity, is responsible for the fission of mitochondria. DLP-1 knockdown by siRNA or the expression of a dominant negative (GTPase defective) DLP-1 (K38A) resulted in the elongation of mitochondrial tubules due to the inhibition of fission [195, 196]. Several post-translational modifications of DLP-1 have been described. Sumoylation of
DLP-1 via the Sumo1 pathway appears to protect it from degradation and may lock it to the mitochondrial surface [197, 198]. DLP-1 and Fis1 (discussed below) are both targets of the mitochondrial ubiquitin ligase MITOL. Ubiquitination via the MITOL pathway results in the degradation of DLP-1 and Fis1 [199, 200]. It has also been shown that DLP-1 can be phosphorylated on Serine 656. This phosphorylation is mediated by cAMP-PKA and it inhibits mitochondrial fission [201]. Dephosphorylation by calcineurin on the other hand, promotes mitochondrial fission [201]. Cdk1/cyclin B has also been shown to phosphorylate DLP-1, but on Ser 585. This phosphorylation activates DLP-1 and promotes fission [202].

Fis1 is a small protein that localizes to the OMM. It has a small C-terminal transmembrane domain with a cytosolic N-terminal domain. The N-terminus is composed of six α-helices of which the central four form two tandem tetratricopeptide repeats (TPRs), which are helix-turn-helix motifs. One surface of this helical bundle forms a concave hydrophobic bundle that is thought to serve as a platform for protein-protein interactions [203-205]. Fis1 is likely one of the receptors for DLP-1 in mammalian cells. In yeast, Dnm1 (the yeast homolog of DLP-1) does not bind Fis1 directly. There are two proteins, Caf4 and Mdv1, which function as adaptor proteins, and bind to both Dnm1 and Fis1 [206]. To date no mammalian homologs of Caf4 and Mdv1 have been identified, therefore it is thought that DLP-1 and Fis1 interact directly. Fis1 overexpression leads to DLP-1 dependent mitochondrial fragmentation in HEK293, COS-7 and HeLa cells [207, 208]. However there is some controversy regarding Fis1 as a receptor for DLP-1, as knockdown of Fis1 by siRNA in HeLa results in the elongation of mitochondria but it does not prevent the recruitment of DLP-1 to the mitochondria [196]. More studies need to be conducted to determine the exact nature of the interaction between DLP-1 and Fis1 in the mammalian system.
Figure 6

Mitochondrial Fission.

A. Players

DLP-1

Fis-1

B. Mechanism
Although mammalian cells do not have Caf4 or Mdv1, there are other proteins that have been identified as having a role in mitochondrial fission. Endophilin B1 is a protein that possesses a BAR domain, a protein domain which has been implicated in sensing changes in membrane curvature. Like DLP-1, it is found in the cytosol and can translocate to the mitochondria, although a direct interaction between endophilin B1 and DLP-1 has not been reported [176]. The knockdown of endophilin B1 in HeLa cells resulted in the elongation of mitochondria, consistent with a reduction in mitochondrial fragmentation. In addition, endophilin B1 was found to play a role in the maintenance of mitochondrial morphology downstream of DLP-1, but the exact mechanism is unclear [209]. GDAP1 may also serve as a regulator of mitochondrial fragmentation. When GDAP1 is overexpressed in COS-7 cells there is an increase in mitochondrial fission and conversely, GDAP1 depletion results in mitochondrial elongation [173]. MTP18, a proposed mitochondrial fission regulator, could also potentially play a role in mitochondrial fission. As in the case of GDAP1, overexpression of MTP18 in COS-7 cells results in fragmented mitochondria while depletion results in elongated ones [210]. Because of this finding and the fact that MTP18 resides in the intermembrane space, it is thought that it might have a role in the fission of the inner membrane [210, 211]. A recent study in Drosophila has identified PTEN-induced kinase 1 (Pink1) and Parkin as proteins that have an impact on the maintenance of mitochondrial morphology [212]. Pink1 is a mitochondrially localized serine/threonine kinase and Parkin is a ubiquitin-protein ligase. Loss-of-function mutations in these genes are associated with recessive familial forms of Parkinsonism [213, 214]. Pink1 acts upstream of Parkin and mutations in the genes encoding these proteins result in large or swollen mitochondria. These phenotypes can be rescued by the overexpression of DLP-1 or loss-of-function mutations in OPA1 and Mfn2. A recent study suggests that Pink1 is able to activate
DLP-1 and that this is mediated by the interaction of Pink1 with Fis1, although the biochemical characteristics of this interaction remain to be defined [215].

ii) The Fission Mechanism

One of the biggest questions in the field of mitochondrial dynamics is the mechanism by which DLP-1 is recruited to the mitochondria. Once localized to the mitochondria DLP-1 molecules interact with Fis1 and form a spiral around the mitochondria. Likely, through the GTPase function of DLP-1, this spiral constricts and the mitochondrion fragments [195, 216] (Fig 6B). However, the question remains as to what determines the specific sites on the mitochondria that will become fission sites, given that Fis1 is homogeneously distributed throughout the outer membrane [193]. The constriction of the inner membrane or both the inner and outer membranes are thought to create indentations in the outer membrane that target the fission machinery to that site [217-219]. With regard to the recruitment of DLP-1, there is evidence indicating that DLP-1 is capable of self-assembling in the cytosol [220]. A recent study suggests that this self-assembly might actually be enough to induce DLP-1 translocation to the mitochondria [221]. A newly described inhibitor of DLP-1, called mdivi-1, was found to prevent the self-assembly of DLP-1 in the cytoplasm and the subsequent mitochondrial fission induced by an apoptotic stimulus [221]. It has also been shown in HeLa cells that during Bax/Bak mediated apoptosis, a mitochondrial intermembrane space protein called DDP/TIMM8a is released into the cytosol through the mitochondrial membrane permeability transition pore (mPTP – discussed later). Once in the cytosol, DDP/TIMM8a associates with DLP-1 and promotes the redistribution of DLP-1 to the mitochondria and subsequent mitochondrial fragmentation [222]. Dynein, a cytoskeletal motor protein has also been implicated in the mitochondrial localization of DLP-1 in HeLa cells. Overexpression of the p50 subunit, which
inhibits dynein function, prevents DLP-1 localization to the mitochondria [223]. Relevant to this thesis, the cytoskeleton has also been implicated in the regulation of DLP-1. Specifically, F-actin depolymerization was found to inhibit the accumulation of DLP-1 in the mitochondrial fraction, induced by the F_0F_1 ATPase inhibitor, oligomycin [224]. The diversity and multitude of the proposed mediators show that the problem of DLP-1 recruitment is essentially unresolved. Because hyperosmolarity induces mitochondrial fragmentation, our work will focus on the machinery of mitochondrial fission; it is acknowledged however, that future work should address the fusion side of the process as well.

**Mitochondrial Fission and Cell Function**

As previously described, mitochondrial fission is necessary for normal cell function as a dominant negative mutation in the fission protein DLP-1 is lethal [174]. Mitochondrial dynamics and in particular mitochondrial fragmentation has a role in several cellular processes including: the organellar distribution and localization of mitochondria, mitochondrial calcium handling and apoptosis [176, 193].

**Mitochondrial distribution**

Localization and distribution of mitochondria need to be regulated for two important reasons. The first is that during mitosis there needs to be a mechanism which ensures that both daughter cells receive enough mitochondria to maintain their viability. The second is that mitochondria need to be distributed evenly throughout the cell, especially in asymmetric cells (e.g. neurons) to ensure that ATP is available locally to all areas of the cell [225, 226]. The distribution of mitochondria in the cell is dependent on both microtubules and microfilaments.
When one or the other is disrupted, mitochondrial movement in neurons is altered but not abolished. If both systems are chemically disrupted simultaneously, mitochondrial movement in the cell ceases altogether [227]. Microtubules are polarized and have a plus-end and minus-end. Plus-end directed movement (also called anterograde movement) moves cargo toward the periphery of the cell, while retrograde movement (minus-end directed) brings payloads toward the cell body [226]. Kinesins are plus-end directed motors that use ATP to move along the microtubules. Two kinesin isoforms, Kif1B and Kif5B, are known to associate with mitochondria and mediate their movement [228, 229]. Dynein is the minus end motor that transports mitochondria back to the cell body [223]. In yeast, mitochondrial movement depends highly on actin. The actin nucleation mediator Arp2/3 can associate with the mitochondria, suggesting that actin polymerization is responsible for the movement of mitochondria [230]. In neurons, the role of actin seems to be anchoring mitochondria to areas of the cell where they are required. For example, a functional actin cytoskeleton is necessary for the immobilization of mitochondria and prevention of microtubule dependent retrograde transport [231, 232]. Mitochondrial fission has also been linked to the transport and localization of mitochondria during mitosis. In HeLa cells the onset of mitosis induces mitochondrial fission by phosphorylating DLP-1, resulting in smaller, more portable mitochondria. This was correlated with the cyclin-dependent kinase (Cdk1/cyclin B)-mediated phosphorylation of DLP-1. Cdk1/cyclin B is involved in the regulation of the cell cycle [202]. With regards to distribution, there is evidence that in neurons mitochondrial fragmentation generates healthy mitochondria of the proper size that can be selectively transported to the regions of the cell (especially the axon) where they are required. Mitochondria that are not functioning properly are moved back to the cell body where they can be repaired or degraded [233, 234]. Moreover, it is likely that the size
of mitochondria affects their efficient trafficking [226]. However, little is known about the coordination of mitochondrial shape and trafficking and this interesting topic needs to be explored further.

*Mitochondrial calcium handling*

Ca$^{2+}$ is one of the most important second messengers in the cell, and the mitochondrion is one of the most important regulators of this key ion. The mitochondrial Ca$^{2+}$ transport-system has a triple role in cellular Ca$^{2+}$ homeostasis: 1) During a cytosolic Ca$^{2+}$ transient, the mitochondria take up Ca$^{2+}$ (directly at ER release sites or from the cytosol) and relay an amplified Ca$^{2+}$ signal to the matrix space, where Ca$^{2+}$ stimulates three key enzymes of the citric acid cycle, thereby satisfying the increased energy demand of the activated cell [235]; 2) In the case of sustained or unusually high cytosolic Ca$^{2+}$ elevations, the mitochondria act as a Ca$^{2+}$ sink: they accumulate large amount of this potentially deleterious ion, thereby ensuring cell survival [236]; 3) Upon extreme Ca$^{2+}$ load or in the presence of other oxidative damage, the so-called membrane permeability transition pore (mPTP) opens, which dissipates the membrane potential and leads to the release of mitochondrial contents [237]. Opening of this large non-selective pathway is intimately related to mitochondrially induced cell death (apoptosis and necrosis) [238, 239]. Cycling of Ca$^{2+}$ across the inner membrane is realized by various transporters: Ca$^{2+}$ uptake, is driven by the membrane uniporter (Fig 7); Ca$^{2+}$ release, under normal conditions, is catalyzed by the coordinated operation of the Ca$^{2+}$/Na$^+$ and possibly the Na$^+$/H$^+$ antiporters [240, 241]. Strangely, the molecular identity of the two former pathways remains elusive. More is known about the mPTP: it is composed of the adenine nucleotide translocator in the IM, the voltage-dependent anion channel (VDAC) in the OM, and some other
Figure 7
Mitochondrial calcium and apoptotic signaling.

- Ca\textsuperscript{2+} efflux
- Ca\textsuperscript{2+} influx
- Ca\textsuperscript{2+} Release
- Ca\textsuperscript{2+} influx

VDAC
Adenine Nucleotide Translocator

Apoptotic Signals

APOPTOSIS
Active caspases

VDAC

Ca\textsuperscript{2+} uniporter

Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger
NHE

APAF-1
Caspase-9
45
proteins, which together constitute a large “transmitochondrial” channel (Fig7). IM permeabilization through the opening of the mPTP may initiate apoptosis. However, the key event in apoptosis induction is the permeabilization of the OM, a process regulated by pro- and antiapoptotic members of the Bcl family (see the next section) [238, 239] (Fig 7). Recent research has revealed interesting relationships between mitochondrial Ca\(^{2+}\) handling and fragmentation; on one hand elevated Ca\(^{2+}\) has been reported to promote DLP-1 recruitment to the mitochondria [242] and to facilitate DLP-1 activity, by inducing its calcineurin-mediated dephosphorylation [201]; on the other hand, DLP-1 mediated fragmentation has been proposed to protect cells from apoptosis. The suggested reason is that in highly interconnected mitochondria, the Ca\(^{2+}\) mediated apoptotic signals are rapidly propagated to the entire network. In contrast, mitochondrial fragmentation prevents the generalized spreading of this signal, thereby limiting apoptosis [243].

**Apoptosis**

Mitochondria play a key role in programmed cell death, primarily by releasing cytochrome C (CytC) and other apoptogenic proteins (Smac/DIABLO and Omi/HtrA2) [244], which then activate the cytosolic caspase cascade. When released into the cytosol, CytC complexes with Apoptosis activating factor 1 (Apaf-1) and caspase-9 to form the apoptosome (Fig 7). Within the apoptosome, caspase-9 is cleaved, which enables it to cut effector caspases, like caspase-3. Once activated, caspase-3 is able to cleave several substrates and induce apoptosis (reviewed in [245]). Natural inhibitors of caspases, called inhibitor of apoptosis proteins (IAPs) are found in the cytosol and strongly inhibit caspase activation. When released
from the mitochondria, Smac/DIABLO and Omi/HtrA2 antagonize IAPs so that CytC can complex with Apaf-1 and caspase-9, and induce the apoptotic caspase cascade [246].

The central question is: how is CytC, a component of the respiratory chain, which resides in the intermembrane space attached to surface of the cristae, released from this location into the cytosol? It seems that there are two requirements for this process: induction of increased mitochondrial outer membrane permeabilization (MOMP) and the remodeling of the cristae (i.e. the widening of the neck of the cristae to allow CytC liberation). [247, 248]. These processes, which may be separately regulated, are not fully understood. OPA1, which is usually associated with mitochondrial fusion, has been proposed to mediate apoptotically-induced cristae remodeling [248]. Regarding the process of MOMP, there are several theories backed by various experimental observations, the most plausible of which will be summarized here. One mechanism involves the opening of the mPTP, which generates a colloid-osmotic imbalance leading to matrix expansion and OM rupture liberating CytC. However, in many cases CytC is released prior to mPTP opening, pointing to alternative mechanisms [249, 250]. While the details are not clear, OM permeability is primarily dictated by the Bcl-2 protein family, the members of which can be placed into 3 functional groups: A) *Apoptosis initiators* such as Bid or Bad; these are the so called BH3-domain only proteins, which activate members of the next group, the effectors; B) *Effectors*, such as Bax and Bak; these are multidomain proteins (BH1-3), which actually carry out OM permeabilization; and C) *Apoptosis inhibitors*, such as Bcl-2, Bcl-XL; these are the so called Bcl-2-like proteins, which interfere with action of the other two groups [251, 252]. According to the current view, apoptotic stimuli activate the initiators, which induce conformational changes or proteolysis in the effectors. The effectors then translocate to the mitochondria and induce OM permeabilization [245]. The exact mechanism of the OM
permeabilization is not fully clear. One (likely) possibility is that Bax and Bak, which are pore-forming proteins multimerize and assemble into large channels in the OM (Fig 7). Alternatively, Bax may induce pore-formation by binding to the OM protein VDAC (Fig 7). Whatever the mechanism is, the assembled large pores allow the efflux of CytC from the intermembrane space [250].

Intriguingly, recent findings suggest molecular interactions between the fission machinery and the pro-apoptotic (effector) Bcl-2 proteins: e.g. Bax and Bak colocalize with DLP-1 on the mitochondria of apoptotic cells [220]. Moreover, Bax interacts preferentially with narrow curvature vesicles, a biophysical characteristic of fragmented mitochondria [253]. However, the biochemical basis of the fission-apoptosis relationship is still largely unknown.

The relationship between mitochondrial fragmentation and apoptosis is controversial. It is not known whether mitochondrial fragmentation is the cause of or the result of MOMP. Two models that link mitochondrial fragmentation and apoptosis will be briefly discussed [245]. It is likely that both models (or a part of each model) are valid in various cell types or upon different apoptotic stimuli. The first proposes that upon receiving an apoptotic stimulus, Bax and DLP-1 translocate to the mitochondria. At the OMM, active Bax participates in DLP-1 mediated fission and cristae remodeling by a mechanism which remains unclear. MOMP occurs after Bax activation and mitochondrial fission, which results in the release of CytC [245]. In favor of this view, during apoptosis Bax translocates from the cytosol to the mitochondrion in COS-7 cells, fibroblasts and murine thymocytes [254, 255], and Bax has been found to associate with Bak and DLP-1 on the mitochondria [256, 257]. In addition, there are studies showing that DLP-1 mediated mitochondrial fission is stimulated by Bax and/or Bak activation [220, 257].
The other model postulates that apoptotic stimuli promote the translocation of Bax and other pro-apoptotic Bcl-2 proteins to the mitochondria where they induce MOMP and the release of soluble intermembrane space proteins into the cytosol. Some of these proteins cause the recruitment of DLP-1 to the mitochondria, which induces mitochondrial fission [245]. In HeLa cells, Bax/Bak dependent MOMP caused the release of DDP/TIMM8a, a soluble intermembrane space protein, from the mitochondria. Once in the cytosol DDP/TIMM8a can bind DLP-1 and facilitate its recruitment to the mitochondria. Once at the mitochondria DLP-1 initiates mitochondrial fragmentation [222]. Some studies provide evidence that apoptosis and fragmentation can be dissociated: in HeLa and COS-7 cells siRNA, the knockdown of both DLP-1 and Fis1 prevents mitochondrial fragmentation but does not inhibit apoptosis induced by Bax/Bak dependent death stimuli such as, ultraviolet light (UV) or staurosporine [258]. Interestingly, the same study showed that DLP-1 and Fis1 downregulation partially inhibited the mitochondrial release of CytC. However, the release of Smac/DIABLO was unaffected and apoptosis still occurred. This suggests that DLP-1 and mitochondrial fission could have a role in the cristae remodeling that could be necessary for CytC efflux from the mitochondria [258].

A recent study suggests that DLP-1 mediated mitochondrial fission is not necessary for Bax/Bak activation but that the physical presence of DLP-1 at the mitochondria is required. Cassidy-Stone et al. [221] showed that inhibition of DLP-1 self-assembly and translocation to the mitochondria by mdivi-1 (a new DLP-1 specific inhibitor), prevents Bax/Bak mediated MOMP. They suggest that DLP-1 acts upstream of or in conjunction with Bcl-2 proteins to directly regulate MOMP [221]. Clearly the apoptosis/fragmentation relationship remains a hotly debated area.
Hyperosmotic stress has also been shown to induce apoptosis via the mitochondrial pathway. In HeLa cells, the knockdown of Bcl-2 and Bcl-XL sensitized the cells to hyperosmotically-induced apoptosis. Conversely, the knockdown of Bax in A549 cells reduced the release of apoptosis inducing factor from the mitochondria, thereby protecting the cells from hyperosmotically triggered apoptosis [259].

Interestingly members of the Rho family small GTPases, which are known to be activated by hyperosmotic stress (discussed previously), have also been implicated in the activation of mitochondrially mediated apoptosis: both Rho and Rac have been reported to upregulate Bax expression [260, 261]. In the case of Rac the following mechanism was suggested: upon apoptosis induction in NIH 3T3 cells, Rac activates p38 MAPK, which in turn induces nuclear translocation of p53. This protein can upregulate Bax expression. Once upregulated, Bax localizes to the mitochondria and promotes the opening of the MOMP. This results in caspase activation and apoptosis. Nonetheless, the role of small GTPases in mitochondrial fragmentation and in the relationship between apoptosis and fragmentation are unknown.
EXPOSITION OF THE BIOLOGICAL PROBLEM: RATIONALE, HYPOTHESIS AND AIMS

Hyperosmotic stress, cellular dehydration and cell shrinkage, conditions encountered under a variety of physiological and pathological states, represent a major threat to cellular integrity. Accordingly, each cell mobilizes a set of regulatory responses, which either lead to adaptation, or - during overwhelming volume changes – to programmed cell death [259]. These responses are either functional or structural. The first category includes the activation of solute transporters and the transcription of osmoprotective genes [122, 127, 145, 146]. The second category is much less explored and understood. Nonetheless, osmotic stress has been described to induce two major structural alterations: reorganization of the cytoskeleton [115] and remodeling of membrane-enclosed organelles, particularly the Golgi apparatus and the mitochondria [164, 165]. Changes in mitochondrial structure and function are likely of key importance, since this organelle provides the energy for all other adaptive responses and it is also the main death/survival switch of the cell.

A single, recent publication has reported that during osmotic stress mitochondria undergo fragmentation [165]. However, the phenomenon of hyperosmolarity-induced mitochondrial remodeling has not been thoroughly characterized, and the underlying mechanisms, as well as the signaling processes that connect osmotic insult to changes in mitochondrial structure remain unknown.

Mitochondria are dynamic organelles that exhibit large shape changes in various metabolic states [193]. In addition, they undergo genuine fusion and fission (fragmentation) cycles [193]. One of the central mediators of mitochondrial fragmentation is dynamin-like protein-1 (DLP-1), which is recruited to mitochondria during the fission process [195, 196].
While the mechanism of recruitment has not been fully elucidated, the cytoskeleton has been implicated in the process: actin depolymerization has been shown to prevent the mitochondrial recruitment of DLP-1 induced by metabolic poisons [224]. Moreover, increasing evidence suggests that the actin skeleton might be a general regulator of mitochondrial structure and function: excessive actin polymerization has been correlated with increased mitochondrial ROS production, aging and induction of apoptosis [262].

Previous studies in our lab have shown that hyperosmotic stress induces robust cytoskeleton remodeling, characterized by actin polymerization and myosin phosphorylation [55, 115]. The hyperosmolarity-induced activation of Rho family GTPases (Rho and Rac) plays a central role in these cytoskeletal responses [55, 116]. Interestingly, recent observations suggest that Rho and Rac are involved in the regulation of the mitochondrial apoptotic pathways as well [260, 261]. However, nothing is known about the potential role of the cytoskeleton and/or the cytoskeleton-regulating Rho GTPases in the hyperosmotically induced fragmentation of mitochondria.

In addition to its major role as an energy supplier, the mitochondrion has emerged as an important player in cellular signaling. Relevant to our topic, hypertonicity has been shown to increase mitochondrial ROS production [157], and mitochondrially derived ROS have been suggested to alter gene expression through the induction of the osmotic response element (ORE) [157, 158]. These findings render the mitochondrion a potential mediator of an important and specific osmoprotective response. However, it remains unknown whether hypertonicity-triggered fragmentation plays a role in ORE regulation.

Finally, the mitochondrion has been implicated in the propagation of the apoptotic signals that are induced by hyperosmotic stress [259]. Mitochondrial fragmentation has been associated
with apoptosis, but the cause-effect relationship between these phenomena is highly controversial (reviewed in [245]). The potential role of mitochondrial fragmentation in osmotic stress-induced apoptosis remains to be elucidated.

With this scenario in mind, we formulated the following hypothesis.

**HYPOTHESIS:** Hyperosmotic stress induces the recruitment of DLP-1 to the mitochondria, and the recruited DLP-1 is responsible for the ensuing mitochondrial fragmentation. Osmotically provoked cytoskeleton remodeling and/or the related activation of Rho-family GTPases are important upstream mediators of DLP-1 recruitment or action. The consequent mitochondrial fragmentation plays important roles in the subsequent effector responses, involved in adaptation or apoptosis (Fig 8).

To address this hypothesis we set the following general and specific aims:

**GENERAL AIM:** To characterize the process of osmotic stress-induced mitochondrial fragmentation, to explore the underlying molecular mechanism, and to define the link between mitochondrial fragmentation and hyperosmolarity-induced adaptive and apoptotic responses.

**SPECIFIC AIMS:**

1) To characterize the effect of hyperosmotic stress on DLP-1, and to define the role of DLP-1 in hyperosmolarity-induced mitochondrial fragmentation.

2) To define the role of the cytoskeleton and Rho family GTPases in hyperosmotically triggered mitochondrial fragmentation.
3) To explore the relationship between osmotically induced mitochondrial fragmentation of apoptosis induction and ORE activation.
Figure 8

Working hypothesis.

Osmotic Stress

- Rho family small GTPases
- Cytoskeletal Reorganization

DLP-1 Recruitment

- Mitochondrial Fission / DLP-1 Action

Downstream Effectors

- Adaptation
- Apoptosis
INTRODUCTION

Alterations in cell volume, a vital parameter tightly regulated by all cells, can occur under various physiological, pathological and therapeutic conditions [14]. Volume changes can be brought about by the polymerization or depolymerization of cellular substrates, the transport of ions and metabolites and exposure to anisoosmotic environments [1]. Significant changes in cell volume represent a major threat to cell integrity, as they can lead to membrane rupture (under hypoosmotic conditions) or disruption of normal metabolism and apoptotic death (during hyperosmotic stress) [14]. Accordingly, several mechanisms have evolved to protect the cell from osmotic stresses; these serve either to restore near-normal cell volume or reinforce the cell structure, allowing it to withstand the mechanical challenge. In the case of hyperosmotic stress, volume restoration is achieved in the short term, by the activation of ion and metabolite transporters and channels [263] and in the longer term by the activation of osmoprotective genes under the control of the osmotic response element (ORE) [14]. The physical protection is achieved by reorganization of the actin cytoskeleton (reviewed in [67]). Previous studies in our lab have shown that Rho, Rac and Cdc42, members of the Rho family small GTPases, are stimulated by hyperosmotic stress and that their activation plays a central role in the subsequent cytoskeleton remodeling [55, 115, 116]. Specifically, Rac and Cdc42 facilitate *de novo* F-actin assembly via the Arp2/3 complex and cortactin [115, 116, 264, 265], while the activation of Rho leads to reduced F-actin severing and enhanced myosin light chain phosphorylation [55]. Collectively these changes promote the formation of a submembranous actin ring, and facilitate myosin-based contractility, thereby increasing cortical rigidity and mechanical resistance [117].

Intriguingly, recent studies suggest that in addition to cytoskeletal reorganization, osmotic stress induces another major structural change: the remodeling of membrane-enclosed
organelles, such as the Golgi apparatus and the mitochondrion [164, 165]. A recent article has reported that in Vero cells hyperosmolarity caused mitochondrial fragmentation [165], however the mechanism and the significance of this process has not been clarified.

Mitochondria are dynamic organelles that undergo fusion and fission cycles [193], and mutations altering the balance between these processes lead to human diseases [168]. While the exact roles of fission and fusion are just emerging, they are thought to be important for normal distribution of mitochondria, the regulation of Ca^{2+} homeostasis and apoptosis [225, 226, 237, 245]. Mitochondrial fusion is mediated by the transmembrane mitofusin GTPases (Mfn1 / Mfn2) and optic atrophy 1 (OPA1), involved in the fusion of the outer and inner mitochondrial membrane respectively [179, 180, 184, 266]. Key components of the mitochondrial fission machinery include the dynamin family GTPase, dynamin-like protein 1 (DLP-1) [195] and its receptor on the outer mitochondrial membrane, Fis1 [207, 208]. During fission, DLP-1 is recruited from the cytoplasm to the mitochondria, but the underlying mechanisms are poorly understood. Posttranslational modification of DLP-1 [197, 199-202] or Fis1 [215] or the release of an intramembrane adaptor protein (DDP/TIMM8a) have been proposed as potential mechanisms [222]. Importantly, the actin skeleton has also been suggested to regulate fragmentation, as F-actin depolymerization prevented the mitochondrial recruitment of DLP-1, induced by the ATPase inhibitor oligomycin [224].

Osmotically induced changes in mitochondrial structure and function may be of key importance, since this organelle provides the energy for all other adaptive responses and it is also the main death/survival switch of the cell. Indeed, hypertonicity-induced, mitochondrionally derived reactive oxygen species (ROS) have been suggested to contribute to the induction of ORE [157]. Furthermore, strong hyperosmotic stress has been shown to activate the
mitochondrial apoptotic machinery [259], and new studies suggest that Rho and Rac might be involved in the regulation of mitochondrial apoptosis [260, 261].

Together these findings raise the possibility that mitochondrial remodeling may be an important new aspect of osmoregulation. However, the molecular mechanism underlying hypertonicity-induced mitochondrial shape change, the relevant signaling pathways, and the potential contribution of the concomitant cytoskeleton remodeling are unknown. Similarly, the functional consequences of osmotically induced mitochondrial fragmentation in adaptation or apoptosis remain to be elucidated.

To address these important issues, we sought to answer the following questions: a) Is DLP-1 recruited to the mitochondrion under hyperosmotic stress, and if so, is it responsible for mitochondrial fragmentation? ; b) Does cytoskeleton remodeling and/or activation of Rho-family GTPases play a mediatory role in the process? ; and c) Does fragmentation (or DLP-1 recruitment) have an impact on the osmotically induced activation of ORE and apoptosis? Our results indicate that hyperosmolarity induces DLP-mediated mitochondrial fragmentation, and this process requires Rac activity. Actin polymerization is not necessary for DLP-1 recruitment per se, but it has a potentiating role in fragmentation. DLP-1 recruitment seems to promote ORE activity, while hyperosmotically induced fragmentation appears to mitigate the initiation of apoptosis during strong osmotic stress.
MATERIALS AND METHODS

Materials and Reagents

All reagents were from Sigma (St. Louis, MO) if not otherwise stated. Enhanced Chemiluminescence kits were from Amersham Biosciences (Piscataway, NJ) or Perkin Elmer (Waltham, MA). The BaculoGold 50X protease inhibitor cocktail added to the lysis buffer was from BD Pharmingen (Franklin Lakes, NJ). Complete-Mini protease inhibitor tablets were from Roche (Laval, QC).

Primary Antibodies

The following commercially available primary antibodies were used: anti-DLP-1 (BD Transduction Laboratories, Franklin Lakes, NJ), anti- F0F1 ATPase subunit α (Molecular Probes, Carlsbad, CA), anti-Rac (Upstate Biotechnology, Lake Placid, NY), anti-Myc (9E-10) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-tubulin (Sigma).

Secondary Antibodies

Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Jackson Immunoresearch (West Grove, PA). FITC, Cy2 and Cy3-conjugated anti-mouse and anti-rabbit antibodies were from Jackson Immunoresearch.

Inhibitors

The following pharmacological inhibitors were used: Latrunculin B, Jasplakinolide, Y-227632, Cytochalasin D, Blebbistatin and SB203580 were from Calbiochem (La Jolla, CA); Taxol, Nocodazole, Colchicine and EHNA were from BioMol (Plymouth Meeting, PA)

Cell Culture and Treatment

LLC-PK1/AT1, cells a kind gift from Dr. R.C. Harris (Vanderbilt University School of Medicine, Nashville, TN) and wild type (WT) LLC-PK1 cells were grown in Dulbecco’s
Modified Eagle Medium (DMEM)(Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum and 1% antibiotic (penicillin and streptomycin, Sigma). Cells were grown in a humidified atmosphere of 5% CO₂ at 37 °C. Unless otherwise stated, cells were grown to 80-100% confluence, serum deprived in serum-free DMEM for 2 hours and then pre-incubated in Iso-NaCl medium (130mM NaCl, 3mM KCl, 1mM MgCl₂, 1mM CaCl₂, 20mM HEPES, 5mM glucose [pH 7.4]) for 10-20 minutes. The osmolalities of the treatment media were confirmed using a vapor point osmometer [64] and very closely approximated the calculated osmolarities of the media. Therefore in our studies we refer to osmolarity as calculated by the concentration of all small molecule osmolytes in the solution. They were then treated as described in the figure legends.

**Plasmids and Transient Transfection**

Constructs encoding for wild type (WT) DLP-1-GFP and dominant negative (DN) DLP-1-GFP (K38A) were kind gifts from Dr. M. McNiven (Mayo Clinic, Rochester, MN) [267]. pDsRed2-Mito (mito-RFP) and mito-GFP are commercially available from Clontech (Mountain View, CA). Constitutively active (CA) Myc-tagged Rac (Rac1Q61L) and Myc-tagged DN Rac (Rac1T17N) were gifts from Dr. G. Bokoch [268]. Myc-tagged PAK1 mutants (CA PAK: PAK1H83,86L/T422E and DN PAK: PAK1H83,86L/K299R) were kindly provided by Dr. A.S. Mak [269]. If not otherwise stated the parent plasmid for each construct was pcDNA3. Transient transfection was performed using FuGene6 (Roche) according to the manufacturers instructions. Cells were transfected with 1μg total of plasmid DNA per well (for a 6-well plate). 2.5 μL of FuGene was used for every 1μg of plasmid DNA.
Stable Expressing DN DLP-1-GFP Cell Line

Wild type LLC-PK1 cells were transfected with DN DLP-1-GFP. After 24h the growth medium was replaced with selection medium (DMEM growth medium supplemented with 1mg/mL G418 (Sigma)) to select stably transfected cells. Surviving cells were plated on 10cm dishes. After 72h GFP expressing colonies were selected with cloning rings and transferred to 24-well plates. The highest GFP expressing colonies were transferred to 6-well plates and eventually the best expressing cells of the 6-well dish were transferred to tissue culture flasks. Cells were grown in selective medium for 12 weeks, after which they were grown in selective medium every second passage.

siRNA Design and Transfection

An siRNA against porcine DLP-1 was designed against the following sequence, 3’-TGAACCTTATTAACCTCAT-5’ and ordered from Ambion (Austin, TX). Cells were grown in antibiotic-free serum-containing DMEM. SiRNA transfection was performed using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) according to the manufacturers instructions. The siRNA was diluted from a 100mM stock to a 25nM final concentration in a 12-well plate and incubated for 48h (If not stated otherwise, a final siRNA concentration of 25nM was used for all experiments). Cells were then serum starved for 2h and treated as described in the figure legends in DMEM without antibiotics or serum.

Immunofluorescence Microscopy

Cells were grown on coverslips in 6-well dishes (unless otherwise stated) until 80-100% confluent. Where indicated mitochondria were labeled with MitoTracker Orange (Molecular Probes) for 30 min with a final dye concentration of 100mM. After treatment the cells were fixed in 4% paraformaldehyde (PFA) for 30 min, washed in phosphate buffered saline (PBS) and
then quenched with 100mM glycine in PBS. The cells were permeabilized with 1% BSA in PBS supplemented with 1% Triton-X 100, followed by blocking with 3% BSA in PBS for 1 hour. The coverslips were then incubated with the primary antibody for 1 hour, then washed with PBS and incubated with a fluorescently-labeled secondary antibody (with DAPI to visualize the nuclei) for 1 hour. After a final wash, the coverslips were mounted onto slides using Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Samples were viewed using an Olympus IX81 microscope (Melville, NY) coupled to an evolution QEi monochrome camera (Media Cybernetics, Silver Spring, MD) or confocally with a Leica DMIRE2 confocal inverted fluorescence microscope coupled to a Hamamatsu Black-Thinned EM-CCD camera and equipped with a spinning disk confocal scan head.

**Aspect Ratio Calculation**

Aspect Ratio (AR) was calculated using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) image analysis software. Mitochondria were defined as objects and the software placed an ellipse of best fit around each object, from which the major and minor axes were determined and the AR calculated (major/minor) (Fig 14A). At least 50 mitochondria from 3 different cells were analyzed per treatment condition for a total \( n \) of 150 or greater.

**Western Blotting and Protein Assay**

Unless otherwise specified, after treatment, cells were washed in ice-cold PBS and then lysed and scraped into Triton-X lysis buffer (100mM NaCl, 30mM HEPES, 20mM NaF, 1mM EGTA, 1% Triton-X 100, pH 7.5) supplemented with 1mM Na3VO4, 1mM PMSF and protease inhibitor. For total cell lysates, cells grown in 6-well plates were lysed in 150\( \mu \)L of supplemented Triton-X lysis buffer. Samples were then diluted in an equal amount of 2X Laemmli buffer, boiled for 5 min and then separated by SDS-PAGE. Equal amounts of protein were then loaded
onto the SDS-PAGE gel. The proteins separated by electrophoresis were then transferred onto nitrocellulose membrane using a Bio-Rad Mini Protean III apparatus (Bio-Rad, Hercules, CA). Blots were blocked in Tris-buffered saline (TBS) containing 4% bovine serum albumin (BSA) for 1 hour and then incubated with primary antibody for at least 1 hour. Primary antibody binding was visualized by incubation with the relevant horseradish peroxidase-coupled secondary antibody (mouse, rabbit) followed by the chemiluminescence method. Protein assays were performed using the Bradford Assay (Bio-Rad) with BSA as a control.

**Mitochondrial Preparation**

Cells were grown on 10 cm dishes (2 dishes per sample) until 80-100% confluent. After treatment, the plates were washed with ice cold HBSS at the same osmolarity of the treatment solution and then 1mL of HBSS supplemented with protease inhibitor at the same osmolarity as the treatment solution was added to each dish. On ice, the cells were scraped with a soft rubber scraper and collected into 1.5mL tubes. The samples were centrifuged at 1500 rpm, 4°C for 5 min to pellet the cells and the supernatant was removed. The cells were resuspended in mitochondrial lysis buffer (10mM HEPES, 220mM mannitol, 70mM sucrose, 1mM EDTA, 1mM DTT, 1mM PMSF; pH 7.4) that was supplemented with sucrose to the same osmolarity as the treatment solution and the 2 tubes for each sample were combined. Each sample was then manually homogenized for 2 min and 40μL of the whole cell lysates removed, mixed with 13μL of 4X supplemented Triton-X lysis buffer; 10μL was removed for protein analysis and the remainder mixed with 43μL of Laemml buffer and boiled for 5 min. Each sample was then supplemented with a 5M sucrose solution, so that each sample was prepared at the highest osmolarity (usually 600mosm extra). Mitochondrial lysis buffer was added as needed to maintain constant volume between the samples. This would ensure that any observations were
due to the treatment and not to preparation in solutions with different osmolarities. The samples were centrifuged at 3,000 x g, 4°C for 5 min to remove unbroken cells, nuclei and large organelles. The supernatant was transferred to new tubes and the pellet discarded. The samples were centrifuged at 11,000 x g, 4°C for 15 min. 40μL of each supernatant was collected and prepared in the same manner as the whole cell lysates. The remaining supernatant was removed and discarded and the pellet, containing the mitochondrial fraction, was resuspended in 60μL of supplemented Triton-X lysis buffer. 10μL was removed for protein concentration analysis and the remainder mixed with 2X Laemmli buffer and boiled for 5 min. The protein concentrations were determined for the whole cell lysates, supernatant and mitochondrial fraction of each sample. Equal amounts of protein (20μg) were loaded onto SDS-PAGE gels, the proteins separated and then analyzed using Western blotting.

**ORE activity assay**

The ORE-Luc reporter plasmid, which contains three OREs coupled to a firefly luciferase enzyme, was a kind gift from Dr. B.C. Ko (University of Hong Kong, Hong Kong) [73]. The thymidine kinase-driven renilla Luciferase vector (pRL-TK; Promega) was used as an internal control.

LLC-PK1 cells were grown on 6-well plates and after 24 hours were transfected with the following constructs (amounts per well) using FuGene6 transfection reagent: 0.5μg ORE-Luc, 0.05μg pRL-TK and 1μg of pcDNA3 (empty vector) or a test construct. After 48 hours, cells were serum starved for 2 hours and then treated hyperosmotically by the addition of 200mosm sucrose or NaCl for 6 hours (in serum free DMEM). Following treatment, the cells were lysed in 250μL of passive lysis buffer (Promega). Lysates were exposed to one freeze/thaw cycle (~80°C for at least 1 hour / thawed at 37°C). After thawing the samples were centrifuged (13,000 rpm at
4°C, 5 min). Firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay kit (Promega) and a Berthold Lumat LB 9507 luminometer. Results are expressed as a normalized ratio, dividing the firefly luciferase activity by that of the renilla luciferase for each sample.

In experiments where both siRNA and ORE-Luc reporter transfection was necessary the following procedure was used. LLC-PK1 cells were plated on 12-well plates, into growth medium containing 25nM siRNA against DLP-1 or a non-related (NR) control RNA. After 24 hours the RNA transfection medium was removed and replaced with fresh medium. The cells were then transfected with 0.05μg ORE-Luc, 0.01μg pRL-TK and 1μg pcDNA3 (per well) using FuGene6. After 24 hours the cells were treated and assay performed as described above.

**Caspase-3 activity assay**

LLC-PK1 cells were grown on 12-well dishes. After 24 hours they were transfected with an siRNA against DLP-1 or a non-related siRNA for 48 hours. The cells were serum starved for 2 hours and then treated hyperosmotically with sucrose in serum-free DMEM (600 mosm extra) for 2 hours. Subsequent to treatment, the cells were washed in sterile ice-cold PBS and lysed in 150μL of lysis buffer (10mM Tris-HCl, 10mM NaH₂PO₄/NaHPO₄, 130mM NaCl, 1% Triton-X 100, 10mM sodium pyrophosphate; pH 7.5, sterile filtered, made per the manufacturer’s directions) for 30 min. 60μL of each sample was added to 1mL of reaction buffer (20mM HEPES, 10% glycerol, 2mM DTT; pH 7.5, made as directed by the manufacturer) and 14.3μL of Ac-DEVD-AMC Caspase-3 (CPP32) fluorogenic substrate (BD Pharmingen) and then incubated in a 37°C water bath for 45 min. Each sample was then placed into a cuvette and read on a PTI DeltaRam system (Photon Technology International, Birmingham, NJ) with an excitation wavelength of 380nm and emission of 440nm. Data was collected as counts emitted per second.
The protein concentration of each sample was determined and the counts for each sample divided by the protein concentration (counts per microgram protein).
RESULTS

Hyperosmotic stress provokes mitochondrial fragmentation.

To determine if hyperosmotic stress induces mitochondrial fragmentation in LLC-PK1 kidney epithelial cells, we loaded these cells with Mitotracker Orange, and exposed them to iso- or hypertonic conditions (Fig 9A). Mitotracker dyes are fluorescent cationic probes that accumulate in the matrix of energized mitochondria, and react with sulfhydryl groups, and thus are fixable. Fig 9A shows that under isotonic conditions the majority of mitochondria exhibited an elongated, tubular shape, and were organized in an interconnected network. Exposure of the cells to a hyperosmotic solution (600 mosM extra) for 15 minutes induced remarkable morphological changes: the probe visualized dot-like, spherical structures with no or minimal connectivity (Fig 9 A). This picture corresponded to a typical tubular-to-punctate (T-P) transition, which is the hallmark of mitochondrial fragmentation [193]. While these observations were in good accord with the single previous report on the effect of hyperosmolarity on mitochondrial shape in Vero cells, (obtained with the same dye [165]), it was important to corroborate these findings with an alternative method, due to the limitations of Mitotracker probes. Specifically, the distribution of these dyes depends on the metabolic state of the mitochondria, particularly on the membrane potential and superoxide production [165], both of which were reported to show major changes during osmotic stress [158, 165]. To substantiate that the observed difference in dye distribution reflected morphological and not simply functional differences, we transfected the cells with mito-GFP or mito- RFP, fusion proteins composed of subunit VIII of cytochrome c oxidase, a component of the respiratory chain, fused to Green (or Red) Fluorescent Protein (Fig 9B and C, respectively). These probes are efficiently targeted to the inner mitochondrial membrane (no cytosolic labeling) where they reside,
Figure 9

A. Mitotracker

B. mito-GFP

C. mito-RFP
Figure 9. Hyperosmotic stress induces mitochondrial fragmentation. (A) LLC-PK1 cells grown on glass coverslips were loaded with a cell permeable mitochondrial dye, Mitotracker Orange for 30 min. The cells were washed to remove excess dye and then treated with isotonic (Iso) or hypertonic (Hyp; sucrose, 600 mosm extra) medium for 15 min. After fixing the cells, the morphology of the mitochondria was visualized by immunofluorescence microscopy. (B) For time-lapse fluorescence video microscopy, cells were transfected with mito-GFP plasmid encoding a mitochondrially targeted GFP construct. The cells were exposed to isotonic medium (Iso) and then treated hypertonically (Hyp; sucrose, 600 mosm extra) and the morphological changes of the mitochondria were followed. Characteristic frames from the video at the indicated time points are presented. (C) Cells on coverslips were transiently transfected with a mitochondrially targeted RFP (mito-RFP) construct. 24 h later the cultures were treated and visualized similarly as in A.
independent of subsequent bioenergetic changes. Fig 9B shows a typical experiment, in which we used time-lapse fluorescence video microscopy to visualize mitochondrial changes in a mito-GFP expressing cell: under isotonic conditions, the imaged cell contained an array of long, tubular mitochondria; within 1 min after switching to the hypertonic medium, the mitochondria became highly kinked. During the course of the next 20 minutes, the mitochondrial network underwent near complete fragmentation: the mitochondria acquired spherical morphology, and lost their connectedness (Fig 9B). Next we sought to test if fixation leaves the normal (isotonic) morphology intact and preserves the hypertonically induced change, when visualized by the fusion protein. This was an important question because in further studies we wished to perform immunofluorescent staining for various proteins and investigate their effect on or their relationship to mitochondria. Therefore we transfected cells with mitochondrially targeted RFP (mito-RFP), and treated them isotonically or hypertonically for 15 min, and then fixed them with paraformaldehyde. As shown in Fig 9C, in the isotonic samples the mitochondria maintained an elongated tubular morphology, while in the hypertonic samples, they were completely fragmented. Taken together, these findings indicate that hyperosmotic stress causes robust fragmentation of the mitochondrial network in LLC-PK1 cells, and that these morphological changes can be consistently detected in live and fixed cells using different methods for the visualization of the mitochondria.

**Hypertonic stress causes the rapid recruitment of DLP-1 to the mitochondria in an osmotic concentration-dependent manner.**

Having demonstrated that hyperosmotic shock induced mitochondrial fragmentation, we wished to identify the mechanism responsible for this phenomenon. Importantly, alterations in
mitochondrial respiration and bioenergetics are accompanied with major shape changes (e.g. doughnut formation), which may not be mediated by the fission apparatus [270, 271]. To assess whether hyperosmolarity induces real mitochondrial fission, and to gain insight into the underlying molecular mechanism, we asked if hyperosmolarity induces mitochondrial translocation of DLP-1, a major component of the fission machinery in LLC-PK1 cells. To address this, we treated cells isotonically or with solutions of varying degree of hyperosmolarity, (using sucrose), and then isolated the mitochondria and performed Western blotting on these mitochondrial fractions using an anti-DLP-1 antibody. To maintain isotonicity or the applied osmotic stress during the whole experiment, the cells were detached and then broken up in a solution of the same osmolarity, which they had been treated with. Furthermore, to ascertain that any change in mitochondrial DLP-1 must have occurred within the cells and not in the homogenization buffer (due to varying osmolarity) during isolation we supplemented each homogenate with a concentrated sucrose solution to set the same final osmolarity (900 mosm) in every sample. The amount of mitochondrially associated DLP1 was normalized to the level of the F₀F₁ ATPase (subunit α), an inner mitochondrial membrane protein. Fig 10A shows that our isolation technique yielded equal amount of mitochondria (F₀F₁ ATPase) from each sample. Importantly, a moderate amount of DLP-1 was associated with mitochondria under isotonic conditions, which gradually increased at elevated osmolarities. Doubling of the extracellular osmotic concentration resulted in approx. 3-fold increase in mitochondrial DLP-1, whereas tripling the osmolarity caused a 10-fold rise compared to the isotonic level (Fig 10B). Seeing that hypertonic treatment induced a rapid morphological change in the mitochondria, we wished to determine whether the kinetics of DLP-1 recruitment might be consistent with the time course of the observed fragmentation. To assess this we treated LLC-PK1 cells with a moderately
Figure 10

A.

![Image of Western blot showing DLP-1 and F0F1 ATPase in WCL and mitochondrial fraction with increasing osmolality (0 to 600 mosm).]

B.

![Graph showing the fraction of mitochondrial DLP-1 vs. extra mosm.](https://example.com/graph.png)
Figure 10. Hypertonicity induces DLP-1 accumulation in the mitochondrial fraction in an osmotic concentration-dependent manner. (A) LLC-PK1 cells were grown on 10 cm dishes and then treated isotonically or hypertonically at the indicated osmolarity for 5 min. Mitochondrial extracts were isolated as described in the Methods. After the cells were manually homogenized, a sucrose solution was added to each sample to correct the final osmolarity of each sample to 900 mosm. Mitochondrial extracts were subjected to SDS-PAGE and the DLP-1 content was determined by Western blotting. Whole cell lysate (WCL) was used to represent the total amount of DLP-1 in the cells. The same membranes were also probed for the mitochondrial protein F$_0$F$_1$ ATPase subunit $\alpha$ as a loading control. (B) Western blots from A were further analyzed by densitometry. The mean density of each DLP-1 band was normalized internally using the corresponding value for the F$_0$F$_1$ ATPase of the same sample. The normalized values of the mitochondrial DLP-1 for each sample were expressed as a percentage of the value of the sample treated with +600 mosm (taken as 100%). Data shown of the graph are mean+/- S.E. (n=3 independent experiments).
hypertonic medium (300 mosm extra) for various times between 1 and 15 min, followed by mitochondrial preparation and Western blotting for DLP-1 (Fig 11A). Remarkably, hyperosmolarity induced a ~2.5-fold increase in mitochondrial DLP-1 within 1 minute, (Fig 11B), i.e. it rapidly induced a near-maximal response, which tended to slightly elevate further (to >3-fold) by 15 minutes. To visualize the distribution of DLP-1 in intact cells, we labeled cells with Mitotracker Orange, treated them isotonically or hypertonically and then stained for endogenous DLP-1. As expected, in the isotonically treated cells the mitochondria exhibited tubular morphology, and a substantial portion of DLP-1 was spread diffusely throughout the cytoplasm (Fig 12A). In addition, a part of the DLP-1 staining was localized to distinct puncta, which were often associated with mitochondria, confirming previous findings [195], as well as our own biochemical data (Fig 12A Iso Box). In the hypertonically treated cells the mitochondria were robustly fragmented and the DLP-1 labeling became entirely particulate. Many of these large DLP-1 aggregates were closely associated with the fragmented mitochondria (Fig 12A), apparently either attaching to the surface mitochondrial particles, or surrounding them. (Fig 12A Hyp Box). To gain insight into the kinetics of DLP-1 redistribution and recruitment, we examined the process using live microscopy. Cells were cotransfected with mito-RFP and wild type (WT) DLP-1-GFP, and single cells were then viewed using a spinning disk confocal microscope. Initially the cells were placed in isotonic solution; as expected, under these conditions they contained elongated mitochondria, which were periodically decorated by a few DLP-1 puncta (Fig 12B and Iso Box). Replacement of the isotonic medium with a hypertonic one (600 mosm extra) induced an almost immediate increase both in the total number and the size of the DLP-1 puncta. Enhanced clustering of DLP-1 was present in the cytosol. In addition, consistent with our Western blot data (Fig 10A) these changes were accompanied by an
Figure 11

A.

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<tr>
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<td>$F_0F_1$ ATPase</td>
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Hyp (300 mosm extra)

B.

Mitochondrial DLP-1 (fold increase)

0 5 10 15 min

300 mosm extra
Figure 11. DLP-1 association with the mitochondrial fraction is rapid and sustained. (A) LLC-PK1 cell monolayers were treated isotonically (Iso) or hypertonically (Hyp; sucrose, 300 mosm extra) for the indicated times. Mitochondria from the cells were then isolated. The DLP-1 and $F_0F_1$ ATPase subunit $\alpha$ content of the samples was detected by Western blotting. The whole cell lysate (WCL) sample was used to indicate overall DLP-1 abundance in the cells. (B) Western blots from A were analyzed by densitometry. The mean density of each DLP-1 band was normalized internally using the corresponding value for the $F_0F_1$ ATPase of the same sample. The normalized values amount of mitochondrial DLP-1 for each sample was expressed as a percentage of the value of the isotonically treated sample (taken as 100%). Data shown on the graphs are mean +/- S.E. (n=3 independent experiments).
Figure 12

Mitochondria

DLP-1

Merge

Box
Figure 12

B. mito-RFP

<table>
<thead>
<tr>
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<th>Iso</th>
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Figure 12. **DLP-1 is recruited to the mitochondria under hyperosmotic stress.** (A) LLC-PK1 cells on coverslips were preloaded with Mitotracker Orange for 30 min and then the excess dye was washed away. Cells were exposed to isotonic (Iso) or hypertonic medium (Hyp; sucrose, 600 mosm extra) for 15 min and then fixed in 4% PFA. Endogenous DLP-1 was labeled with an anti-DLP-1 monoclonal antibody followed by a Cy2 conjugated secondary antibody. The cells were visualized by immunofluorescence microscopy. Representative areas (dotted lines) of the merged images were magnified further in the bottom row. (B) LLC-PK1 cells grown on coverslips were cotransfected with wild type (WT) DLP-1-GFP and mito-RFP plasmids for 48 h. Successfully transfected cells were identified and the effect of hypertonic treatment (600 mosm extra sucrose) was followed by spinning disc confocal microscopy. Pictures were taken every 90 sec for 30 min. Frames taken at the indicated time points of the movie are shown. Representative areas (dotted lines) of the merged images were magnified further in the bottom row. The arrows in the magnified pictures point to preexisting DLP-1 puncta in isotonic conditions to which cytosolic DLP-1 localizes upon hyperosmotic treatment. The asterisks indicate newly formed DLP-1 punctae visualized upon hyperosmotic treatment.
increased association of DLP-1 with the mitochondria (Fig 12B Hyp Box). Apparently, DLP-1 was recruited from the cytosol to DLP-1 puncta that had been already present under isotonic conditions (arrows), as well as to some originally DLP-1-free areas (asterisk). The mitochondria showed extensive beading and during the next 5-20 minutes underwent robust fragmentation.

Taken together, our biochemical and imaging data show that hyperosmolarity induces rapid translocation of DLP-1 to the mitochondria, the extent of which is proportional to the applied osmolarity. Hyperosmolarity triggers DLP-1 aggregation, which (at least partly) appears to take place in the cytosol before or concomitantly with the recruitment of DLP-1 to the mitochondria. DLP-1 may preferentially accumulate on the mitochondrial surface at preexisting puncta.

**Hypertonicity-induced mitochondrial fragmentation is mediated by DLP-1 activity.**

To substantiate that DLP-1 activity was in fact responsible for mitochondrial fragmentation during hypertonic stress, we transfected cells with WT DLP-1-GFP (WT DLP-1) or dominant negative (DN) DLP-1-GFP (DN DLP-1) and then stained them with Mitotracker Orange (Fig 13). In DN DLP-1 a critical lysine has been mutated to alanine (K38A), which renders the protein GTPase-defective and thereby eliminates its capacity to catalyze membrane fission. Overexpression of WT DLP-1 had no major effect on mitochondrial morphology under isotonic conditions (albeit sometimes it seemed to promote a slight fragmentation) (Fig 13A), nor did it influence the hypertonically-induced mitochondrial shape change (Fig 13 A and B). In contrast, expression of DN DLP-1 (which appeared to moderately increase the density of the mitochondrial network under isotonic conditions) efficiently protected the mitochondria from hyperosmotically-provoked fragmentation (Fig 13A). In the presence of DN DLP-1, a large
Figure 13

A.

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| WT DLP-1-GFP | ![Image]
| Hyp    | ![Image]
| Iso    | ![Image]
| DN DLP-1-GFP | ![Image]
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Figure 13

B.

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mito-RFP

GFP

C.

Mitochondrial Fractions

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DN DLP-1
DLP-1

F₀F₁ ATPase
Figure 13

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DLP-1

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Tubulin

E.  

Mitotracker

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83
Figure 13. Dominant negative DLP-1 and DLP-1 downregulation by siRNA prevent hyperosmotically induced mitochondrial fragmentation. (A) LLC-PK1 cells were grown on coverslips and transfected with either WT or DN DLP-1-GFP. The cells were then preloaded with Mitotracker for 30 min. After exposure to isotonic conditions (Iso) or hypertonic stress (Hyp; sucrose, 600 mosm extra) for 15 min, the cells were fixed and then visualized by fluorescence microscopy. Transfected cells were identified by their GFP fluorescence. Although the WT DLP-1-GFP protein exhibited similar intracellular localization as the endogenous protein, it is noteworthy, that the dominant negative mutant formed bright clusters of aggregated proteins in the cytoplasm. The contour of the DN DLP-1-GFP expressing cells is marked with white dotted lines. (B) LLC-PK1 cells on coverslips were transiently transfected with mito-RFP and either with WT or DN DLP-1-GFP. After hypertonic treatment (sucrose, 600 mosm extra) for 15 min the cells were fixed and visualized by fluorescence microscopy. (C) LLC-PK1 cells stably transfected with DN DLP-1-GFP construct (DN DLP-1 LLC-PK1) or the wild type cells (WT LLC-PK1) were challenged with isotonic or hypertonic medium (sucrose, 600 mosm extra) for the indicated times. The mitochondria of the cells were isolated and analyzed by Western blotting. The endogenous DLP-1 and DN DLP-1-GFP fusion protein were distinguished on anti-DLP-1 blots based on the molecular weight. Subunit α of the F₉F₁ ATPase was used as a loading control. (D) Wild type LLC-PK1 cells were transfected with 25 or 50 nM of siRNA construct against the porcine DLP-1 (siDLP-1) or a non-related negative control (si non-related) for 48 hours. Whole cell lysates were prepared and analyzed by Western blotting for DLP-1 content. Tubulin was used as a loading control. (E) LLC-PK1 cells grown on coverslips were incubated with a non-related (siNR) or the DLP-1 siRNA (siDLP-1) construct for 24 h and then transfected with mito-RFP for another 24 h. The cells were then treated isotonically (Iso) or hypertonically.
(Hyp; sucrose, 600 mosm extra) for 15 min and fixed in 4% PFA. Mitochondrial morphology was visualized by using fluorescence microscopy.
number of mitochondria were able to maintain elongated, tubular shape (Fig 13A). Similar results were obtained when mitochondrial morphology was followed by mito-RFP, confirming that DN DLP-1 indeed efficiently reduced mitochondrial fragmentation (Fig 13B). Curiously, the basal distribution of WT vs. DN DLP-1 showed remarkable differences: while the WT protein, similar to endogenous DLP-1, exhibited a diffuse or finely punctuate pattern, DN DLP-1 was primarily organized in large clusters in many cells. Albeit diffuse (cytosolic) labeling was also present, the clusters were so bright that it was difficult to assess changes in the distribution of the non-clustered DLP-1 (Fig 13B). To overcome this difficulty we generated a cell line stably expressing DN DLP-1 (see below), which enabled us to study the movement and effects of DN DLP-1 using biochemical approaches. Mitochondrial preparations obtained from this cell line revealed that hyperosmolarity induced robust translocation of DN DLP-1 to the mitochondria, without inhibiting the translocation of endogenous DLP-1 (Fig 13C). Finally, we eliminated DLP-1 using siRNA (Fig 13D and E). Transfection with the specific (but not the unrelated) siRNA construct caused a near complete downregulation of DLP-1 (Fig 13E), which resulted in the formation of long, interconnected mitochondria (Fig 13D). Importantly, the absence of DLP-1 conferred substantial resistance against osmotically provoked fragmentation (Fig 13D).

Taken together, these results imply that hyperosmolarity-induced mitochondrial fragmentation is mediated by DLP-1. The osmotically induced translocation of DLP-1 to mitochondria does not require the GTPase activity of DLP-1 and does not significantly interfere with the translocation of the endogenous protein. DN DLP-1 inhibits mitochondrial fragmentation, presumably by forming multimeric complexes with endogenous DLP-1, thereby reducing its activity.
Quantification of morphological changes in mitochondria.

While these qualitative data were very suggestive, we felt that quantitative approaches should be used to verify and extend our results. There are two main reasons why quantitation of fragmentation was necessary: the first is the heterogeneity of the normal mitochondria, which—as the name of the organelle suggests—always exist as a mix of tubular (mitos) and particulate (chondrion) forms, complicating the objective evaluation of the change in selected (i.e. transfected) cells. The second is that even though hyperosmolarity exerts obvious effects, it may not induce complete fragmentation in every cell, and similarly even a potent protective effect may not prevent fragmentation of every mitochondrion. Therefore, we wished to work out a quantitative criterion, based on which a cell (containing many mitochondria) can be regarded as “protected” vs. “non-protected” against osmotic fragmentation. By this approach, we wished to validate our qualitative classification, and enable ourselves to reliably discern whether different pharmacological or genetic treatments can indeed alter mitochondrial shape or have an impact on the osmotic effects. To achieve these goals, we used Aspect Ratio (AR) measurements, which proved to be a reliable method in previous studies [224]. To measure the AR, image analysis software fits an ellipse around a particular mitochondrion (Fig 14A). The lengths of the major and minor axes are then determined and their ratio (major/minor) calculated, yielding the AR. For a perfect circle the AR is 1. In fact this method gives a conservative estimate, meaning that in two specific cases it would actually underestimate the true AR of an elongated mitochondrion. The first is when the mitochondrion curves, and thus the short axis of the ellipse fitted around the curved shape is greater than the true width of the mitochondrion (Fig. 14B). The second case is when one is confronted with branching mitochondria, and there is no clear “main mitochondrion” from which the branch could be separated. In this case our strategy was to split
Figure 14

A. [Image of a curved mitochondrion with fitted ellipse]

B. [Diagram showing minor axis and MAJOR axis of a fitted ellipse]

C. [Graph showing aspect ratio for different conditions]

D. [Bar graph showing aspect ratio for different conditions]

E. [Histogram showing distribution of mitochondria by aspect ratio]
Figure 14

F. mito-RFP

G. Mitotracker
Figure 14. Quantitative evaluation of mitochondrial morphology (fragmentation) by measurement of Aspect Ratio (AR). (A) The AR of a mitochondrion was calculated by fitting an ellipse around the object and then dividing the length of the major axis of the ellipse by that of the minor axis. The picture shows a typical mitochondrion from a control cell labeled with mito-RFP. (B) A small fraction of the mitochondria exhibit a curved shape, causing the software to underestimate the length and overestimate the width of the mitochondrion, and consequently the AR by fitting an ellipse, as shown on the scheme. For further details see the Results section. (C) Quantitation of mitochondrial morphology in cells from experiments shown in Fig 13B. At least 50 mitochondria in 3 different cells (total n ≥ 150) were examined per treatment group and each individual measurement was plotted on a scatter graph to demonstrate the variability of the ARs within each group. The numbers on the graph indicate the mean values. (D) The average AR for each treatment group from the experiment shown on C was calculated and plotted (mean +/- S.E. n > 150). (E) The ARs in each group were binned (bin size = 1) and plotted on a histogram. Using these data we defined non-fragmented (protected) mitochondrial morphology as follows: If 10% of the mitochondria in a cell had an AR greater than 4, the cell was classified as protected against mitochondrial fragmentation (indicated by the dashed line on the graph). (F,G) Quantification of experiments shown on Fig 13 A and B, respectively. Cells were transfected with WT or DN DLP-1 and treated isotonically or hypertonically, as described in the legends for Fig 13. Mitochondria were visualized by mito-RFP (F) or Mitotracker (G). Cells were classified as protected (i.e. cells with long mitochondria) based on the criteria above. At least 100 cells were evaluated for each group and the percentage of cells with elongated mitochondria (protected) was calculated in each experiment. The graphs show mean + S.E. of 3 independent experiments.
the mitochondria into three or more individual objects depending on the number of ramifications at the branch point. To establish our criteria, for all AR measurements at least 50 mitochondria/cell were analyzed in 3 cells per treatment condition, and all the mitochondria pooled together for an $n$ of at least 150. We calculated the AR for WT and DN DLP-1 transfected cells that were treated iso- or hypertonically, and plotted the individual measurements on a scatter graph and the averages on bar diagrams (Fig 14C,D). The wide range in the distribution of the AR in the WT DLP-1-expressing, isotonic samples reflects the normal variability of mitochondria in healthy cells (Fig 14C). The WT hypertonic samples had a greatly reduced variability in their AR, which corresponds to major mitochondrial fragmentation, yielding a more uniform shape. Importantly, transfection with DN DLP-1 prevented the reduction of the variability amongst the mitochondria; indeed ARs higher than 10 were still observed, similar to the WT isotonic samples. As shown in Fig 14D, the average AR in the WT cells (5.75) showed a more than 3.5-fold reduction under hypertonic conditions, whereas the slightly higher average AR of the DN hypertonic samples (6.89), showed only a $< 1.4$-fold decrease. To establish our criteria based on which we classified cells into protected vs. non-protected categories, the data were plotted on a histogram (Fig 14E). Remarkably, in the WT hypertonic sample less than 1% of the mitochondria had an AR of greater than 4. Therefore we considered, that if a cell has at least 10% of its mitochondria with an AR greater than 4 (i.e. it contains at least ten-fold more long mitochondria than it would have under the usual hypertonic conditions), a biologically significant protective effect can be established. In order to assess a large number of cells quickly and reliably, it was important to validate our visual classification; using visual inspection, we sorted cell in two categories: those with long mitochondria (i.e. protected) and those without. Importantly, when we randomly selected and analyzed cells from
the former group, we invariably found that they had at least 10% (and often many more) mitochondria with an AR > 4. (It is worth noting that our intervals were wide enough in both direction: a 10-fold higher frequency of long mitochondria was defined as the lower threshold for protection; on the other hand intact, isotonically treated cells usually had >50% of their mitochondria with an AR > 4. Thus significant partial fragmentation could occur even in “protected” cells, which nonetheless preserved many of their long mitochondria.

Using these criteria, visual inspection of > 100 cells per condition revealed that under hypertonic conditions, only 12% of the cells, cotransfected with WT DLP-1 and mito-RFP contained some long mitochondria, whereas in the DN DLP-1 group, 70% of the cells had long mitochondria, and thus showed protection against fragmentation (Fig 14F). These findings were confirmed in Mitotracker stained cells that were treated hyperosmotically. In the WT DLP-1 group 25% of the cells contained non-fragmented mitochondria, while over 80% of the cells in the DN DLP-1 group were protected against fragmentation (Fig 14G). Taken together, these quantitative data validate our qualitative observations, and confirm that the GTPase activity of DLP-1 is required for hypertonicity-induced fragmentation.

**Actin polymerization induces mitochondrial fragmentation while actin depolymerization partially protects against hyperosmolarity-induced mitochondrial fragmentation.**

To gain insight into the mechanism whereby hypertonicity can induce DLP-1-mediated mitochondrial fragmentation, we initially investigated whether the actin cytoskeleton plays a role in this process. The rationale behind this assumption was that previous studies in our lab have shown that actin undergoes a dramatic reorganization during periods of hypertonic stress [115], and that disruption of F-actin has been shown to protect CV1-4A cells against mitochondrial...
fragmentation induced by oligomycin or Cyclosporin A [224]. The latter study also reported that F-actin disruption prevents the translocation of DLP-1 to the mitochondria [224]. Because hyperosmolarity induces actin polymerization in LLC-PK1 cells [115], we first investigated whether increased actin polymerization per se might induce mitochondrial fragmentation. To this end, we treated cells with Jasplakinolide (Jas), a drug that induces strong F-actin polymerization. Jas provoked robust mitochondrial fragmentation similar to hypertonic treatment (Fig 15A).

Next we tested whether drugs that inhibit actin polymerization would have an impact on hyperosmolarity-triggered fragmentation. We used Latrunculin B (Lat B), which sequesters actin monomers and Cytochalasin D (Cyt D), which caps the barbed (growing) ends of actin filaments. Without exerting much effect under isotonic conditions, both drugs reduced hyperosmotically-induced fragmentation. This partial protective effect was revealed both by the aspect ratios and by counting the number of cells with long mitochondria (Figs 15B,C,D,E,F). Specifically, we found that after hyperosmotic challenge, 29% and 58% of the mitochondria had an aspect ratio > 4 in the presence of Lat B and Cyt D, respectively (Fig 15D) (This compares to the values of < 1%, in the absence of these drugs.). Accordingly, about 48% and 42% of hypertonically exposed cells exhibited mitochondrial protection in the presence of Lat B and Cyt D, amounting to a substantial (albeit partial) protection compared to the 13% of hypertonic control cells with protected mitochondria (Fig 15F).

We then asked whether the attenuation of fragmentation by the depolymerizing drugs was due to mitigated recruitment of DLP-1 to the mitochondria. To assess this we pretreated cells with Lat B or Cyt D, exposed them to hypertonic conditions, isolated their mitochondria, and determined the DLP-1 content of these mitochondrial fractions by Western blotting. There was no noticeable difference in the amount of DLP-1 in the hypertonic control versus the Lat B and
Figure 15

A.

![Images of different conditions: Iso, Hyp, Jas, Lat B, Lat B + Hyp]

B.

![Bar chart showing aspect ratios for different conditions: Iso, Hyp, Jas, Lat B, Lat B + Hyp]

C.

![Bar chart showing aspect ratios for different conditions: Iso, Hyp, Jas, Lat B, Lat B + Hyp]
Figure 15

D.

E.

F.
Figure 15. Actin polymerization induces mitochondrial fragmentation, while actin depolymerization partially protects against hyperosmotically induced mitochondrial fragmentation. LLC-PK1 cells grown on coverslips were transfected with mito-RFP. Subsequently cells were pretreated with 1μM Jasplakinolide (Jas), 10μM Latrunculin B (Lat B) or 10μM Cytochalasin D (Cyt D) for 30 min and then treated isotonically or hypertonically (sucrose, 600 mosm extra) for 15 min as indicated. (A). Mitochondrial shape was visualized by fluorescence microscopy. (B) From the previous experiment the ARs for at least 50 mitochondria in 3 cells were calculated for each treatment group. Each data point was plotted for every group on a scatter graph. The numbers indicate the mean values. (C) The average AR for each group in the experiments shown above was calculated and presented as mean±SE (n ≥ 150). (D) The ARs for each groups were binned (bin size = 1) and the data plotted as a histogram to evaluate the distribution of the ARs in the different treatment groups. Protection was defined as described on Fig 13. Biologically significant protection against fragmentation is indicated on the graph by the line (cells with at least 10% of all mitochondria with an AR > 4). (E) Isotonic (Iso), hypertonic (hyp) and Jasplakinolide (Jas) treatment groups of the above experiment were visually evaluated and at least 100 cells classified as protected or fragmented. The percentage of cells with elongated (protected) mitochondria was calculated and the results from separate experiments were pooled and expressed as mean +/- SE (n = 3 independent experiments). (F) Groups were pretreated with Lat B, Cyt D or vehicle only (Cntrl) and then challenged isotonically (Iso; open bars) or hypertonically (Hyp; dark bars). Cells were examined visually and classified as protected or fragmented. At least 100 cells were counted per group. The data are presented as the percentage of cells that have long mitochondria with SE (n = 3 independent experiments).
Cyt D treated hypertonic samples, indicating that DLP-1 still translocated to the mitochondria under hyperosmotic stress (Fig 16).

Besides the actin skeleton, microtubules have also been suggested to participate in mitochondrial shape determination, and the microtubular motor protein dynein has been implicated in the recruitment of DLP-1 to mitochondria [223]. To address the potential role of this system we used the tubulin depolymerizing and polymerizing drugs (colchicine, nocodazole, taxol) as well a dynein inhibitor, (erythro-9-(2-hydroxy-3-nonyl)adenine), EHNA. None of these appeared to interfere with the basal shape or the hyperosmotic-induced fragmentation of mitochondria (results not shown).

Taken together these results show that actin polymerization induces mitochondrial fission while actin depolymerization partially protects against mitochondrial fragmentation induced by hyperosmotic exposure. However, actin depolymerization does not seem to prevent the hyperosmotic recruitment of DLP-1 to the mitochondria, suggesting that actin polymerization may have a permissive role in the function (rather than the recruitment) of DLP-1 when it is localized to the mitochondria.

**Constitutively Active (CA) Rac, but not Rho, induces mitochondrial fragmentation while DN Rac prevents the hyperosmotic-induced fragmentation of mitochondria.**

Members of the Rho family small GTPases, in particular Rho and Rac, are known to be activated by hyperosmotic stress [55, 116]. Previous work in our lab has shown that Rho and Rac have major roles in hyperosmotically-induced cytoskeletal reorganization [115], including actin polymerization and myosin phosphorylation. Rho and Rac have also been implicated as players in the mitochondrial apoptotic signaling pathway [260, 261]. Cognizant of these facts,
Figure 16

<table>
<thead>
<tr>
<th></th>
<th>Cntrl</th>
<th>Lat B</th>
<th>Cyt D</th>
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<tbody>
<tr>
<td></td>
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<td>Hyp</td>
<td>Iso</td>
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Figure 16. Actin depolymerization does not prevent the translocation of DLP-1 to the mitochondria during hyperosmotic stress. LLC-PK1 cells were pretreated with vehicle (Cntrl), Latrunculin B (Lat B) or Cytochalasin D (Cyt D) for 30 min and then treated isotonically or hypertonically (sucrose, 600 mosm extra) for 15 min. Mitochondria were isolated and the samples were analyzed by Western blotting for DLP-1 and F₀F₁ ATPase subunit α content. One representative blot of 3 similar experiments is shown.
and the involvement of the cytoskeleton in the modulation of hyperosmotically-induced mitochondrial shape changes, we conducted experiments to test whether the Rho family GTPases contribute to mediation of mitochondrial fragmentation in response to hyperosmotic stress. Initially we assessed the impact of the Rho pathway, using transfection with CA and DN Rho constructs and various pharmacological inhibitors for different Rho effectors. While active Rho appeared to promote perinuclear condensation of mitochondria in some cells, we did not observe any major effects on mitochondrial fragmentation. Similarly, although both Rho kinase and its downstream target myosin light chain are activated by hypertonic stress [55], neither pharmacological inhibition of ROK by Y-27632 nor elimination of myosin-based contractility by blebbistatin had any discernable effect on osmotic fragmentation (these negative data are not shown but are summarized in Table 2).

Strikingly different results were obtained when we examined the Rac pathway. To do this, initially we cotransfected cells with Myc-tagged CA Rac along with mito-RFP (Fig 17A). CA Rac induced dramatic mitochondrial fragmentation: the average AR (1.47) was even less than that of the hypertonic samples, and the variability in size disappeared (Fig 17E,F,G). Visual classification of many cells revealed that only 25% of the cells transfected with CA Rac had elongated mitochondria, a major reduction compared to the 80% found in the isotonically treated control. Similar qualitative and quantitative results were obtained when the cells were analyzed using Mitotracker (Fig 17B). Next we tested the effect of DN Rac under iso- and hypertonic conditions. DN Rac had no major impact on mitochondrial shape distribution or AR in isotonically treated cells, but it exerted strong protection against the hypertonically induced changes (Figs17A-G): there was only a slight drop in the average AR (Fig 17E,F), and 56 % of the mitochondria maintained an AR greater than 4 (Fig 17G). Visual classification of cells
Table 2

<table>
<thead>
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<th>Osmotically Sensitive Pathway</th>
<th>Effectors</th>
<th>Method of Interference</th>
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<td>Rho</td>
<td>CA Rho</td>
<td>Minor effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DN Rho</td>
<td>No obvious effect</td>
</tr>
<tr>
<td></td>
<td>ROK</td>
<td>Y-27632</td>
<td>No obvious effect</td>
</tr>
<tr>
<td>Myosin Activity</td>
<td></td>
<td>Blebbistatin</td>
<td>No obvious effect</td>
</tr>
<tr>
<td>Rac</td>
<td>Rac</td>
<td>CA Rac</td>
<td>Induced major fragmentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DN Rac</td>
<td>Significant protection against fragmentation</td>
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<td></td>
<td>PAK</td>
<td>CA PAK</td>
<td>No obvious effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DN PAK</td>
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</tr>
<tr>
<td></td>
<td>p38</td>
<td>SB203580</td>
<td>No obvious effect</td>
</tr>
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</table>
Table 2. Effect of inhibition of Rho Family GTPases and their effectors on mitochondrial morphology. The table summarizes the results of our series of experiments aiming to discern the role of Rho, Rho kinase (ROK), myosin, Rac and p38 MAP kinase, in the regulation of mitochondrial fragmentation. Constitutive active (CA) and dominant negative (DN) mutant DNA constructs (48 h transfection) or pharmacological inhibitors were used to interfere with specific signaling molecules, and their effect on mitochondrial morphology was analyzed as above. A final concentration of 20μM was used for Y-27632, Blebbistatin and SB203580 treatment.
Figure 17

A. mito-RFP

CA Rac | DN Rac iso | DN Rac hyp

Myc

B. Mitotracker

CA Rac | DN Rac iso | DN Rac hyp

Myc

C. mito-RFP
Cells with long mitochondria(%)  

Cntrl | CA Rac | DN Rac

D. Mitotracker
Cells with long mitochondria(%)  

Cntrl | CA Rac | DN Rac
Figure 17

E.

![Graph showing aspect ratio for different conditions]

F.

![Bar graph showing aspect ratio for different conditions]

G.

![Histogram showing number of mitochondria per aspect ratio]

Legend:
- Isotonic
- Hypertonic
- CA Rac
- DN RacIso
- DN RacHyp
Figure 17. Constitutively active Rac induces mitochondrial fragmentation and dominant negative Rac prevents hypertonically-induced mitochondrial fragmentation. (A and B) LLC-PK1 cells were grown on coverslips and then cotransfected with mito-RFP and either with Myc tagged constitutively active (CA) or dominant negative (DN) Rac. The cells were incubated in isotonic (iso) or hypertonic (hyp, sucrose, 600 mosm extra) medium for 15 min and then fixed in 4% PFA. Mitochondrial morphology was detected by fluorescence microscopy using mito-RFP (A) or Mitotracker (B). The expressed Rac protein was visualized with an anti-Myc primary antibody and a Cy2 coupled secondary antibody (Myc, lower row) (C and D).

Quantitation of experiments shown in A and B. The mitochondrial morphology of at least 100 cells was evaluated. The percentage of cells with elongated mitochondria in each treatment group in 3 independent experiments was calculated. The graph shown mean ± SE of n=3. (E) The ARs for at least 50 mitochondria in 3 cells per treatment group in experiments as in A (mito-RFP transfected cells) were calculated and each individual AR plotted on a scatter graph. The numbers indicate the average values. (F) The average AR from the graph in C is shown (mean±SE.). (G) The ARs for each treatment group from the above experiment were binned (bin size = 1) and the results presented as a histogram. Protection was defined as described on Fig 13 and is indicated on the graph by the line (cells with at least 10% of all mitochondria with an AR > 4).
corroborated these data: 58% of the cells possessed long mitochondria, which signifies a substantial protection compared to the 15% present in the hypertonically treated cells in the absence of DN Rac (Fig 17C,D). Again, these data were confirmed using Mitotracker, in which case mitochondria do not contain exogenously expressed proteins markers (Fig 17D).

Two of the well-known downstream effectors of Rac, classical p21-activated kinases (PAKs) and p38 MAPK were shown to be stimulated by osmotic stress [86, 93]. We therefore tested whether these kinases might be involved in the osmotic fragmentation of mitochondria. Constitutively active PAK1 did not mimic the effect of CA Rac on mitochondria, and SB-203580, a potent inhibitor of p38 did not seem to alter the hypertonicity-induced fragmentation (not shown, but summarized in Table 2). These findings render it unlikely that Rac or osmolarity act through classical PAKs or p38.

Taken together, our results suggest that Rac, but not Rho, activation is associated with dramatic mitochondrial fission, and Rac activity is required for hypertonicity-induced mitochondrial fragmentation.

**Functional consequences of DLP-1 recruitment and mitochondrial fragmentation during osmotic stress.**

As mentioned in the Introduction, mitochondria have been suggested to participate in two critical responses to osmotic stress: the initiation/execution of apoptosis [259] and activation of the osmotic response element [157]. However, the involvement of mitochondrial fragmentation *per se* in these hypertonicity-triggered processes remains unknown. The following experiments were conducted to address the role of fragmentation and/or DLP-1 recruitment in these fate-determining responses, critical for death or survival.
A) The siRNA knockdown of DLP-1 causes increased caspase-3 activation in response to hyperosmotic stress.

To determine the role of mitochondrial fragmentation in apoptosis, cells were transfected either with non-related siRNA or siRNA directed against DLP-1, and 48 hours later exposed to isotonicity or strong hypertonicity for an extended period of time (2 h). Efficient downregulation of DLP-1 was verified by Western blots (Fig 13E). Apoptosis was quantified by measuring the activity of caspase-3 in extracts obtained from the corresponding lysates. Caspase-3 activity was used as it reflects the overall apoptotic process, since this enzyme is the convergence point of the extrinsic and intrinsic (mitochondrial) apoptotic pathways. Under isotonic conditions there was no difference between the basal caspase-3 activity of the two groups. Osmotic shock induced a 40-fold increase in the caspase-3 activity of the control siRNA-treated cells, while it triggered a 2-times higher, (80 fold) increase in the DLP-1-downregulated cells (Fig 18A). Visual inspection of mitochondria verified that downregulation of DLP-1 exerted substantial protection against fragmentation even under the harsh and long-lasting (600 mosm extra for 2 hours) hypertonic exposure used to induce apoptosis (Fig 18B). These results indicate that the downregulation of DLP-1 actually augments osmotically provoked apoptosis. This suggests that the prevention of fragmentation or the absence of DLP-1 per se increases the susceptibility of LLC-PK1 cells to hypertonicity-induced cell death. These data imply that mitochondrial fragmentation may be a protective mechanism during osmotic shock (see Discussion).

B) The absence of DLP-1 reduces the hyperosmotic induction of the osmotic response element.

Generation of mitochondrial reactive oxygen species (ROS) has been suggested to
Figure 18

A. Caspase-3 Activity (Fold Increase)

<table>
<thead>
<tr>
<th></th>
<th>Iso</th>
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B. Imaging of Caspase-3 Activity

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Figure 18. DLP-1 downregulation by siRNA increases the activation of caspase-3 in response to hypertonicity. (A) LLC-PK1 cells transfected with either a non-related (siNR) or DLP-1 specific (siDLP-1) siRNA construct were treated isotonically or hypertonically (sucrose, 600 mosm extra) for 2h. The cells were then lysed and a fluorometric caspase-3 activity assay was performed as described in the Methods. The fluorescence of each sample was normalized to protein concentration. Results are presented as fold increase compared to control (non-related siRNA, isotonicity treated group). The graph shows mean +/- SE (n=6). (B) LLC-PK1 cells grown on coverslips were transfected with non-related or DLP-1 specific siRNA for 24h. Subsequently these cultures were transfected with mito-RFP for another 24h. The cells were treated isotonically or hypertonically (sucrose, 600 mosm extra) for 2h then fixed in 4% PFA. Mitochondrial morphology was viewed by fluorescence microscopy.
contribute to the hypertonicity-induced activation of the osmotic response element [157]. To determine if mitochondrial fragmentation has a role in the hyperosmotic activation of the ORE, we cotransfected cells with the ORE-Luc reporter plasmid and either the empty pcDNA3 vector or the DN DLP-1 construct. After hyperosmotic treatment with either NaCl or sucrose, we saw no significant difference between ORE reporter activity in the control and DN DLP-1 groups (Fig 19A), albeit there was a trend towards a marginally reduced activation. To substantiate these data and to exclude the possibility that inefficient cotransfection of the reporter plasmid and the DN DLP-1 construct could be responsible for the lack of effect, we repeated these experiments on our DN DLP-1-expressing stable cell line (see also Fig 19C). Parent and DN DLP-1 LLC-PK1 cells were transfected with ORE-Luc reporter and treated iso- or hypertonically. Again we observed no significant difference in the ORE reporter activity between the wild type and DLP-1 expressing cells (Fig 19B), although the latter showed slightly lower values.

An intriguing recent publication has reported that translocation of either WT or GTPase deficient DLP-1 can reduce mitochondrial respiration [272]. This finding suggests that DLP-1 can exert significant mitochondrial effects independent of its capacity to induce fragmentation. As mentioned earlier and shown on Fig 13C, DN DLP-1 does translocate to the mitochondria, and does not prevent the translocation of endogenous DLP-1. Thus it was conceivable that the absence of DLP-1 might have different effects than the overexpression of the DN construct, despite the fact that both techniques are able to reduce mitochondrial fragmentation. To address this possibility we downregulated DLP-1 in LLC-PK1 cells using siRNA, followed by transfection with the ORE-Luc reporter construct (Fig 19E). Elimination of DLP-1 did not affect basal ORE activity, but significantly reduced its hyperosmolarity-induced activation: in cells
Figure 19

A. ORE Luc

Relative luciferase activity

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Transient transfection

B. ORE Luc

Relative luciferase activity

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Stable expressing cell line

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DN DLP-1

DLP-1

tubulin
Figure 19

D. Relative Luciferase Activity

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E. DLP-1
Figure 19. Absence of DLP-1 reduces the hyperosmotic activation of the osmotic response element. (A) LLC-PK1 cells were transiently transfected with ORE-Luc construct and the pRL-TK internal control and either with pcDNA3 (empty vector) or DN DLP-1 plasmid. The cultures then were treated isotonically (Iso) or hypertonicity (Hyp; sucrose (suc), 200 mosm extra or NaCl, 200 mosm extra) for 6h. Firefly and renilla luciferase activities from the cell lysates were measured by luminometry using a Promega Dual Luciferase Reporter Assay kit. The ORE-Luc activity was normalized to renilla luciferase activity. Results are presented as fold increase compared to the pcDNA3 transfected isotonic control (n=2 independent experiments). (B) Wild type (WT) LLC-PK1 cells and LLC-PK1 cells stably expressing the DN DLP-1 construct were cotransfected with the ORE-Luc and pRL-TK reporter plasmids. The cells were treated and luciferase activity measured as in A. Results are presented as fold increase compared to the isotonic group in the wild type cells (n=3). (C) Expression of the DN DLP-1 in the stable cell lines. Wild type and the stably transfected DN DLP-1 LLC-PK1 cells were treated isotonically or hypertonicity (sucrose, 600 mosm extra) for the indicated times. Whole cell lysates were prepared and the samples analyzed by Western blotting using anti-DLP-1 and anti-β-tubulin antibodies. The dominant negative mutant has a higher molecular weight than the endogenous protein as a result of the GFP tag. (D) LLC-PK1 cells were transfected with non-related (siNR) or a DLP-1 specific (siDLP-1) siRNA for 24h, followed by transfection with the ORE-Luc reporter construct for 24h. The cells were treated isotonically or hypertonicity (sucrose, 200 mosm extra) for 6h. Luciferase activity was measured as in A. The results are presented as fold compared to the isotonically treated non-related siRNA controls. Graphs show mean+/− SE (n=4). (E) Samples from the total cell lysates from the previous experiment were analyzed by
Western blotting and probed for DLP-1 to demonstrate the effectiveness of the knock down by siRNA transfection.
treated with non-related vs. DLP-1-specific siRNA, ORE was activated 6.7-fold and 4.7-fold, respectively, i.e. the activation was reduced by 30% (Fig 19D). This decrease is similar to the contribution attributed to mitochondrial ROS generation [157].

Taken together these results suggest that DLP-1 contributes to the efficient activation of ORE by hypertonic stress, an effect that might be dependent more on the recruitment of DLP-1 to the mitochondria, than on its mitochondrion-fragmenting activity (see Discussion).
DISCUSSION

While cellular stresses are known to reprogram essentially all vital functions from metabolism to cell division, their effects on the membrane dynamics of intracellular organelles and the underlying molecular mechanisms remain poorly defined. Since organellar, and particularly mitochondrial remodeling is a key factor in adaptation and apoptosis, we intended to explore the effect of osmotic stress on mitochondrial dynamics. Our current studies show that cellular dehydration induces robust tubular-to-particulate transition of the mitochondrial network in kidney epithelial cells. The only previous work, which reported the phenomenon of hyperosmolarity-induced mitochondrial fragmentation, has not characterized the underlying mechanism, but the authors suggested that the process might be due to the inhibition of mitofusins, and thereby reduced mitochondrial fusion [165]. While the participation of this process cannot be excluded, our results provide strong evidence that the osmotically induced activation of the fission machinery is an indispensable and critical mechanism for mitochondrial fragmentation. This conclusion is based on our biochemical and morphological findings that hyperosmolarity triggers rapid and osmotic concentration-dependent recruitment of DLP-1 to the mitochondria, and that inhibition of DLP-1’s function, either by GTPase-deficient (dominant negative) DLP-1 or by gene silencing, strongly suppresses cell shrinkage-induced mitochondrial fragmentation. We found that DN DLP-1 is also recruited to the mitochondria upon osmotic stress, implying that its GTPase activity is dispensable for translocation but necessary for fragmentation.

The mechanism(s) of DLP-1 recruitment to the mitochondria, and the responsible signaling events are major unresolved issues of the field. In various cell types several mechanisms have been proposed, most of which involve posttranslational modification of DLP-
1. In HeLa cells, DLP-1 has been found to be phosphorylated on serine 585 by Cdk1/cyclin B, and this reaction promoted mitochondrial fission during cell cycle progression [202]. Whether phosphorylation was responsible for recruitment or primarily increased the activity of DLP-1 remains to be elucidated. An inactivating phosphorylation of DLP-1 on serine 656 by cAMP-dependent protein kinase (PKA) has also been reported. This inhibition was relieved by calcineurin-mediated dephosphorylation [201]. In our system, we found no evidence for hyperosmolarity-induced phosphorylation or dephosphorylation of DLP-1, either by probing DLP-1 immunoprecipitates with multiple anti-phospho- Ser, Thr or Tyr-specific antibodies or by observing any noticeable difference in the electrophoretic mobility of DLP-1 (not shown). It remains to be determined whether these negative data truly reflect the absence of change in phosphorylation or are due to the limitation of the immunological approach. Ubiquitination [199, 200] and sumoylation [197] of DLP-1 have also been suggested to have an impact on its mitochondrial binding or disengagement. While hyperosmotic stress has been reported to induce such modifications of certain proteins [14], again we found no indication that hypertonic exposure would promote either reaction with regards to DLP-1. Another suggestion is that DLP-1 is recruited to the mitochondria by proteins released from the mitochondrial intermembrane space. This study proposed that the Bax/Bak dependent release of DDP/TIMM8a from the intermembrane space allows this protein to bind to DLP-1 in the cytoplasm and facilitates its recruitment to the mitochondria [222]. However, we found no association between DLP-1 and DDP/TIMM8a during hyperosmotic stress (not shown). Indeed this mechanism seems unlikely, given the fact that DLP-1 recruitment is very fast (≈ 1min), and is clearly detectable at moderate osmolarities, i.e. under conditions, which do not involve OM permeabilization and DDP/TIMM8a release.
Recently, an intriguing alternative mechanism has been postulated, which claims that DLP-1 multimerization (self-assembly) in the cytoplasm is necessary for its subsequent translocation to the mitochondria. DLP-1 is known to polymerize into long spirals that are tailored to fit mitochondria [273]. Inhibition of DLP-1 self-assembly with a newly discovered DLP-1 inhibitor, mdivi-1, prevented DLP-1 recruitment during staurosporine-induced apoptosis [221]. Our findings are consistent with a self-assembly-driven translocation mechanism during osmotic shock. We observed that upon hyperosmotic treatment, DLP-1 exhibited rapid clustering in the cytoplasm, prior to its translocation. At later time points such DLP-1 clusters (presumably self-assembled multimers) were detected at the mitochondria, localizing either to preexisting DLP-1 puncta or to new areas on the mitochondrial surface. Since cellular dehydration and shrinkage strongly facilitates macromolecular crowding [274], and crowding is known to enhance protein aggregation [275], augmented DLP-1 self-assembly is a plausible molecular mechanism to account for hyperosmolarity-induced DLP-1 recruitment.

The actin cytoskeleton has also been implicated in DLP-1 translocation and mitochondrial fission induced by metabolic poisons [224]. The involvement of the cytoskeleton as a mediator of osmotically provoked mitochondrial fragmentation was an attractive possibility, since, as demonstrated by us and others, hyperosmolarity induces cytoskeleton remodeling and a net increase in F-actin [7, 115, 276, 277]. Indeed, we found that pharmacologically induced actin polymerization was sufficient to provoke fragmentation, and inhibition of polymerization reduced osmotically triggered fission. However, the polymerization inhibitors failed to prevent the mitochondrial recruitment of DLP-1. This interesting finding implies that the primary role of actin polymerization during osmotic stress is not the recruitment of DLP-1 to the mitochondria; rather F-actin may have a permissive or potentiating effect on the activity of DLP-1, once it is
localized at the mitochondrial membrane. Consistent with this notion, we found that hypertonicity increased the association of actin with mitochondria (not shown). The exact mechanism whereby actin can increase the efficiency of DLP-1-mediated fission remains to be determined. It is worth noting, however, that increased actin polymerization has been correlated with enhanced mitochondrial ROS production and susceptibility to apoptosis, i.e. effects that also occur upon hyperosmotic stimulation [262].

Since both Rho and Rac have been shown to be activated by hyperosmolarity, and they are major upstream mediators of the ensuing cytoskeletal responses (e.g. increased actin polymerization, decreased severing and myosin phosphorylation), we investigated whether they might be involved in the fission process as well [55, 116]. Constitutively active Rho did not significantly alter mitochondrial morphology. In contrast CA Rac caused robust fragmentation, while DN Rac suppressed the osmotically triggered shape change. The significance of these intriguing findings go beyond the scope of osmotic signaling, since they suggest that Rac might be a hitherto unrecognized, important regulator of mitochondrial morphology.

What could the mechanism of Rac-induced mitochondrial fragmentation be? Some possibilities are summarized on Fig 20. Increased actin polymerization, predominantly through the Rac-dependent activation of the Arp2/3 complex [278] is a plausible contributor. Using pharmacological and genetic approaches we excluded the involvement of two other osmosensitive and cytoskeleton-regulating Rac targets, PAK1 and p38 [86, 279]. PAK5, however, a mitochondrially localized enzyme, which can affect mitochondrial morphology [280, 281], remains a candidate. Integrin engagement has been reported to cause Rac-mediated mitochondrial ROS production [282] via an unknown mechanism. Increased ROS may itself potentiate the induction of fragmentation. Rac is also known to collapse the vimentin
Figure 20

Possible mechanisms by which Rac may regulate mitochondrial fragmentation.

Explanations of the proposed mechanisms can be found in the Discussion and General Overview and Future Directions sections.
intermediate filament network [283], which has been proposed to maintain elongated mitochondrial morphology [284]. Further, Rac has been reported to increase the synthesis of the proapoptotic protein Bax [260]. It has been proposed that Bax could interact directly with DLP-1 and might facilitate its action [257]. Finally, there is an intriguing possibility, that could potentially integrate many important players into a coherent picture. Rac binds to the adaptor protein IRSp53, which in turn activates WAVE2 and thus Arp2/3 [285]. However, IRSp53 has other functions too: it contains a BAR-like domain, which - in the presence of Rac - can induce deformation (budding) of lipid membranes. Remarkably, IRSp53 can also bind to actin, which increases its membrane-bending activity, and can associate with dynamin as well [286]. Thus, if IRSp53 or a similar BAR domain-containing protein translocated to the mitochondria, it could (theoretically) gather all the implicated players (Rac, actin, DLP-1) into a membrane-deforming complex. Future studies are warranted to address this possibility. It also remains to be elucidated whether Rac activation or simply basal Rac activity is required for the osmotically promoted fragmentation.

Next we investigated the functional significance of DLP-1 in an apoptotic and an adaptive response to hypertonicity. It has been shown that the mitochondrion contributes to the regulation of hyperosmotic stress-induced apoptosis [259], but the role of fragmentation in the process has not been addressed. We found that DLP-1 knockdown by siRNA increased the susceptibility of cells to osmotically provoked apoptosis, as revealed by augmented caspase-3 activity. This finding seems to be contrary to recent studies, which showed that the downregulation of DLP-1 or prevention of its translocation to the mitochondria reduced or delayed staurosporine-induced apoptosis [220, 221, 287]. On the other hand our data point in the same direction as the work of Szabadkai et al. [243], who showed that the presence of DLP-1
mitigates ceramide-induced, Ca\textsuperscript{2+}-mediated apoptosis. The authors argued that fission prevents
the spreading of intramitochondrial proapoptotic signals (e.g. Ca\textsuperscript{2+} load) to the entire
mitochondrial network. This raises the interesting possibility that the role of DLP-1 may depend
on the type of apoptotic stimulus, or more precisely, on the apoptotic mechanism involved.
Specifically, staurosporine initiates the process by inducing (Bax/Bak-dependent) OM
permeabilization and cristae remodeling, factors that act from outside, whereas Ca\textsuperscript{2+} opens the
mPTP, intramitochondrially. Osmotic stress does not generate a Ca\textsuperscript{2+} signal, but does induce
ROS production [158], which can also open mPTP. Moreover, cristae remodeling (a process,
which is necessary for CytC release, and is promoted by DLP-1) may not be an issue under
hypertonic stress, when the folds of the inner membrane are distorted anyway, due to
mitochondrial shrinkage. Future work should address whether DLP-1 plays differential roles in
the regulation of apoptosis, depending on the underlying mechanism. In any case, our results
suggest that the presence of DLP-1 is protective against hyperosmotically-provoked apoptosis.

Activation of the osmotic response element (ORE) is an adaptive mechanism that enables
the cell to turn on osmoprotective genes. The mitochondrion has been implicated in ORE
regulation, as mitochondrially derived ROS have been suggested to contribute to ORE activation
during hypertonic stress [157]. To assess the potential role of mitochondrial fragmentation in
ORE activation, we used cells transiently or stably transfected with DN DLP-1. These cells
exhibited similar or marginally less ORE stimulation compared to the controls, suggesting that
fragmentation *per se* does not play a major role in ORE regulation. However, when DLP-1 was
eliminated by siRNA, ORE activation was significantly reduced. This interesting finding raises
the possibility that DLP-1 might regulate ORE, independent of fragmentation. Intriguing recent
observations might provide an explanation for this phenomenon. Bras *et al.* [272] found that
association of DLP-1 with mitochondria impairs electron transport and slows down respiration. This effect is independent of the fission-inducing capacity of DLP-1, as it can be provoked by DN DLP-1 as well. Slower electron flow through the ETC increases mitochondrial ROS production [272]. Indeed these authors found that recombinant WT or DN DLP-1 induced increased ROS generation in isolated mitochondria. It is therefore conceivable that osmotically induced DLP-1 translocation contributes to mitochondrial ROS production, which in turn may facilitate ORE. This hypothesis should be tested by direct measurements of hypertonically provoked mitochondrial ROS formation in the presence and absence of DLP-1. These experiments are underway.

In summary, our work provides evidence that osmotic stress induces DLP-1-mediated, cytoskeleton- and Rac-dependent fragmentation of mitochondria, which has an impact on adaptive and apoptotic responses. Remodeling of intracellular organelles may be a central factor in the overall response to the stress that ultimately determines death or survival.
GENERAL OVERVIEW AND FUTURE DIRECTIONS

Considering the significance of our studies from a more general perspective, perhaps the most important aspect of our work is that it attempts to link mechanochemical changes with organelle dynamics, and provides some insight into the underlying molecular mechanisms (see Fig 21). Cellular mechanics (which includes mechanosensation, mechanotransduction and mechanoprotection) is increasingly recognized as a major regulator of cell structure and function [288]. Regarding mitochondria, recent studies indicate that mechanical forces are transmitted to this organelle through the cytoskeleton, causing its displacement [289]; that fluid sheer stress induces Ca\(^{2+}\) release from mitochondria in cardiomyocytes [290] and that engagement of integrins, the chief mechanoreceptors has an impact on mitochondrial Ca\(^{2+}\) handling and ROS production [282, 291]. Supplementing these functional aspects, our studies indicate that a complex mechanical and chemical stimulus, hyperosmotic stress, exerts a dramatic effect on mitochondrial structure as well. Moreover, this organellar remodeling participates in the ensuing adaptive/apoptotic responses.

We have shown that hyperosmolarity induces robust mitochondrial fragmentation and characterized this process quantitatively. We have also provided evidence that this effect is mediated by the mitochondrial recruitment and subsequent action of DLP-1.

As mentioned before, a general outstanding problem within the field is that the upstream signaling events that cause DLP-1 recruitment to the mitochondria in mammalian systems are not clear. Regarding osmotic stress, the first (“most upstream”) question is: What are the triggering parameters that initiate DLP-1 recruitment? There are several parameters that change during hyperosmotic treatment, including membrane curvature [42, 43], ionic strength, cell volume, osmotic concentration, macromolecular crowding [1, 50, 51], and put stress on cell
Hyperosmotic stress induces the DLP-1 mediated, F-actin modulated, Rac-dependent fragmentation of mitochondria. DLP-1 may play a role in ORE activation or apoptosis either directly, or through its capacity as a mediator of mitochondrial fragmentation.
constituents [60] including the cytoskeleton [66, 67]. All these can then act directly or initiate specific signaling processes, e.g. via membrane receptors [13]. Here we would like to consider some of these possibilities and the ways in which one can address them.

While our studies show that changes in F-actin polymerization affect mitochondrial morphology, actin depolymerization does not appear to prevent the translocation of DLP-1 to the mitochondria during osmotic stress. Rather, F-actin seems to regulate the activity of DLP-1 at the mitochondria. While this is an interesting finding that requires further study, it makes it unlikely that the actin skeleton is an initiator of recruitment. On the other hand, macromolecular crowding is an appealing possibility. The observed DLP-1 clustering is consistent with the assembly of large DLP-1 complexes, and the cytosolic self-assembly of DLP-1 has been proposed to be necessary (and perhaps sufficient) for its mitochondrial recruitment of [221].

Another intriguing possibility is mitochondrial shrinkage, which might initiate the process “from within”. This possibility adds further physiological relevance to our studies, since mitochondrial volume is known to change under a variety of metabolic conditions [271]. Interestingly, a recent study [215] suggests that Pink1, a Ser/Thr kinase attached to the outer surface of the inner membrane cristae, regulates DLP-1 recruitment, presumably by acting on Fis1. We found that hypertonicity exerts immediate changes on mitochondrial shape, which manifest (prior to fragmentation) in the shrinkage and kinking of mitochondria. It is very likely that these effects would alter cristae organization and the relationship between the OM and IM. Such events may act on Fis1, and (directly or through signaling) might alter its affinity for DLP-1.

The potential role of mitochondrial shrinkage, cytosolic ionic strength, osmotic concentration and molecular crowding in mitochondrial fission can be separately investigated in
cells in which the plasma membrane has been selectively permeabilized for ions or macromolecules using ionophores (e.g. nystatin) or mild detergents (e.g. digitonin). Previously our lab applied these methods to selectively manipulate the above parameters and study their effect of the cytoskeleton [55].

Another parameter that could have a role in hyperosmotic-induced mitochondrial fragmentation is altered membrane curvature (either that of the plasmalemma or the mitochondrion). It has been suggested that members of the BAR family of proteins (so named after their bin, amphiphsin, rvs domain) lie parallel to membranes and are capable of detecting changes in membrane curvature [42, 43]. Interestingly endophilin B1, which has been implicated in the process of mitochondrial fission is a BAR family protein [209]. Although no direct interaction between DLP-1 and endophilin B1 has been reported [176], it is attractive to think that it may have a role in the hyperosmotic recruitment or activation of DLP-1. As mentioned in the Discussion section, another candidate is IRSp53, a WAVE-interacting protein, that contains a BAR-like domain. IRSp53 can interact with Rac, dynamin and actin and can induce Rac-dependent membrane budding [286]. It will be worthwhile to address the potential involvement of endophilin B1 and/or IRSp53 in the mitochondrial recruitment of DLP-1, using gene silencing.

Another possibility for DLP-1 recruitment is its post-translational modification. As mentioned, activating and deactivating phosphorylation [201, 202], as well as ubiquitination and sumoylation [197, 199, 200] have been suggested to regulate DLP-1 association with mitochondria. Using antibody-based approaches, we could not verify any of these changes under hypertonic conditions; this issue should be revisited by applying more definitive methods. Primarily $^{32}$P labeling, followed by DLP-1 immunoprecipitation and autoradiography should be
performed to test whether hypertonicity alters $P_i$ incorporation into DLP-1.

Mitochondrial dynamics occurs as a balance between the processes of fission and fusion. While our studies have provided evidence for the role of mitochondrial fission, potential changes in mitochondrial fusion should not be ignored. To assess this one should use tools that interfere with the fusion machinery (e.g. CA and DN Mfn1 and 2 and OPA-1 and specific siRNAs), to test whether these manipulations alter the effect of hyperosmolarity on mitochondrial shape and the subsequent responses (e.g. ORE activation and apoptosis).

Perhaps our most novel finding was that CA Rac induces dramatic fragmentation of mitochondria, whereas DN Rac suppressed hyperosmotically-induced fragmentation. This observation suggests that Rac may be a potentially key regulator of mitochondrial morphology, which could have far-reaching consequences. The plethora of possibilities whereby Rac might exert its effect on the mitochondria has been discussed in the previous section (see Fig 20). Here we describe only the next, most important experiment. The major question is whether Rac acts by inducing DLP-1 recruitment to the mitochondria and whether the absence of Rac interferes with DLP-1 translocation. To address this, we will downregulate Rac by siRNA and test whether this alters hypertonicity-provoked DLP-1 translocation to the mitochondrial fraction.

Finally we examined the involvement of DLP-1 in two functional consequences of osmotic shock, ORE activation and apoptosis. These outcomes were chosen because the mitochondrion has been implicated in both events: mitochondrially derived ROS participates in the activation of the ORE [157] and the Bax/Bak-dependent mitochondrial release of cytochrome c propagates the apoptotic signal initiated by severe hypertonic stress [259]. We found that the elimination of DLP-1 significantly reduced ORE activation. The siRNA exerted a much more pronounced effect than the inhibition of DLP-1 activity with the DN construct. This raises the
possibility that it is the presence, rather than the fission activity, of DLP-1 that is important for ORE activation. This is consistent with a recent report [272], showing that endogenous DLP-1, independent of its GTPase activity, causes dysfunction of the mitochondrial respiratory chain and consequent ROS generation. However, it remains to be tested whether the absence of DLP-1 indeed reduces osmotic stress-induced ROS production, and if so, whether this is the critical factor in mitigating ORE activation. To address this point, we will measure mitochondrial superoxide release with the mitochondrion-specific ROS indicator Mitosox, under iso- and hypertonic conditions, in the presence and absence of DLP-1. Hyperosmotic shock was also shown to depolarize mitochondria [165], an effect with major consequences in terms of metabolism and apoptosis. Measuring changes in the membrane potential in DLP-1 expressing and deficient cells will help elucidate whether fission or the presence of DLP-1 contributes to this functional effect as well. In any case, our studies are among the first to detect fragmentation-independent effects of DLP-1 on mitochondria.

Typically (but not univocally), mitochondrial fragmentation has been associated with the induction or propagation of apoptosis [245]. In fact fragmentation was claimed to be responsible for, independent of, or protective against apoptosis [245]. Our findings suggest that the presence of DLP-1 is protective against hyperosmotically-induced apoptosis. Specifically, we have shown that DLP-1 knockdown by siRNA caused increased caspase-3 activation in response to hypertonic treatment. We do not know whether this effect was due to reduced fission or the absence of DLP-1 itself. This can be determined by using our DN DLP-1 expressing stable cell line. Reduced activation of ORE in DLP-1 deficient cells may itself be a predisposing factor to apoptosis. In any case, our results contribute to the ongoing debate by showing that fragmentation can be dissociated from apoptosis, and that DLP-1 can be anti-
apoptotic. Based on our data, we propose that the role of the DLP-1 in apoptosis (as a mediating or mitigating factor) depends on the particular apoptotic mechanism (See Discussion).

Taken together, our studies show that osmotic stress has a strong impact on mitochondrial dynamics, that DLP-1 is a major player in this process, and that its effect appears to be adaptive or protective against the deleterious consequences of stress. As usually happens in research, we have provided a few answers, but generated many more questions, the investigation of which will further the field of osmotic signaling.
References


