**Final report 104220-Balazs Sümegi**

It has been shown that mitochondrial fragmentation contributes to disease development and progression including diabetes- metabolic diseases, cardiovascular, neurodegenerative, inflammatory diseases, cancer and several other diseases (~1000 PubMed articles). There are two possible ways to prevent mitochondrial fragmentation (1) inhibition of mitochondrial fission or (2) activation of mitochondrial fusion.
Previous data showed the discovery of a mitochondrial fission inhibitor (mdivi-1) which has protective effect in certain diseases models (1,2) Inhibition of fission has positive effect on the short run, but during the long run it may cause problems with autophagy, development, and cell cycle regulation according to knockout mice data (3). Recent works show that mdivi-1 is not a specific Drp1 inhibitor, but it inhibits complex I and modify mitochondrial ROS production which may contribute to the protective effects observed in disease models (Dev Cell. 2017 Mar 27;40(6):583-594.e6.). That is, it has similar properties as metformin. Therefore up to this point there is no well-characterized molecule which can regulate mitochondrial fragmentation.

**Activation of mitochondrial fusion by Opa1 activator**. It is well documented that ROS initiate mitochondrial fragmentation and contribute to the development and progression of various diseases. Therefore, it would be very useful to find a way to reverse mitochondrial fragmentation. We provided evidence that BGP-15, as well as several other small molecules, prevented oxidative stress-induced mitochondrial fragmentation in several cell lines and in an in vivo PAH model. BGP-15 protects against ROS-induced mitochondrial damage and reduces ROS-induced mitochondrial ROS production (Sumegi et al. PLoS One. 2017 Jan 3;12(1):e0169372). The prevention of ROS-induced mitochondrial fragmentation by BGP-15 requires the existence of active outer and inner membrane fusion machinery; suppression of Mfn1/2 and OPA1 eliminates the effect of BGP-15 (Fig.1.). To the best of our knowledge, BGP-15 is the first small synthetic molecule which activates mitochondrial fusion.
Thus, activation of mitochondrial fusion by synthetic small molecules provides new possibility to prevent or slow down the progression of oxidative stress related diseases.
Active mitochondrial fusion is important not only to maintain mitochondrial integrity, but also to activate mtDNA replication by elevating mtDNA copy number (4). In addition, we provided evidence that BGP-15 activated the hydrolysis of GTP, which can be important since dynamin GTPases hydrolyze GTP to provide energy to constrict membranes. That is, BGP-15 acts in a unique fashion to trigger OPA1 GTP hydrolysis, which may represent the molecular mechanism underlying the constriction of mitochondrial cristae membranes and facilitate the fusion of inner mitochondrial membranes.

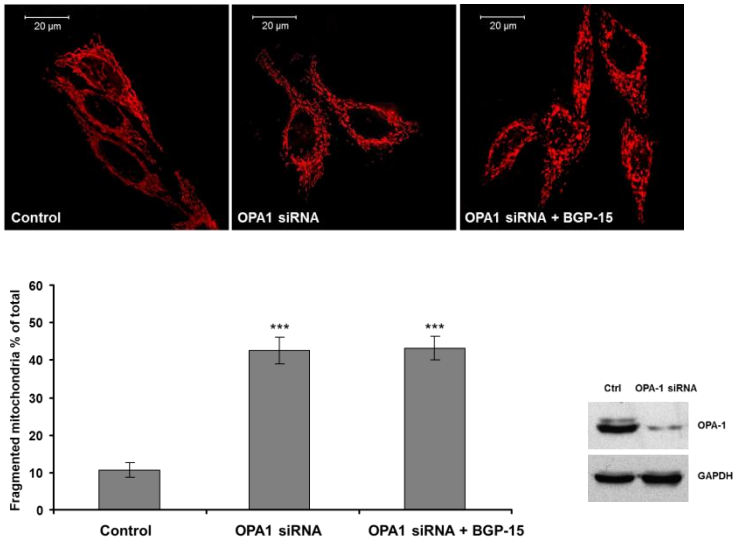


**Fig. 1. Opa1 is required for BGP-15 facilitates mitochondrial fusion.**

Therefore, it is very likely that activation of the mitochondrial fusion by synthetic small molecules affecting OPA1 function can be a novel and effective way for the prevention of mitochondrial fragmentation and to prevent or slow down the progression of mitochondrial diseases.

BGP-15, a hydroxylamine derivative, prevents insulin resistance in humans and protects against several oxidative stress-related diseases in animal models. In addition to the activation of optic atrophy 1 (OPA1), BGP-15 required active Mfn1 and Mfn2 to induced mitochondrial fusion. BGP-15 activates Akt, S6K, mTOR, ERK1/2 and AS160, and reduces JNK phosphorylation which can contribute to its protective effects.

Furthermore, BGP-15 protects lung structure, activates mitochondrial fusion, and stabilizes cristae membranes *in vivo* determined by electron microscopy in a model of pulmonary arterial hypertension. These data provide the first evidence that a drug promoting mitochondrial fusion in *in vitro* and *in vivo* systems can reduce or prevent the progression of mitochondria-related disorders.

**Indirect way for the prevention oxidative stress induced mitochondrial fragmentation: PARP-1 regulates the ATF4-MKP-1-JNK/p38 MAPK retrograde pathway**.

Oxidative stress induces DNA breaks and PARP-1 activation which initiates mitochondrial reactive oxygen species (ROS) production and cell death through pathways not yet identified. Here, we show the mechanism by which PARP-1 influences these processes via PARylation of activating transcription factor-4 (ATF4) responsible for MAP kinase phosphatase-1 (MKP-1) expression and thereby regulates MAP kinases. PARP inhibitor, or silencing, of PARP induced MKP-1 expression by ATF4-dependent way, and inactivated JNK and p38 MAP kinases. Additionally, it induced ATF4 expression and binding to cAMP-response element (CRE) leading to MKP-1 expression and the inactivation of MAP kinases (Fig. 2). In contrast, PARP-1 activation induced the PARylation of ATF4 and reduced its binding to CRE sequence in vitro. CHIP-qPCR analysis showed that PARP inhibitor increased the ATF4

occupancy at the initiation site of MKP-1. In oxidative stress, PARP inhibition reduced ROS-induced cell death, suppressed mitochondrial ROS production and protected mitochondrial membrane potential on an ATF4 and MKP-1 dependent way.

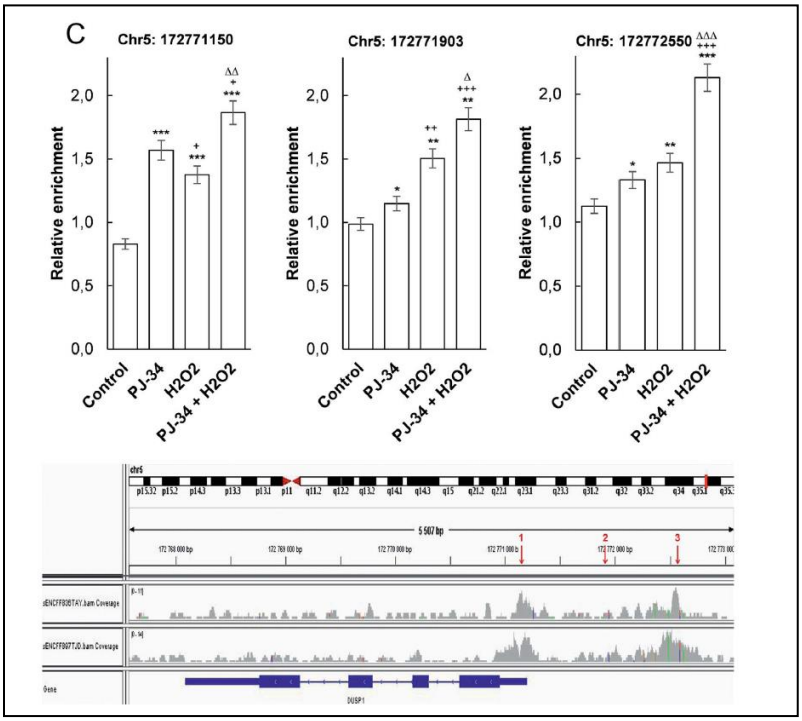


***Fig. 2. Effect of PARP inhibition on ATF4/CREB2's PARylation and binding to the MKP-1/Dusp1 promoter region.*** *WRL-68 cells were treated or not with 0.3 mM H2O2 with or without PJ-34for 30 min. (Upper figures) ChIP-qPCR analysis was performed to measure the capacity of ATF4 binding to MKP-1 Dusp1 promoter region position 1 (Chr5:172771150+/−150 nt), 2 (Chr5:172772550+/−150 nt), 3 (Chr5:172771903). MKP-1 Dusp1 promoter region positions derived from the graphical view of ATF4 ChIP-seq binding peaks (tag densities). (Lower figure). Data of three parallel experiments are presented as representative blots and bar diagrams of means±SEM. * p<0.05 between H2O2 and H2O2+PJ-34 groups;† p<0.05 compared to the untreated control group; a.u. arbitrary units.*

Basically identical results were obtained in WRL-68, A-549 and T24/83 human cell lines indicating that the aforementioned mechanism can be universal. Here, we provide the first description of PARP-1-ATF4-MKP-1-JNK/p38 MAPK retrograde pathway, which is responsible for the regulation of mitochondrial integrity, ROS production and cell death in oxidative stress, published in: Free Radic Biol Med. 2017 Jul;108:770-784.. This pathway can be important in the regulation of mitochondrial fission and fusion since JNK interact and activates mitochondrial E3 ubiquitin ligase 1 (Mul1) (*Mitochondrion. 2016 May;28:49-53.*) which ubiquitinates and facilitates the proteolyses of Mnf2 mitochondrial fusion protein. Therefore, the inactivation of JNK by PARP inhibitor can prevent oxidative stress induced mitochondrial fragmentation and this retrograde pathway can contribute to the protective effects of PARP inhibitors in disease models.

**PARP inhibitor prevents mitochondrial fragmentation in oxidative stress**.

In cell culture system we showed that hydrogen peroxide induces mitochondrial fragmentation in several cell lines and that PARP inhibitor (HO-3089) attenuated mitochondrial fragmentation (Fig. 3.). Since PARP is a nuclear enzyme therefore these observations are the consequences of some indirect mechanism namely through the

PARP1 dependent regulation of MKP-1/Dusp1, PI-3K-Akt system or by other mechanism (Free Radic Biol Med. 2017 Jul;108:770-784; Free Radic Biol Med. 2010 Dec 15;49(12):1978-88.; J Biol Chem. 2005 Oct 21;280(42):35767-75.). This question is studied in the following sections.

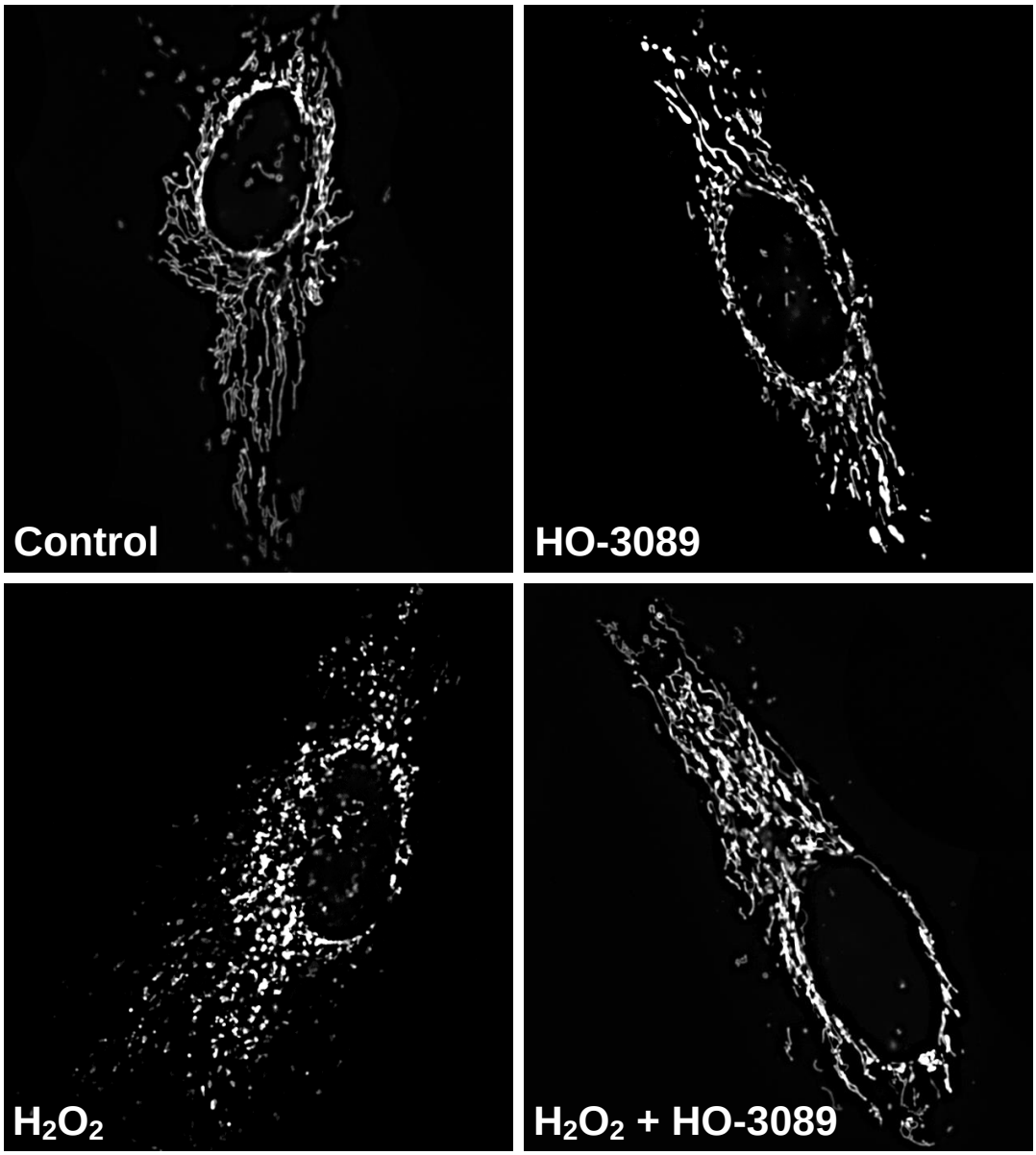


***Fig. 3.*** *PARP inhibitor (HO-3089) protects against oxidative stress-induced mitochondrial fragmentation in WRL-68 cells. (A) HO-3089 (10 μM) protects against oxidative stress-induced (50 μM $H_2O_2$) mitochondrial fragmentation in WRL-68 cells; fragmented mitochondria were shorter than 2 μm and filamental mitochondria were longer than 5 μm. Mitochondria were labeled by mitochondria directed-ERFP.*

**Possible role of PKCdelta in PARP mediated inhibition of mitochondrial fragmentation in oxidative stress**. It was previously shown that PKCdelta (PKCδ) binds

Drp1, a major mitochondrial fission protein, and phosphorylates Drp1 at Ser 579, thus increasing mitochondrial fragmentation in neurones. Phosphorylation of Drp1 Ser 579 by PKCδ promotes the translocation of Drp1 to the mitochondria under oxidative stress. This study indicates that PKCδ activation upregulates mitochondrial fission and contributes to neurological pathology (5). In our experimental system, we find that PKCdelta suppression has only a minor effect on the mitochondrial fragmentations, and together with PARP inhibitor it has no any effect (Fig. 4).

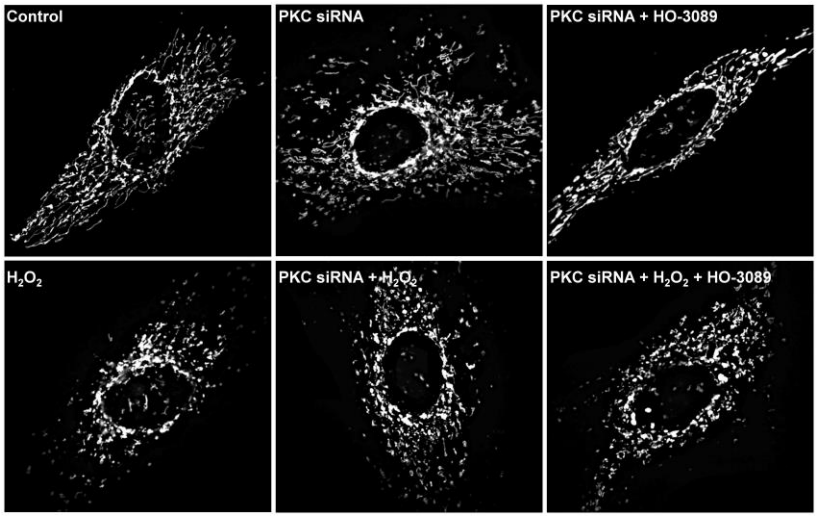


***Fig. 4. Role of PKCdelta suppression on the mitochondrial fragmentation and on the PARP inhibition mediated protection in oxidative stress in HeLa cells.*** *HeLa cells were treated by 25 μM hydrogen peroxide in control and PKCdelta suppressed cells, and in some cases PARP inhibitor (HO-3089) were added in 10 μM. Mitochondria were visualized by 50 nM MitoTracker Red by fluorescent microscope*.

In the presence of hydrogen peroxide PKCdelta suppression did not change the fragmentation of the mitochondria, but prevented the protective effect of PARP inhibitor. Since PKCdelta can activate NF-kappaB, which can activates Opa1 expression therefore we need further study to clarify the regulatory mechanisms. Manuscript under preparation.

**2, Determining the effect of PARP activation/inhibition on high glucose-increased $Ca^{2+}$-ERK1/2-Drp1 mediate mitochondrial fission**. We are close to the completion of the study. Analyzing the role of hyperglycemia on mitochondrial fragmentation we used **C2C12** -an immortalized mouse myoblast cell line. Elevated glucose level induced mitochondrial hyperpolarization, enhanced ROS production and increased mitochondrial fission at least it is published in the literatura. In our system, the same level of hyperglycemia did not induced mitochondrial fragmentation C2C12 cells (Fig. 5.). However, the addition of Palmitate induced the production of cytosolic and mitochondrial reactive oxygen species (ROS) and triggered aberrant endoplasmic reticulum (ER) Ca2+ release (6). Our data show the induction of mitochondrial fragmentation in hyperglycemia plus palmitate treatment (Fig. 5). BAPTA-AM which suppresses intracellular calcium level prevented mitochondrial fragementation, and a novel anti-diabetic drug candidate BGP-15 which activates Opa1 attenuates hyperglycemia and palmitate induced mitochondrial fragmentation (Fig. 5).

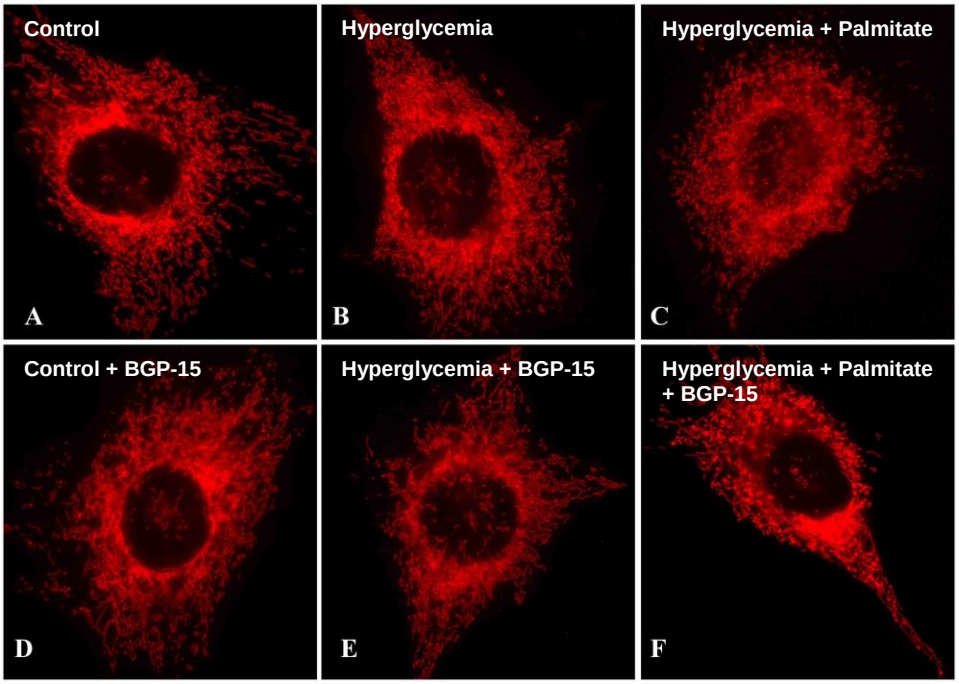


**Fig. 5.**

This observation is in a good correlation with in vivo data showing that BGP-15 reduces insulin resistane in animal models and in human clinical studies (7). In addition BGP-15 protects again oxidative stress induced mitochondrial destabilization cell death (8).

It is well-known that diabetes 2 induces mitochondrial fission leading to fragmented mitochondria in model systems and human studies, and that elevated mitochondrial fragmentation disrupts active insulin signaling (9). That is, active mitochondrial fusion machinery and active Akt are prerequisites for GLUT4 translocation and glucose transport. BGP-15 induced GLUT4 transport to cell membrane requires active mitochondrial fusion machinery and active Akt using the overexpressed plasmide of Glut4-EGHP. These data show the anti-diabetic effect of BGP-15 mediated via the mitochondrial fusion machinery. These data suggest that mitochondrial fusion activators can be a novel type of anti-diabetic drug candidates. Manuscript under preparation.

**3, To determine the effect of PARP activation/inhibition on mechanism of Akt mediated mitochondrial protection and mitochondrial fission/fusion pathway**.
We provided data that a new small heat shock protein called HSP16.2/HSPB11 – discovered by our lab- has mitochondrial protective effect through Akt activation. HSPB11 augments inhibitory phosphorylation of DLP1 thereby reduces mitochondrial fission that eventually leads to reduced apoptosis (Journal of Cancer 6 (5), 470, 2015).
BGP-15 activates Akt and induces the phosphorylation of some related kinases, mTOR, S6K and AS160, and this process can play important role in the protection of mitochondrial membrane systems. Therefore, we analyzed the effect of Akt suppression on BGP-15 induced fusion. Our data show that suppression of Akt induced mitochondrial

fragmentation (Fig. 6.). Oxidative stress-induced mitochondrial fragmentation was significantly reduced by BGP-15 in normal cells; however, BGP-15 could not prevent or reduce ROS-induced mitochondrial fragmentation in Akt suppressed cells. These data show that active Akt is required for mitochondrial fusion, and the BGP-15-mediated prevention of mitochondrial fragmentation requires Akt. We also studied the effect of BGP-15 on the MAPKs; BGP-15 significantly activated ERK1/2 phosphorylation and reduced JNK1 phosphorylation (10).

In addition, we provided evidence that overexpression of mutated catalytically active Akt is sufficient to prevent ROS-induced mitochondrial fragmentation under our experimental conditions. These observations also demonstrated that the protective effect of PARP inhibitor in mitochondrial fragmentation can at least partially rely on the PARP inhibitor induced activation of Akt (J Biol Chem. 2005 Oct 21;280(42):35767-75.) .

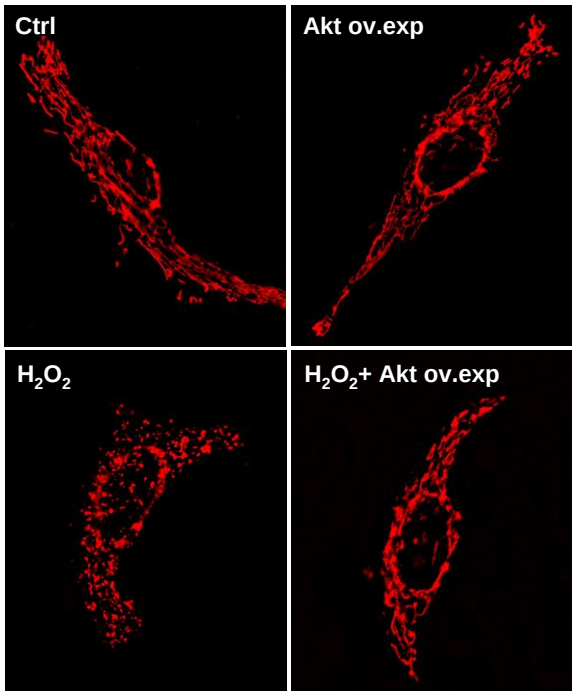


**Fig. 6. Fig. 1.** Overexpression of continuously active recombinant Akt (T308D-S473D-Akt) protects against oxidative stress-induced mitochondrial fragmentation in WRL-68 cells. The oxidative stress was induced by 50 μM $H_2O_2$ for 4 hours.

**4, To determine the role of cyclophilin D and MPT in the regulation of oxidative stress induced mitochondrial fission/fusion process.** It is well-known that collapsing the mitochondrial membrane potential leads to mitochondrial fission (Antioxid Redox Signal. 2011 May 15;14(10):1939-51.). We analyzed LPS-induced mitochondrial fragmentation cyclophilin D (CypD) suppressed cell, or on cells derived from CypD-knock-out mice. Our data showed that LPS induces significant changes in the expression of 2866 genes and in 206 pathways including: mitochondrial dysfunction, defective oxidative phosphorylation, NRF2, NO, ROS, Hif1α, TLRs, TNFα and DAMPs pathways in wild type mice. The disruption of CypD (Ppif -/-) reduces LPS-induced alterations in gene

expression, and pathways including reduction of TNFRs, TLRs, Il-1, Il-8, Il-10, Jak/Stat and MIF as well as improved survival, attenuates oxidative liver damages, and related NO and ROS pathways. CypD deficiency diminishes the suppressive effect of LPS on mitochondrial biogenesis -nuclear and mitochondria encoded genes- and mtDNA quantity which could be the critical point in improving survival. This short summary of the effects of CypD disruption shows that CypD have a much more complex role on gene expression and 206 pathways including the expression of mitochondrial biogenesis (Fig. 7.) and has complex effect on the expression of mitochondrial fission and fusion proteins.

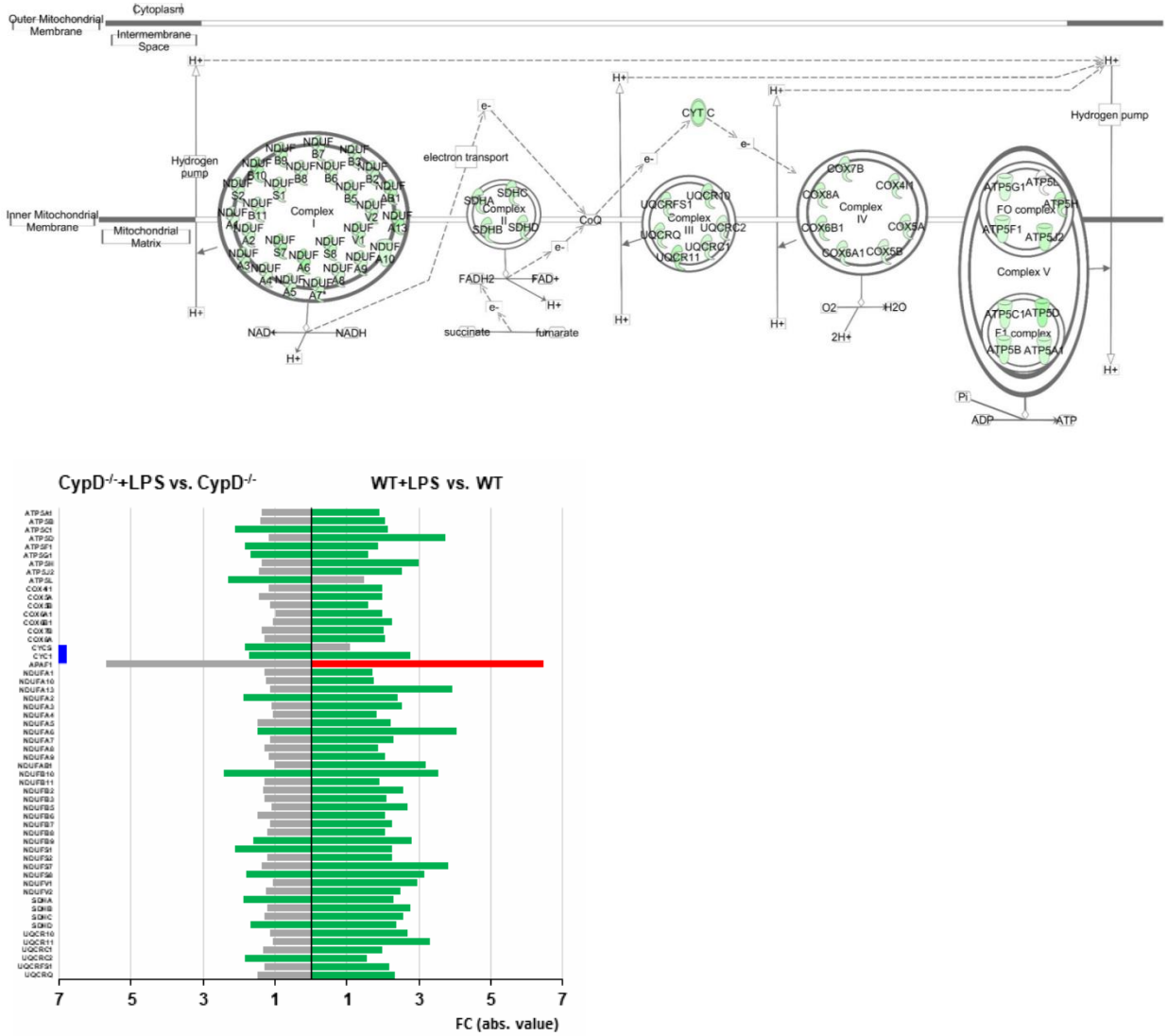





**Fig. 7. CypD disruption attenuates LPS-induced suppression of mitochondrial biogenesis –nuclear encoded components.** A. IPA analysis of the LPS-induced DEGs on nuclear DNA encoded genes of oxidative phosphorylation in WT mice. The green signs represent significantly downregulated genes, and only those genes were showed which were changed significantly. B. Effect of CypD disruption on the DEGs in nuclear DNA encoded genes of oxidative phosphorylation. Red bars show significantly upregulated genes while green bars represent downregulated genes, and gray bars show genes not significantly affected.

Our study shows that mPT can lead to necrotic cell death but normally induces ROS related signaling which can regulate metabolism, kinase cascades and activation, or inhibition, of transcription factors -NRF2, NF-kappaB and several others- leading to gene expressions, explaining the high number changes in gene expression and inflammatory reprogramming. Disruption of CypD prevents mPT and so attenuates the above mentioned complex changes including maintaining more stable mitochondrial structure and less ROS related signaling. One of the most dramatic effects of CypD-KO was the protection of mitochondria and oxidative phosphorylation which must have important effect on the survival of animals. These data show that CypD-KO has much more complex effects in gene expressions and signaling as we expected before; it protects mitochondrial structure, mitochondrial biogenesis, oxidative phosphorylation and the mitochondrial genome. Intrestingly we found that similar pathway changes occur in the liver of mice endotoxemia model and in the blood of septic patients, and in both cases prevention of mitochondrial dysfunction is an important cause for survival. In the case of mice model, we showed that prevention of mPT highly reduces mitochondrial dysfunction and highly promotes survival, indicating that specific mPT inhibitors could be potentially useful target in sterile shock and in sepsis.(Critical role of mitochondrial permeability transition in reprogramming and in disease progression of the immunosuppressive phase of endotoxemia. Manuscript under preparation.)

**5, Determining the role of mitochondrial fission/fusion pathways in heart disease model.** *Doxycycline protects against ROS-induced mitochondrial fragmentation and ISO-induced heart failure.* Tetracyclines have complex biological effects beside their anti-bacterial action, including the modification of mitochondrial protein synthesis, metabolism and gene-expression. Long clinical studies were performed with tetracyclines without significant side effects. Doxycycline (DOX), a major tetracyclin antibiotic performed protective effect in heart failure animal models without being known its exact molecular mechanism. Here, we provide the first evidence that DOX reduces oxidative stress-induced mitochondrial fragmentation and depolarization in H9C2 cardiomyocytes. Reactive oxygen species (ROS) play major pathological role in the development and progression of heart failure. Under pathophysiological conditions, like myocardial ischemia damaged mitochondria become the main sources of endogenous ROS production. In mitochondria **oxidative stress causes a shift toward fission which will lead to mitochondrial fragmentation**. Protecting mitochondria from oxidative stress and the regulation of mitochondrial dynamics by drugs that shift the balance toward fusion could be a novel therapeutic approach for heart failure. Fusion and fission are regulated by dynamin-like guanosine triphosphatases (GTPases): Mfn-2, OPA-1 and Drp-1. In our isoproterenol (ISO) -induced heart failure model, DOX reduced left ventricular hypertrophy, left-ventricular wall thickness, and attenuated the reduction in ejection fraction. DOX reduced oxidative/nitrosative stress, reduced plasma BNP levels, myocardial fibrosis and beneficially altered the expression of MFn-2, OPA-1 and Drp-1. On the basis of our findings we raised the possibility that DOX could be a novel therapeutic agent in the future treatment of heart failure. Published in: **PLoS One. 2017; 12(4): e0175195**.

We showed in spontaneously hypertensive rat (SHR) that PARP inhibitor shifting kinase cascades in a favorable way activating PI-3K-Akt pathway, suppressing MAP

kinase pathways (Biochim Biophys Acta. 2014 Jul;1842(7):935-44.; PLoS One. 2014 Jul 11;9(7):e102148. ; PLoS One. 2017 Mar 24;12(3):e0174401. ) and so reducing oxidative stress induced fragmentation as well as mitochondrial biogenesis which is demonstrated below by EM in SHR rats (Fig. 8.).

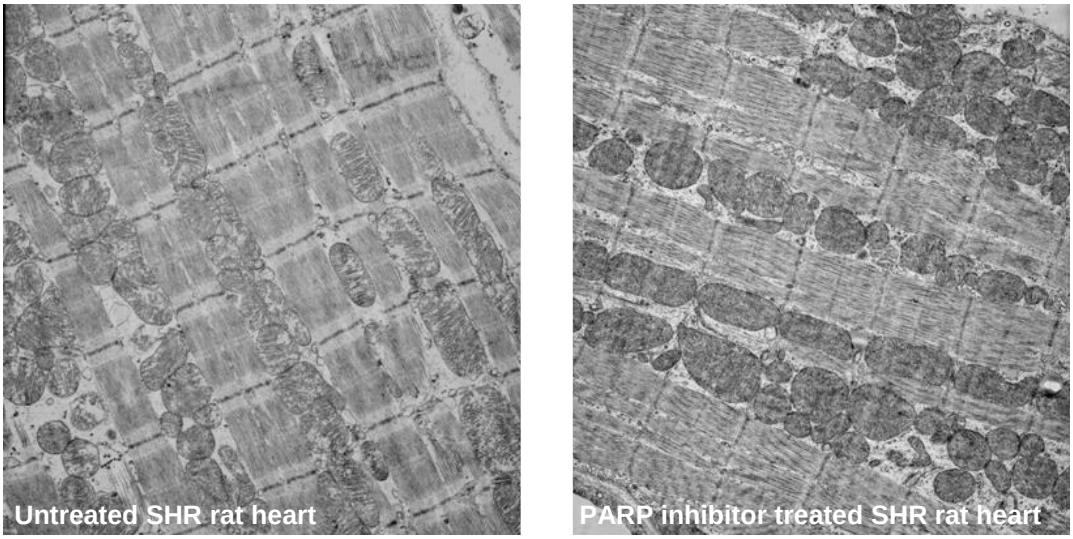


***Fig. 8. Effect of PARP inhibitor (L-2286) treatment on the mitochondrial structure in SHR rats.***
*Representative electron microscopic sections from myocardium of SHR heart and SHR rats treated by PARP inhibitor.*

## Most important discoveries.

1. Identification of an Opa1 activator (BGP-15) which promotes Opa1-Opa1 interactions and prevents ROS-induced mitochondrial fragmentation. BGP-15 is protective in several animal models, non-toxic in human studies and an insulin sensitizer in human Phase II studies.
2. Identification of a novel PARP-1-ATF4-MKP-1-JNK/p38 MAPK retrograde pathway, which regulate mitochondrial stability and explain the inhibition of ROS-JNK- the Mff-required mitochondrial fission.
3. Discovery of novel kinase –Akt; p38 MAP kinase-Gp78 E3 ubiquitin ligase ER-mitochondria association and fragmentation, potential role of PKCdelta- pathways in PARP inhibition prevented mitochondrial fragmentation in oxidative stress.
4. We provide evidence that active mitochondrial fusion pathway requires the Glut4 transport in C2C12 mule cells, and that BGP-15 activates Glut4 transport to the cell surface.
5. Evidence were presented that PARP-inhibitor prevented mitochondrial fragmentation in cardiomyocytes in animal models.

References

1. N. Xie, C. Wang, Y. Lian, H. Zhang, C. Wu, Q. Zhang, A selective inhibitor of Drp1, mdivi-1, protects against cell death of hippocampal neurons in pilocarpine-induced seizures in rats, Neuroscience letters 545 (2013) 64-68.;

2. X. Ma, Y. Xie, Y. Chen, B. Han, J. Li, S. Qi, Post-ischemia mdivi-1 treatment protects against ischemia/reperfusion-induced brain injury in a rat model, Neuroscience letters 632 (2016) 23-32.

3. N. Ishihara, M. Nomura, A. Jofuku, H. Kato, S.O. Suzuki, K. Masuda, H. Otera, Y. Nakanishi, I. Nonaka, Y. Goto, N. Taguchi, H. Morinaga, M. Maeda, R. Takayanagi, S. Yokota, K. Mihara, Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice, Nature cell biology 11(8) (2009) 958-966.

4. Ishihara N, et al. Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat. Cell Biol. 2009 Aug;11(8):958-66.

5. Qi X, Disatnik MH, Shen N, Sobel RA, Mochly-Rosen D. Aberrant mitochondrial fission in neurons induced by protein kinase C{delta} under oxidative stress conditions in vivo. Mol Biol Cell. 2011 Jan 15;22(2):256-65.

6. Exp Mol Med. 2017 Feb 3;49(2):e291. Oxidative stress and calcium dysregulation by palmitate in type 2 diabetes.

7. The HSP co-inducer BGP-15 can prevent the metabolic side effects of the atypical antipsychotics. Cell Stress Chaperones. 2012 Jul;17(4):517-21.; Literáti-Nagy B, Péterfai E, Kulcsár E, Literáti-Nagy Z, Buday B, Tory K, Mandl J, Sümegi B, Fleming A, Roth J, Korányi L. Beneficial effect of the insulin sensitizer (HSP inducer) BGP-15 on olanzapine-induced metabolic disorders. Brain Res Bull. 2010 Nov 20;83(6):340-4

8. Sumegi K, Fekete K, Antus C, Debreceni B, Hocsak E, Gallyas F Jr, Sumegi B, Szabo A. BGP-15 Protects against Oxidative Stress- or Lipopolysaccharide-Induced Mitochondrial Destabilization and Reduces Mitochondrial Production of Reactive Oxygen Species. PLoS One. 2017 Jan 3;12(1):e0169372.

9. A. del Campo, V. Parra, C. Vasquez-Trincado, T. Gutierrez, P.E. Morales, C. Lopez-Crisosto, R. Bravo-Sagua, M.F. Navarro-Marquez, H.E. Verdejo, A. Contreras-Ferrat, R. Troncoso, M. Chiong, S. Lavandero, Mitochondrial fragmentation impairs insulin-dependent glucose uptake by modulating Akt activity through mitochondrial Ca2+ uptake, American journal of physiology. Endocrinology and metabolism, 306 (2014) E1-E13

10. Aliz Szabo, Katalin Sumegi, Katalin Fekete, Eniko Hocsak, Balazs Debreceni, Gyorgy Setalo Jr., Krisztina Kovacs, Laszlo Deres, Andras Kengyel, Jozsef Mandl, Miklos Nyitrai, Mark A Febbraio, Ferenc Gallyas Jr., Balazs Sumegi. Activation of mitochondrial fusion provides a new treatment for mitochondria-related diseases. Biochem Pharmacol. In press, 2018.

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Activation of mitochondrial fusion provides a new treatment for mitochondria-related diseases

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