Mitochondrial Light Switches: Optogenetic Approaches to Control Metabolism

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Abstract

Developing new technologies to study metabolism is increasingly important as metabolic disease prevalence increases. Mitochondria control cellular metabolism and dynamic changes in mitochondrial function are associated with metabolic abnormalities in cardiovascular disease, cancer, and obesity. However, a lack of precise and reversible methods to control mitochondrial function has prevented moving from association to causation. Recent advances in optogenetics have addressed this challenge, and mitochondrial function can now be precisely controlled *in vivo* using light. A class of genetically-encoded, light-activated membrane channels and pumps has addressed mechanistic questions that promise to provide new insights into how cellular metabolism downstream of mitochondrial function contributes to disease. Here, we highlight emerging reagents – mitochondria-targeted light-activated cation channels or proton pumps – to decrease or increase mitochondrial activity upon light exposure, a technique we refer to as mitochondrial light switches, or mt^SWITCH. The mt^SWITCH technique is broadly applicable, as energy availability and metabolic signaling are conserved aspects of cellular function and health. Here, we outline the use of these tools in diverse cellular models of disease. We review the molecular details of each optogenetic tool, summarize the results obtained with each, and outline best practices for using optogenetic approaches to control mitochondrial function and downstream metabolism.
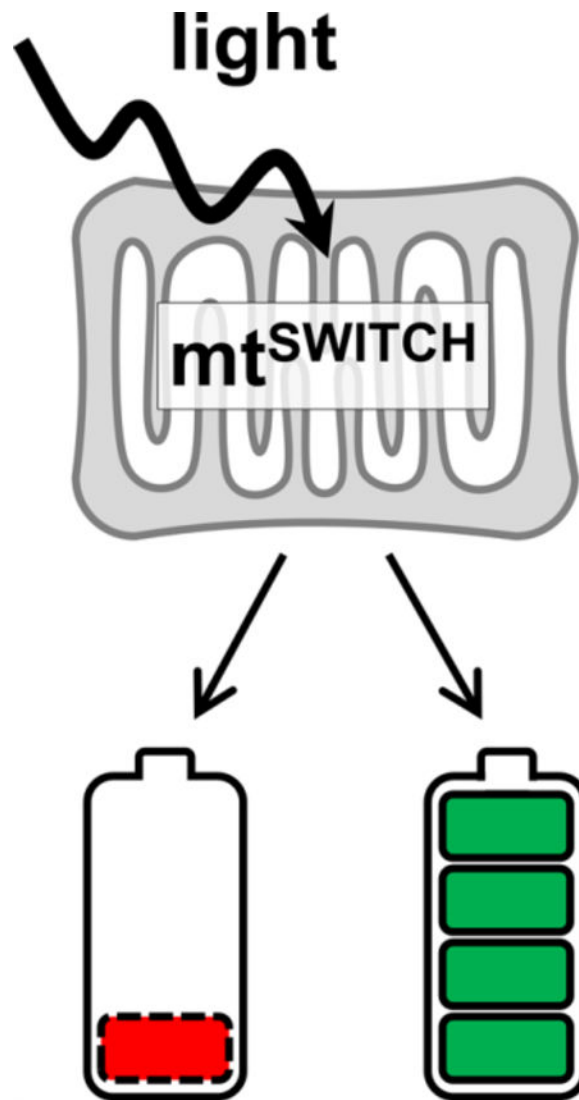
Graphical Abstract

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Mitochondrial light switches (mt$^{\text{SWITCH}}$) are novel optogenetic tools to precisely, reversibly and instantaneously decrease or increase mitochondrial function using light. Light-sensitive membrane channels or proton pumps confer this control by altering the electrochemical gradient that drives mitochondrial function, the protonmotive force. This advance allows unprecedented, fundamental control over metabolism and physiology, and will establish causal mechanisms in models of metabolic diseases by providing experimental isolation of mitochondrial function.

Keywords

hypoxia; AMPK; calcium signaling; Parkinson's; bioenergetics; apoptosis; mitophagy; diabetes

INTRODUCTION

An increasingly aged population and the prevalence of metabolic diseases represents a global health care crisis [1–3]. Basic science research focused on understanding metabolism

is therefore critical to identify novel molecular targets and mechanisms to develop new therapies. At the core of metabolism are mitochondria, the organelles responsible for making energy available to cells. Mitochondria are metabolically flexible and can adapt to changing cellular conditions and energy needs. Mitochondria affect many cellular decisions, ranging from adaptation and survival to death. Much is still unknown about how mitochondrial function regulates diverse and complicated signaling *in vivo.*

Mitochondria make ATP [4], regulate calcium (Ca^{++}) signaling [5], coordinate redox signaling and reactive oxygen species (ROS) production [6], modulate cell death through apoptosis [7–9], and integrate metabolic signaling across cells and tissues [10]. These factors underlie cellular signaling programs such as unfolded protein responses [11], autophagy [12], and stress adaptation [9]. In this way, mitochondria are essential cellular signaling hubs that impact all areas of cellular life. Each of these processes and pathways is regulated by the driving force of mitochondrial function, the protonmotive force (PMF).

Mitochondrial protonmotive force (PMF)

The mitochondrial PMF is an electrochemical gradient across the mitochondrial inner membrane (IM) made up of a charge separation, or membrane potential, denoted Ψ_m, and a chemical pH separation denoted pH. The PMF is like a battery, in that potential energy is stored for eventual release to do work. The PMF is created by the electron transport chain (ETC) in the IM when electrons from metabolic substrates from food are passed along the chain and protons are pumped from the mitochondrial matrix to the intermembrane space (IMS) as oxygen is consumed (Figure 1). The battery power of the PMF can then drive ATP synthase to phosphorylate ADP to ATP, the cellular energy source. The PMF also drives other mitochondrial functions such as metabolite and ion transport. In particular, the abundant intracellular cation potassium (K^+) is subject to the PMF, as well as Ca^{++} [5, 13]. These ions can flux across the IM in regulated ways to impact cellular metabolism and physiology.

The PMF is central to mitochondrial function and is tightly regulated under different metabolic conditions. For example, substrate and oxygen availability [10, 14] or energetic demand from exercise [15–17] can alter the PMF. Metabolic conditions can vary between tissues, and accordingly there are tissue differences in mitochondrial PMF [18, 19]. The PMF **can also be** differentially regulated within individual mitochondria, with separate matrix cristae compartments able to experience different driving forces [20]. These differences across tissues, metabolic states, and within mitochondria may be responses **to** adjust metabolism to specific cellular and tissue demands. Despite the clear observations and underlying premise suggesting an important role for PMF in cellular metabolism, specific mechanisms through which changes in PMF cause downstream effects are still not fully understood. Nevertheless, regulation of the PMF is important for mounting physiologic responses to stress and to changes in energy availability for organisms to adapt and to survive. When the PMF is not regulated properly to respond to these changes or stresses, cellular damage can occur [11].

Significant insights have evolved from studying downstream functions of endogenous proteins that dissipate the PMF (Figure 1). A major example of these proteins is the

uncoupling proteins (UCP). UCPs are named for their ability to uncouple the reactions of the ETC from ATP synthesis [21, 22]; when protons can leak back into the mitochondrial matrix without driving ATP synthesis through ATP synthase, uncoupled respiration results. To maintain PMF, the ETC increases activity resulting in increased oxygen consumption. The results of uncoupling can range from total energy collapse when using pharmacologic uncouplers, to regulated oxidative stress reduction and energy-sensing signaling to adjust metabolism to meet energy demand through UCPs [23, 24].

Proteins other than UCPs can uncouple mitochondria as well, such as the adenine nucleotide transporter (ANT), which can transport protons into the matrix in a regulated manner [25, 26]. There are also many substrate and ion transporters that use the energy from the PMF to drive their function, such as the mitochondrial phosphate carrier and the glutamate/aspartate exchanger [27, 28]. Cation channels in mitochondria are also subject to the PMF, a driving force for cation accumulation into the matrix. Some examples of cation channels include the ATP-sensitive potassium channel, sodium-activated potassium channels, and the calcium uniporter [29–32]. These channels can regulate responses to different metabolic conditions and to stresses [33, 34]. A large conductance, non-selective channel known as the mitochondrial permeability transition pore (mPTP) is also present in mitochondria and can lead to complete PMF collapse [35, 36]. Though the molecular details of the mPTP are debated, its presence is observed under many regulated metabolic processes and in disease models [37, 38]. While there are many proteins that regulate and respond to the PMF (Figure 1), studying how the PMF controls metabolism and vice versa can be challenging.

Historically, there have been limited experimental approaches to modulate the PMF. Small molecule protonophores, such as FCCP, can be used to shuttle protons across the IM, resulting in PMF dissipation. However, this approach lacks tissue specificity, reversibility, and selectivity. Protonophores can act on cell membrane potential and gradients across other biologic membranes such as those present in the lysosome or in secretory vesicles. Protonophores also lack tissue specificity and reversibility such that FCCP cannot be selectively targeted to one tissue of an organism, and there is no acute means to remove the activity. In addition, until recently there has been no way to specifically increase the PMF in isolation, except by fueling upstream metabolism to provide electrons to the ETC. Because of these difficulties, interpreting physiologic responses to PMF interventions can generate misleading conclusions or opposite conclusions in different models (thoroughly reviewed elsewhere [6]). To circumvent some of these limitations, a mitochondria-targeted light-activated protonophore, mito-photo-DNP, was created. Mito-photo-DNP conferred better spatiotemporal control, allowing specific regions of cells containing mitochondria to be targeted with light for PMF dissipation. [39, 40]. This advance paved the way for optogenetic approaches targeted to mitochondria.

THE MITOCHONDRIAL LIGHT SWITCH TECHNIQUES (mt^SWITCH)

Optogenetics

Optogenetics is the expression of light-sensitive proteins to control an aspect of cell physiology, such as ion gradients across the plasma membrane of cells. Optogenetics flourished in neuroscience, where depolarizing or hyperpolarizing the cell membrane

potential of neurons in response to specific wavelengths of light was used to functionally dissect neural networks. This led to novel insights regarding physiologic regulation of behavior by the nervous system, with approaches designed to test necessity and sufficiency of precise neuronal inputs [41–44]. There are many widely used optogenetic tools such as the cation-selective Channelrhodopin (ChR2), anion-selective Halorhodopsin, and several specific, directional proton pumps like bacteriorhodopsin (bR), the related delta-rhodopsin (dR) [45], Arch and its derivatives [46], and Mac [47]. While the diversity of optogenetic tools is rapidly expanding, we will focus on two classes that were recently targeted to mitochondria to modulate the PMF: ChR2 and proton pumps.

ChR2 and proton pumps can alter the PMF by fostering ion transport across membranes, but there are important differences between ChR2 and proton pumps that contribute to unique aspects of their function. Proton pumps are unidirectional, and can selectively move protons even against a concentration gradient, with directionality determined by orientation of the pump in the membrane [47–49]. ChR2 is less selective, and allows cations (H^+, K^+, Ca^{++}) to move in either direction according to that ion's driving force across a membrane [50, 51]. Notably, proton specific pumps [47] avoid the potential influence of K^+ or Ca^{++} ion flux that may occur with ChR2. Another difference between ChR2 and proton pumps is that ChR2 has an extended desensitization time when the channel remains open [52], whereas proton pumps quickly cease activity in the absence of light [53]. Different proteins in this family can also have different optimal light activation wavelengths, allowing combination of proteins using different irradiation colors [48, 49]. Using proteins with different wavelengths of light would allow differential manipulation of physiology at different times or in different tissues, depending on the color and duration of irradiation. Regardless of their differences, each of these pumps or channels are structurally similar, consisting of 7 transmembrane helices arranged around a covalently bound cofactor all-*trans* retinal, which is required for photocurrents [48, 51].

Subcellular targeting sequences can be used to direct optogenetic tools toward specific cellular compartments, like vesicles or lysosomes [51, 54]. Orientation in membranes matters for unidirectional proton pumps, and different organelle targeting sequences can be used to orient proton pumping in a selected direction. [54]. Proton pumps translocate protons toward their N terminus, which is natively from the cytoplasm to outside the cell [55, 56]. Based on these principles of targeting and directionality, several pumps and channels were targeted to the mitochondrial IM to increase or decrease the PMF in response to light. These optogenetic tools can dissipate the PMF by moving protons from the IMS to the mitochondrial matrix, resulting in respiratory uncoupling. Conversely, proton pumps can pump protons directionally across the IM from the matrix to the IMS to generate a PMF and energize mitochondria. Collectively, we will refer to these tools as the mitochondrial light switch technique (mt^{SWITCH}), as the reagents are switches that can either selectively "turn off" or "turn on" mitochondrial function through PMF manipulation using light. Targeting optical tools to mitochondria can be challenging. There are many mitochondria-targeting sequences (MTS) that can be used to direct proteins to mitochondria [57], and different targeting strategies can orient proteins differently in the IM [54] (Figure 2). It is important to note that a targeting sequence may work for one approach, but it may not be sufficient to

target a different protein to mitochondria. Therefore, proper localization and function should be confirmed.

Once successfully targeted to mitochondria optogenetic tools can be used to study several broad phenomena of cellular metabolism downstream of bioenergetics. Using light allows rapid reversibility and spatial restriction of illumination to confer precise spatiotemporal control of the PMF, a major advance in experimental control. The following sections summarize the mitochondria-targeting details and the findings using individual constructs of the mt^{SWITCH} technique.

mitoChR2

An early example of a mt^{SWITCH} approach targeted ChR2 to mitochondria (mitoChR2) [52], with cation flux across the IM and PMF collapse occurring in response to light. Targeting was through N-terminal fusion to 4 repeats of the well-characterized 29 amino acid COX8A MTS (116 amino acids total) [52, 58, 59]. Our laboratory has found that targeting approaches using a single COX8A targeting sequence are insufficient to direct ChR2 or proton pumps to mitochondria and result instead in plasma membrane targeting, a finding that has been corroborated by others [52, 60]. Mitochondrial targeting using the COX8A repeated sequence was achieved only after deleting a small leading section of the ChR2 sequence (24 amino acids) thought to contain a plasma membrane targeting sequence [52]. Once targeted to mitochondria, topological studies confirmed the orientation of mitoChR2 in the IM (Figure 2A). Confocal fluorescent images of mitoChR2 tagged with YFP combined with protease protection and fluorescence quenching assays confirmed mitoChR2 localization in the IM. mitoChR2 was also functionally characterized in cells to demonstrate PMF dissipation *in vivo* [52].

In response to 460–480 nm light, mitoChR2 depolarized Ψ_m light-dose dependently in cells [52]. In addition, using a stabilized step function opsin version of this construct allowed closure of the channel using 590 nm light. Using these two colors of light conferred precise, reversible control over PMF dissipation with mitoChR2. This study further showed that Ψ_m changes were not due to mPTP formation, providing extra evidence that mt^{SWITCH} functions independent of endogenous mitochondrial PMF changes.

Mitochondria can modulate cellular Ca^{++} signaling through uptake of Ca^{++} into the matrix driven by the PMF. When activated, mitoChR2 modulated Ca^{++} signaling and respiration in cells. Light activation decreased Ca^{++} accumulation in the mitochondrial matrix, and increased oxygen consumption rate in respiring cells, which is the expected effect of increased ETC activity following PMF dissipation [61]. Decreases in both Ψ_m and Ca^{++} uptake mimicked treatment with a protonophore, showing mitoChR2 can completely depolarize mitochondria in cells. These changes in bioenergetics and Ca^{++} handling have broad implications for many experimental models. Ca^{++} signaling is intertwined with many cellular processes that also involve mitochondria [5, 62, 63], and optogenetic approaches will help define these molecular mechanisms with better spatial and temporal control.

Next, mitoChR2 was used to control downstream physiologic functions of metabolism that require mitochondrial function. Muscle contraction relies on Ca^{++} signaling and energy

availability, both of which can be altered by mitoChR2. The activation of mitoChR2 almost completely prevented cardiomyocytes from contracting in culture. The loss of cardiomyocyte beating was therefore likely due to dysregulated Ca^{++} signaling and loss of ATP production downstream of mitoChR2 activation. In another model, mitoChR2 prevented the well-characterized increase in PMF and resultant ATP production in pancreatic β-cells in response to glucose stimulation [64]. These two metabolically distinct effects of muscle contraction and ATP production in β-cells shows the fundamental control mt^{SWITCH} proteins could have in diverse models. Finally, subcellular illumination showed that mitoChR2 functioned only where irradiated, observed through decreased Ψ_m and Ca^{++} uptake only in the illuminated area. This study demonstrates the precise spatiotemporal control of optical dissipation of PMF using mt^{SWITCH} techniques to alter metabolism [52].

ABCB-ChR2

Another approach to selectively dissipate the PMF again targeted ChR2 to mitochondria using a different strategy. Similar to initial targeting strategies for mitoChR2 [52], ChR2 did not reach mitochondria using one, two or three repeats of the COX8A MTS [60]. ABCB-ChR2 was successfully targeted using the large transmembrane ABCB10 MTS [65]. Again, confocal images of ABCB-ChR2 tagged with YFP and immunolabeling confirmed mitochondrial targeting. The ABCB10 MTS would orient ChR2 with the same topology as mitoChR2 [65] (Figure 2A&B), however, orientation in the membrane should not alter the effect of ChR2 as the channel has no directionality. Indeed, the activation of ABCB-ChR2 also resulted in PMF dissipation in cells [60].

Mitochondrial depolarization can result in apoptotic cell death in many contexts [66, 67]. This optogenetic approach allowed precise control to better understand the link between PMF and cell death. ABCB-ChR2 depolarized mitochondria, and cell viability with increasing light exposure was then measured. Light activation of ABCB-ChR2 caused increased cell death and decreased viability. These effects were reversible using Z-VAD-FMK, a specific caspase-dependent apoptosis inhibitor, indicating cell death was occurring through apoptosis. Observation of mitochondrial cytochrome c release confirmed the involvement of apoptosis in the light-induced cell death, as cytochrome c release is a hallmark of mitochondria-driven apoptosis [66].

Mitochondrial fitness is often signaled through the PMF. Broadly, healthy mitochondria will have a PMF, while mitochondria without a PMF are dysfunctional. Dysfunctional mitochondria are removed through a process called mitochondrial autophagy, or mitophagy. Mitophagy is a mitochondrial quality control process and could be a protective mechanism in different metabolic disorders [68, 69]. Activation of ABCB-ChR2 could then be used to trigger or affect mitophagy through PMF loss. Light activation of ABCB-ChR2 resulted in increased markers of mitophagy, mediated by Parkin and subsequent LC3 accumulation at mitochondria. Consistent with the idea that the activation of mitophagy may protect against stress, ABCB-ChR2 activation exhibited a preconditioning effect; when mitochondrial PMF was dissipated with light, cells were more resistant to subsequent PMF dissipation and cell death. This result indicates that altering the PMF can lead to cellular adaptation for stress

resistance. This study highlights the use of mt^{SWITCH} techniques to identify molecular details of the consequences of metabolic shifts in cell fate determination [60].

mtON

While mitochondria-targeted ion channels have been used to dissipate the PMF, PMF generation requires a unidirectional proton-selective pump. The eukaryotic proton pump Mac was expressed in mitochondria and named mtON for its ability to generate a PMF and "turn on" mitochondrial function. Unlike channelrhodopsins, proton pumps like Mac are unidirectional proton transporters, allowing directional control with different targeting sequences. mtON was targeted to the IM by fusion with the MTS and part of the coding region of the IMMT1 protein [70–72]. Importantly, this MTS contains a transmembrane region, required for orientation of mtON to pump protons from the matrix to the IMS to energize the PMF (Figure 2C). Protease protection studies and detailed confocal fluorescent image scans of single mitochondria expressing mtON indicated IM targeting and expected topology oriented to pump protons out of mitochondria. Full characterization of mtON in isolated mitochondria revealed increased PMF in response to light, confirming the direction of proton pumping [71].

The proton pump Mac can generate proton gradients in response to light [47, 49]. Likewise, mtON in isolated mitochondria caused a light dose-dependent energized PMF in response to 550–590 nm light that matched the PMF generated by the ETC [71]. Increased PMF was observed through both Ψ_m and pH components. These results indicated a PMF was generated using only the energy from light, bypassing the ETC, as no source of electrons (metabolic substrates to generate electron donors for respiratory function) were required. To further test this, oxygen consumption was measured in isolated mitochondria fueled with substrates to drive respiration. When activated, mtON decreased reliance on oxygen to make ATP, demonstrating that mtON can supplement ETC activity by using light rather than oxygen and electrons. This showed that mt^{SWITCH} tools can be used to control the PMF independent of oxygen or substrate availability.

In addition to supplementing ETC activity, mtON was able to compensate for lost ETC activity. In a whole-animal *C. elegans* model, animals exposed **to** ETC toxins survived more when mtON was active compared to controls, indicating mtON activation can overcome dysfunctional respiration. These results were confirmed using a mitochondrial mutant strain with decreased animal locomotion, a phenotype rescued by mtON activation. Impaired energetic function of mitochondria is signaled throughout cells and organisms, and AMP-activated protein kinsase (AMPK) is a molecular energy sensor responsible for some of this signaling [73]. mtON silenced AMPK activation under starvation conditions and decreased animal locomotion when starved. These acute changes in AMPK signaling could have broad and lasting impacts on metabolism and physiology [74–76].

One example of a lasting impact downstream of AMPK and other signaling pathways is the ability to adapt to stress. mtON was used to study hypoxia-adaptation as a readout of stress resistance. In *C. elegans* and in mammals a short exposure to hypoxia is protective against a later, more damaging exposure [34, 77]. The protection conferred therein can be suppressed by mtON activation, suggesting that a decreased PMF during the short hypoxia is required

for its protective effect. These results are consistent with those using ABCB-ChR2 [60]; PMF dissipation triggers stress resistance prophylactically. The mtON system highlights how temporal control of the PMF can reveal fundamental requirements for stress resistance in models of mitochondrial and metabolic dysfunction [71].

mito-dR

Another attempt to polarize the PMF targeted the proton pump delta-rhodopsin (dR) to mitochondria. mito-dR was expressed in *Drosophila* and proposed to increase the PMF in response to 550–590 nm light [78]. mito-dR function was assessed in dopaminergic nerve terminals by observing Ψ_m, ATP levels, and Ca^{++} handling in tissues, each after long-term light exposure, indicating mito-dR can affect energetics in fly nervous tissue. In addition, oxidized lipids and mitochondrial redox state were assayed, and mito-dR decreased signs of oxidative stress. In many contexts, however, decreased rather than increased PMF is associated with alleviated oxidative stress [6, 79, 80], especially in Parkinson's disease models [81–84].

Mitochondrial dysfunction is involved in models of Parkinson's disease [85–87]. mito-dR was originally used in a cell model of Parkinson's disease dysfunction [88]. In a *Drosophila* knockout model of Parkinson's disease, mito-dR decreased aggregated α-synuclein levels, a protein causally implicated in Parkinson's pathology involving mitochondria [85–87]. Light activation of mito-dR also increased flight and movement behavior associated with protection against neurodegeneration, indicating that mito-dR acts to improve dopaminergic neuron function *in vivo* [78]. This study shows how mt^{SWITCH} techniques can be used in simple models to better understand the fundamental principles of neurodegenerative metabolism.

In comparison to other mt^{SWITCH} approaches, there is limited evidence surrounding the topology and pumping direction of mito-dR. Similar to Mac and bR, dR is a unidirectional proton pump that translocates protons toward its native N terminus [45, 89]. To generate mito-dR, dR was fused to a single COX8A targeting sequence on the N terminus to achieve expression in mitochondria [88]. With this targeting strategy, the dR N terminus is be predicted to be in the matrix. No topology studies or functional measures in isolated mitochondria or cells were carried out, however, so the direction of mito-dR proton pumping is unclear. While proton pump activity of dR was confirmed in bacteria, the activity of mito-dR remains to be fully characterized [78] (Figure 2D). This uncertainty together with observations that decreased PMF (rather than increased PMF) results in decreased oxidative stress is a significant caveat in the interpretation of results from mito-dR.

Other Optical Tools in Mitochondria

While the focus of this article is on new optogenetic approaches to control the PMF, there are other optogenetic tools used in mitochondria to compliment mt^{SWITCH} techniques. For example, mitochondrial position within cells can be reversibly controlled using light-induced recruitment of cytoskeletal motor proteins [90, 91]. Mitochondrial tethering to endoplasmic reticulum can also be controlled using light [92]. Using modified fluorescent proteins, ROS can be generated upon light exposure, modeling mitochondrial ROS production in a tightly

controlled manner [93–95]. These ROS generating proteins have been targeted to different compartments in cells, and specifically to different compartments in mitochondria to assess nuances of mitochondrial redox signaling [96]. Similarly, combining optogenetics and pharmacology to generate mitochondrial ROS revealed redox communication between mitochondria and chromosomes in the nucleus [97]. This study highlights the broad reach of mitochondrial signals throughout the cell. Each of these mitochondria-targeted optogenetic tools is likely to confer precise spatiotemporal control over different aspects of mitochondrial biology, and each could be used along with the mt^{SWITCH} techniques to study mitochondria in powerful new ways using light.

PERSPECTIVES

mt^{SWITCH} has been used to control mitochondrial function and resulting metabolism in diverse ways thus far. The technique is poised to become a powerful and efficient means to study metabolism in many new contexts. As is true for all optogenetic approaches, mt^{SWITCH} approaches must include experimental controls for light exposure and, where appropriate, cofactor supplementation. In mammalian cell systems all-*trans* retinal may be present at sufficient levels, but exogenous supplementation may increase optogenetic effects [52]. Some systems such as *C. elegans* and *Drosophila* require exogenous all-*trans* retinal supplementation for functional optogenetics [41], which must be controlled for due to potential confounding effects, such as antioxidant capabilities of the cofactor [98]. Off-target effects can be controlled for either using non-functional optogenetic protein controls [52, 78], or by combining experiments both with and without supplemented all-*trans* retinal [71]. Controlling for light exposure is also important, especially when using blue excitation wavelengths that are damaging to biological samples [99]. This is particularly relevant to mitochondria [100] which contain many light-absorbing cytochromes and flavins. Flavins can create ROS when exposed to blue light [101], which can cause damage or alter redox signaling that heavily impacts metabolism [102]. In addition to off-target effects from light or all-*trans* retinal, protein expression artifacts can be kept to a minimum in living systems by using CRISPR/Cas9 approaches to drive single-copy expression, decreasing the likelihood of overexpression artifacts.

When appropriately controlled, mt^{SWITCH} is a powerful toolkit to investigate how precise changes in mitochondrial function regulate metabolism and downstream physiology. Selective dissipation of the PMF overcomes the significant challenges of using protonophores, especially when used tissue- or cell-specifically. Increasing the PMF with light is a significant advance to understand metabolism, as other means to increase the PMF necessarily fuel the ETC or inhibit ATP synthesis; both interventions have myriad other effects on metabolism. Fueling mitochondria to increase the PMF would result in changed glycolysis and Krebs cycle flux, two pathways that are regulated and that participate in cellular signaling [10]. Inhibiting ATP synthase increases PMF by removing the ability to dissipate the PMF through ATP synthesis. This inhibition could result in non-physiologic responses such as cell damage or death due to lack of energy availability, or conversely adaptive signaling to respond to the energetic crisis [103, 104]. Using mt^{SWITCH} to increase the PMF avoids these confounding variables. Together, mt^{SWITCH} can be combined to take

unprecedented control of mitochondrial function and metabolism – decreasing or increasing the PMF in isolation – to determine cause and effect in complicated metabolic models.

mt^{SWITCH} techniques have been used in diverse cell and organism models to lay the foundation of mitochondrial optogenetics going forward (Figure 3). The studies reviewed here show the feasibility of employing mt^{SWITCH} and highlight the broad range of diseases that may be impacted. ATP availability, Ca^{++} signaling, AMPK signaling, and oxidative stress are each implicated in many diseases and pathologies, and each are cellular outputs affected by mt^{SWITCH}. Using mitoChR2 to learn more about glucose-stimulated ATP production may advance understanding in models of diabetes and metabolic syndromes. mitoChR2 could also inform myopathy research through its ability to control muscle contraction. ABCB-ChR2 control of apoptotic cell death and mitophagy could guide future experimental models to understand aging and cellular senescence. Energizing mitochondria with mtON to modulate AMPK signaling and hypoxia resistance could be translated to models of ischemia reperfusion injury, with outcomes of heart attack and stroke dependent on PMF changes that are not fully understood [105, 106]. mito-dR showed that metabolic control will be important in understanding the fundamentals of neurodegenerative disease progression and protection.

Using mt^{SWITCH} to establish causal interactions in these metabolic models of disease will lead to discovery of new pathways to target therapeutically. The most wide-ranging unanswered question mt^{SWITCH} can address is the following: How do mitochondria truly behave *in vivo*, and how does that behavior impact cellular life? This general question is integral to many models of health and disease, from understanding the benefits of exercise, to understanding how metabolic shifts impact aging tissue, tumor microenvironments, or adaptations to obesity. Light as an experimental tool confers precise control in both space and time that no other reagent can manage in mitochondria, especially *in vivo*. The tools reviewed here will be essential to advance our understanding of complex cellular functions by increasing the spatial and temporal resolution of metabolic control.

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Abbreviations:

mt^{SWITCH}	mitochondrial light switches
Ca^{++}	calcium
ROS	reactive oxygen species
PMF	protonmotive force
IM	inner mitochondrial membrane
Ψ_m	mitochondrial membrane potential

pH	pH gradient
ETC	electron transport chain
IMS	intermembrane space
K$^+$	potassium
UCP	uncoupling protein
ANT	adenine nucleotide transporter
mPTP	mitochondrial permeability transition pore
ChR2	channelrhodopsin 2
bR	bacteriorhodopsin
dR	delta-rhodopsin
MTS	mitochondrial targeting sequence
AMPK	AMP-activated protein kinase

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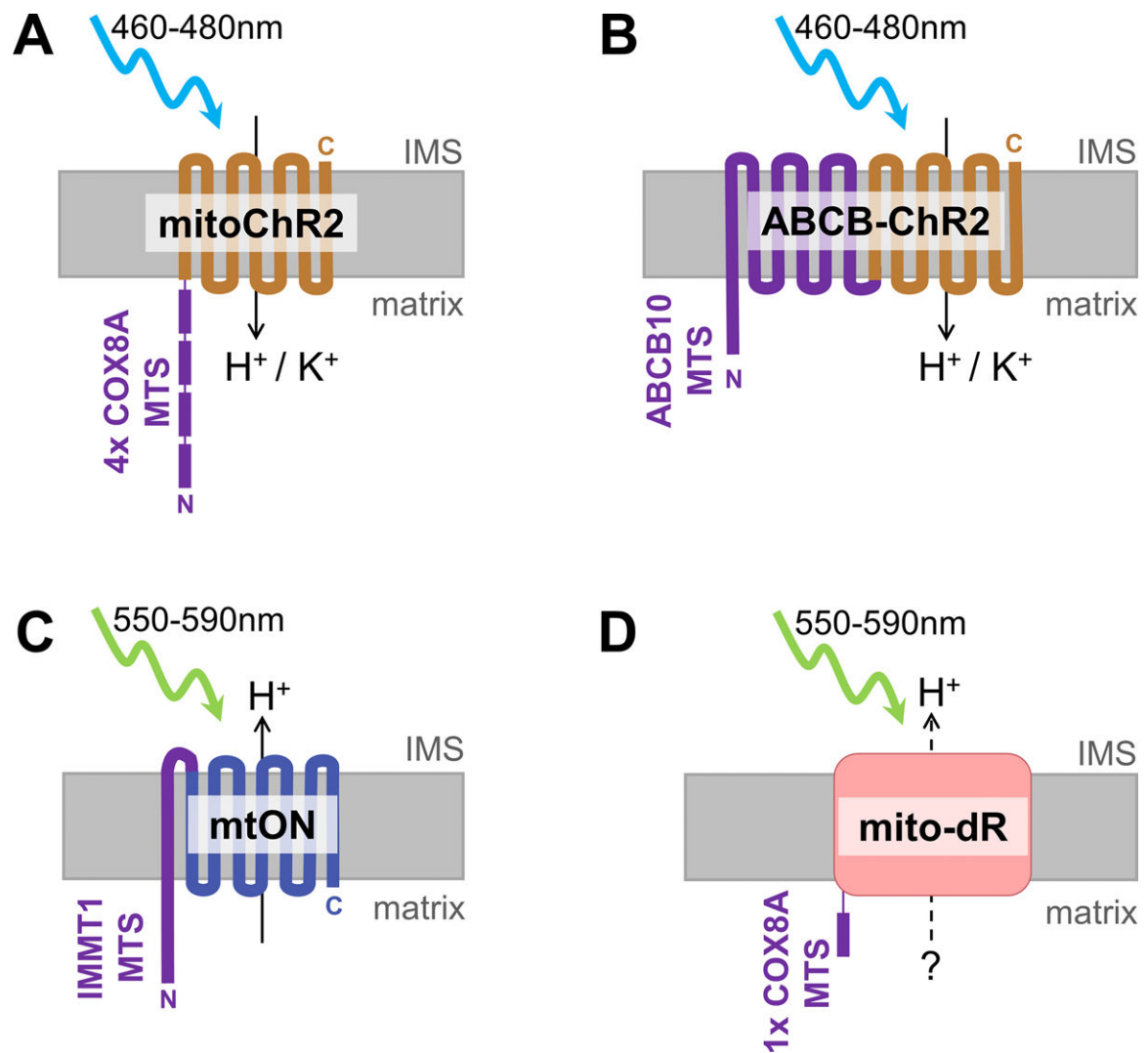
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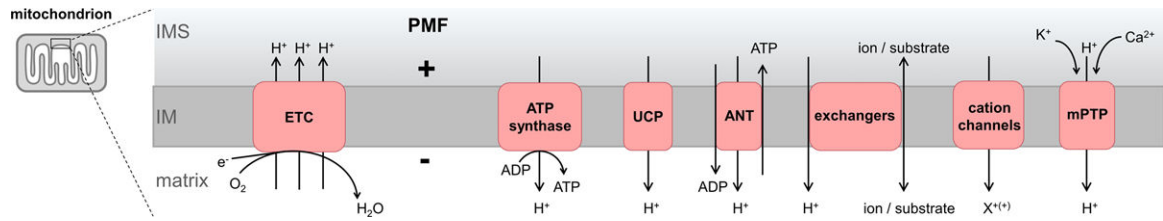
Figure 1. Endogenous protonmotive force (PMF) regulation.
The mitochondrial electron transport chain (ETC) pumps protons from the mitochondrial matrix to the intermembrane space (IMS) to establish the PMF. The PMF drives ATP synthase to phosphorylate ADP to ATP. Other proteins in the inner membrane (IM) regulate mitochondrial function through proton transport which affects the PMF. Uncoupling proteins (UCP) are regulated to dissipate the PMF. Similarly, adenine nucleotide transporters (ANT) can also dissipate the PMF in a regulated manner. Several metabolite and ion exchangers also use the PMF to drive their functions. Cation channels in mitochondria can allow cation $(X^{+(+)})$ accumulation into the matrix. The mitochondrial permeability transition pore (mPTP) is a large conductance channel that can cause total PMF collapse in different physiologic and pathologic conditions.

A 460-480nm

IMS

mitoChR2

matrix

C

4x COX8A MTS

H⁺ / K⁺

N

B 460-480nm

IMS

ABCB-ChR2

matrix

C

ABCB10 MTS

H⁺ / K⁺

N

C 550-590nm

H⁺

IMS

mtON

matrix

C

IMMT1 MTS

N

D 550-590nm

H⁺

IMS

mito-dR

matrix

1x COX8A MTS

?

Figure 2. mt^SWITCH constructs increase or decrease the PMF in response to light.
mt^SWITCH constructs are shown with their respective mitochondria-targeting sequences (MTS, shown in purple) in the mitochondrial inner membrane. Light-activated proteins are shown in either orange or blue to indicate that they decrease or increase the PMF, respectively. Topology of light-activated proteins are indicated where ions are moved either from the intermembrane space (IMS) to the matrix, or vice versa. N and C termini of peptides are indicated in each construct. **A)** mitoChR2 uses the COX8A mitochondria-targeting sequence (MTS) repeated in tandem four times to achieve mitochondrial expression. mitoChR2 is activated by 460–480 nm light, and results in decreased PMF. ChR2 is capable of passing protons (H^+) as well as other cations, potassium (K^+) the most prevalent in the cytosol. **B)** ABCB-ChR2 uses the ABCB10 MTS to direct ChR2 to mitochondria, and is topologically oriented the same as mitoChR2. ABCB-ChR2 is also activated by 460–480 nm light and results in decreased PMF. **C)** mtON is targeted to mitochondria by the IMMT1 MTS and some of the transmembrane protein sequence to flip the topology to direct proton-specific translocation from the matrix to the IMS. The native N terminus of the light-activated proton pump is therefore located on the IMS side, opposite of
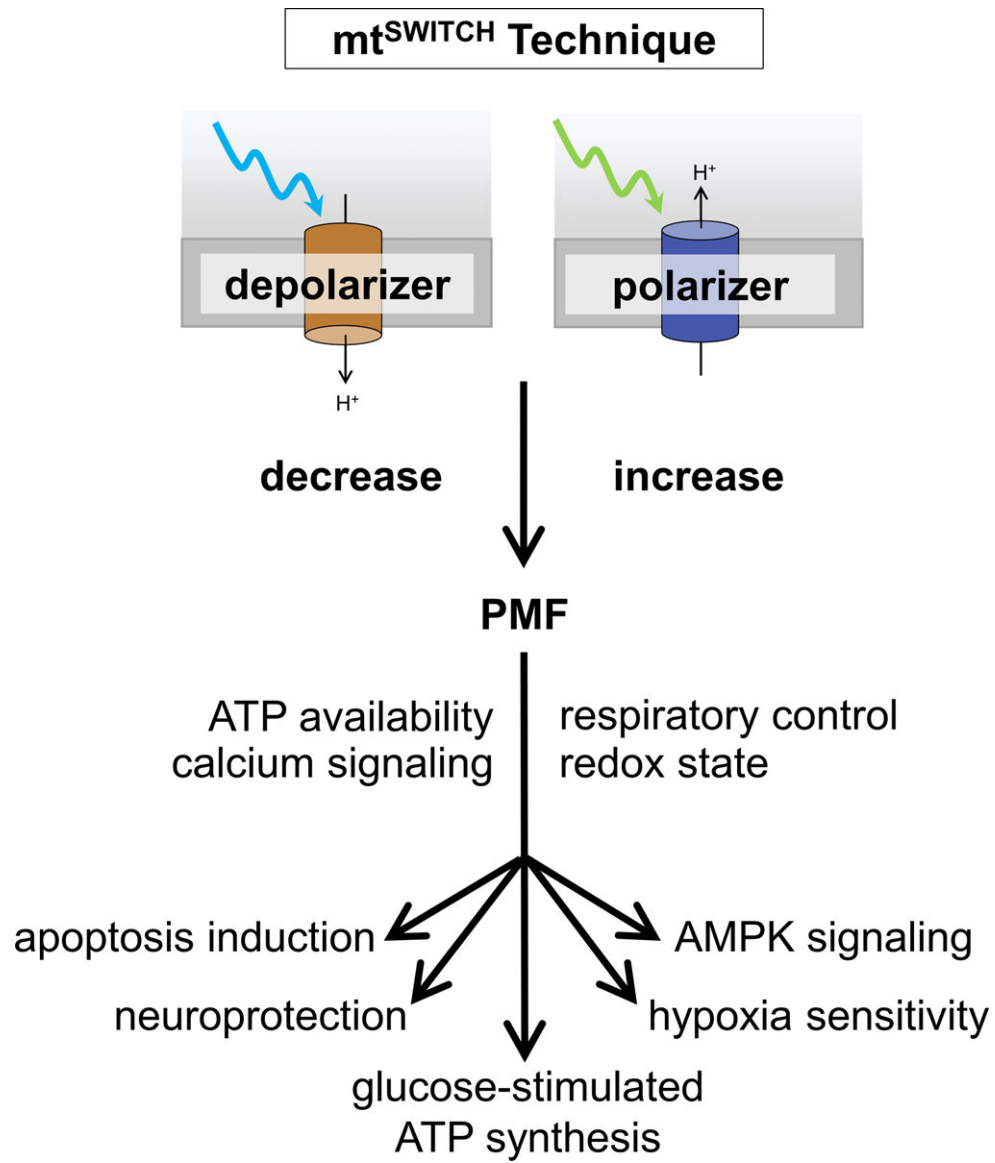
the MTS N terminus. mtON is activated by 550–590 nm light and results in increased PMF. **D)** mito-dR uses one COX8A MTS, and the topology and direction of proton translocation is unclear in mitochondria. mito-dR is activated by 550–590 nm light and is proposed to increase the PMF.

Figure 3. The mt^{SWITCH} technique affects broad metabolic outputs through PMF manipulation.
mt^{SWITCH} constructs directly alter mitochondrial functions that impact downstream metabolism. Decreasing or increasing the PMF results in altered ATP production, O_2 consumption by the electron transport chain, Ca^{++} handling and signaling, and cellular redox state. Each of these factors affect cellular signaling. Changing these parameters has impacted physiologic outputs such as apoptosis and mitophagy, glucose-stimulated ATP production in β-cells, AMPK signaling and hypoxia resistance in living animals, and biochemical and behavioral outcomes in neurodegeneration models. These are just a few validated examples of the impact mt^{SWITCH} will have on many fields of research to understand disease and how metabolism impacts it.