### 15P.10 Simultaneous ratiometric imaging of ATP and $Ca^{2+}$ concentrations inside single living cells

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Calcium ion regulates many cellular processes. Previous study that employed firefly luciferase as a reporter of intracellular ATP concentration has revealed that histamine-induced increase of intracellular $Ca^{2+}$ concentration induces increase of ATP concentration of both cytoplasm and mitochondria [1]. Because oligomycin inhibits the increase of ATP, $Ca^{2+}$ must promote ATP synthesis in mitochondria. However, this experiment used ensemble of cells, and therefore it is still unclear how $Ca^{2+}$ dynamics affects to intracellular ATP concentration at single cell level. We have recently reported a series of genetically-encoded Förster resonance energy transfer (FRET) indicator for ATP, called ATeam, which is based on CFP, YFP and ε subunit of bacterial $F_oF_1$-ATP synthase [2]. Ratiometric FRET imaging of cells expressing ATeam has enabled us to monitor ATP concentration of cytoplasm, nucleus or mitochondria at single cell level. However, it has been difficult to image intracellular ATP together with $Ca^{2+}$ because there are significant overlaps of both excitation and emission spectra between ATeam and ratiometric calcium indicators (eg. Fura-2). Here, we report a red-shifted ATeam (GO-ATeam) that has GFP and OFP as a donor and an acceptor of FRET, respectively, instead of CFP and YFP. Because GO-ATeam is excited with longer wavelength light than the previous ATeam, it is compatible with a UV-excitable ratiometric calcium indicator, Fura-2. We simultaneously imaged ATP and $Ca^{2+}$ concentrations of the same single living cells by loading Fura-2 calcium indicator into the cells expressing GO-ATeam. When $Ca^{2+}$ spark was induced in the cells by histamine, mitochondrial also ATP elevated following $Ca^{2+}$. Intracellular $Ca^{2+}$ typically returned to a basal level within 10 minutes after histamine stimulation. In contrast, most of cells retained high level of mitochondrial ATP for a much longer time (sometimes more than 30 min). This result strongly supports the idea that intracellular $Ca^{2+}$ promotes long-term activation of energy metabolism in mitochondria [1].

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### 15P.11 Outer mitochondrial membrane protein degradation by the proteasome/ubiquitin system

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Protein turnover is used for regulatory processes and to eliminate superfluous, denatured or chemically inactivated polypeptides. Mitochondrial proteins may be particularly susceptible to damage induced by reactive oxygen species and several pathways of mitochondrial proteostasis have been illuminated. However, in contrast to matrix and inner mitochondrial membrane protein degradation, little is known about the turnover of integral outer mitochondrial membrane (OMM) proteins or the mechanisms involved. Our previous studies have demonstrated that the OMM proteins are under control of the ubiquitin (Ub)/proteasome system. Our new data indicate that in addition to the proteasome, various membrane steps of Ub-dependent degradation of the OMM proteins, including retrotranslocation through mitochondrial membrane(s) (through the activity of AAA-ATPase p97), are similar to those of the endoplasmic reticulum-associated degradation pathway. As an example we found a striking increase in ubiquitinated proteins, including Mfn2 and Tom20, of mitochondria isolated from cells expressing a dominant negative mutant of AAA-ATPase p97, p97QQ. Since expression of p97QQ inhibits retrotranslocation of polyubiquitinated proteins from the ER, these data suggest that p97 might function in a similar manner in retrotranslocation of polyubiquitinated proteins from the OMM. We have also identified a family of the OMM-associated E3 Ub ligases that are likely to control ubiquitination and degradation of the OMM proteins. One of these proteins, IBRDC2, an IBR-type RING-finger E3 Ub ligase, regulates the levels of Bax and protects cells from unprompted Bax activation and cell death. Downregulation of IBRDC2 induces increased cellular levels and accumulation of the active form of Bax. The ubiquitination-dependent regulation of Bax stability is suppressed by IBRDC2 down regulation and stimulated by IBRDC2 overexpression, both in healthy and apoptotic cells. These findings suggest the existence of an Ub- and IBRDC2-dependent apoptosis checkpoint safeguarding mitochondria from Bax-dependent damage and thus cell from unprompted apoptosis.

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### 15P.12 The influence of estradiol, epinephrine and cAMP on mitochondria energization and intracellular free $Ca^{2+}$ concentration in lamprey hepatocytes

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The aim of the study was to clarify the role of epinephrine, estradiol and isobutiryl-cAMP in regulation of mitochondrial membrane potential ($\Delta\psi_{mit}$) and intracellular free $Ca^{2+}$ concentration in lamprey hepatocytes during metabolic depression connected with starvation. TMRM (tetramethylrhodamine 0.5 μM) and $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide 1 nM) were used with laser confocal microscopy (Leica TCS SP5) and flow cytometry (Backman Coultrer EPIXC-XL) to investigate $\Delta\psi_{mit}$ in isolated hepatocytes. For measurement of $Ca^{2+}_i$ concentration hepatocytes were loaded by 5 μM FURA 2-AM and were measured by spectrofluorophotometer Shimadzu RF-1501. In autumn lampreys (*Lampetra fluviatilis L.*) return from the Baltic sea to the river Neva where they spend all winter switching off exogenic feeding. In spring after the spawn lampreys die, because they are monocyclic animals. The liver of adult lamprey is a unique natural model witch demonstrates both the processes of metabolic depression (from November until March) and activation (April-May). $\Delta\psi_{mit}$ decreased from October till December. Under the treatment of epinephrine ($10^{-5}$ M) in the middle of November the energization of mitochondria in hepatocytes increased. At the end of November the influence of epinephrine passed away, which seems to be connected with down-regulation of membrane receptors. However, at that time the introduction of isobutiryl-cAMP ($10^{-7}$ M) sharply increased the fluorescence intensity TMRM and $DiOC_6$. In this period estradiol not influenced on $\Delta\psi_{mit}$. Intracellular free $Ca^{2+}$ concentration increased from 60 nM in October to 130 nM in February. In period of metabolic depression (the Atkinson charge in lamprey liver did not exceed 0.2-0.3) cytosolic $Ca^{2+}$ was very high while intracellular calcium stores

(endoplasmic reticulum, Golgi complex, mitochondria) was empty. $Ca^{2+}_i$ concentration did not change after treatment of epinephrine and estradiol from November till February. However in October and March estradiol ($10^{-5}$ M) stimulated rapidly increase of $[Ca^{2+}]_i$ (from 60 to 145 nM). Thus in winter during metabolic depression $\Delta\psi_{mit}$ decreased while $Ca^{2+}_i$ concentration increased in lamprey hepatocytes, but in autumn under the epinephrine and cAMP influence energetic suppression was reversible and increase of $Ca^{2+}_i$ after estradiol treatment was observed.

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### 15P.13 Fluorescent visualization of NAD(P)H oxidoreductase activity in the outer mitochondrial membrane and in cytosol on acute tissue slices

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Free cytosolic and membrane-bound NAD(P)H oxidoreductases play an important role in immune response, detoxication of drugs and xenobiotics, and in signaling. However, the physiological and pathological roles of NAD(P)H oxidoreductases of the outer mitochondrial membrane are not generally recognized even in spite of their capability to produce ROS and regulate the permeability transition pore opening under certain conditions [1]. The main reason for this is the absence of methods for efficient discrimination between cytosolic (microsomal) and outer mitochondrial oxidoreductases. Recently we developed a flow-cytometry-based method for the semiquantitative assessment of the activity of NADH and NADPH oxidoreductases in the outer mitochondrial membrane and cytosol [2]. The method is based on the capability of a range of NAD(P)H oxidoreductases to reduce lucigenin to highly fluorescent water-insoluble dimethylbiacridene by two-electron reduction (DT-diaphorase) or through two consecutive steps of one-electron reduction with an intermediate cation radical (NADH cytochrome $b_5$ reductase, NADPH cytochrome P450 reductase) [3, 4]. The discrimination of oxidoreductases appeared to be possible due to the fact that oxidoreductases of the outer mitochondrial membrane changed the apparent mechanism of lucigenin reduction (from 1-e to 2-e) as spontaneous oxidation of cation radical by cytochrome *c* oxidase was blocked [2]. The method proposed allowed one to assess and rapidly compare the activity of six groups of NAD(P)H oxidoreductases in different cell lines. However, the method required the use of detached or isolated cells and prohibited a comparison of lines of cells of irregular shape or different size. Here we present a modification of this approach, which allows the assessment, visualization, and discrimination of activities of various NAD(P)H oxidoreductases using acute tissue slices. The approach is suitable for tissues composed of cells of different types, size, and shape (brain, kidney, heart). Costaining with Mito Tracker Red, Hoechst, etc. allows specifying the localization of oxidoreductase activity. The approach can be helpful in studies of the role of NAD(P)H oxidoreductases in the range of physiological and pathological processes.

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### 15P.14 Upregulation of human selenoprotein H in murine hippocampal neuronal cells promotes mitochondrial functional performance and biogenesis

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Selenoprotein H (SelH) is one of the 25 known selenoproteins. Previous studies have shown that overexpression of SelH in murine hippocampal neuronal HT22 cell line ameliorates neuronal death after UVB irradiation by reducing ROS production and by blocking mitochondrial initiated apoptotic cell death pathway. The objective of this study was to examine the effects of SelH on mitobiogenesis and mitochondrial function. Three experiments were performed. 1) Protein levels of peroxisome proliferator-activated receptor- coactivator (PGC)-1 and -1β (PGC-1 and PGC-1β), nuclear respiratory factor-1 (NRF-1), mitochondrial transcription factor A (mtTFA), and cytochrome *c* were measured using Western blot analyses; mitochondrial respiration and oxygen consumption were measured using oxygraph; and mitochondrial mass was determined using mitotracker coupled with cell imaging. 2) Both SelH- and vector-tranfected HT22 cells (SelH-HT22 and vector-HT22, respectively) were irradiated with 7 J/cm$^2$ UVB and the above mitochondria-related markers were measured. 3) Selenite was added to the culture media and PGC-1, NRF-1 and mitochondrial respiration were measure in HT22 cells treated with or without UVB irradiation. Our results demonstrated that transfection of human SelH gene into neuronal HT22 cells significantly increased the translational levels of PGC-1 and NRF-1, two key factors that regulate mitochondrial biogenesis. As expected, mitochondrial cytochrome *c* content was elevated, mitochondrial respiration was enhanced and mitochondrial mass was increased in the selH-HT22 compared to vector-HT22 cells. Supplementation of selenite increased the levels of mitobiogenesis regulation factors. We conclude that overexpression of SelH promotes mitobiogenesis and improves mitochondrial functional performance. These effects can also be achieved by supplementation of selenite.

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### 15P.15 Monitoring mitochondrial $[Ca^{2+}]$ dynamics with fluorescent dyes and targeted proteins

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The dynamics of $[Ca^{2+}]$ in the mitochondrial matrix has received much attention in the last 20 years because of its importance in a large variety of critical cellular processes, from energy production to apoptosis. Measurements of mitochondrial $[Ca^{2+}]$ have been made using two different methods: fluorescent $Ca^{2+}$-sensitive dyes such as rhod-2 or similar, and fluorescent or luminescent targeted proteins such as aequorin, pericam or camaleons. Unfortunately, data obtained with each of these approaches are very different, both qualitatively and quantitatively, and the reasons for the discrepancies are still unclear. While studies using fluorescent dyes report maximum $[Ca^{2+}]_M$ values of 2-3 mM [1], data obtained with targeted luminescent and fluorescent proteins indicate that $[Ca^{2+}]_M$ can reach much higher values, up to tenths or hundreds of micromolar [2, 3]. Moreover, the