Develop chemical biology tools to dissect SIRT3 functions and mitochondrial homeostasis of cancer cells

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(a) Background of research

Mitochondria play a critical role in cellular metabolism, particularly in ATP production through cellular respiration. To maintain metabolic balance and prevent the generation of harmful reactive oxygen species (ROS), which are produced during impaired respiration, a homeostasis of mitochondrial function and quality is essential (1). Mounting evidence suggested that disrupted mitochondrial homeostasis underlies the pathology of various chronic medical conditions including cancer (1). Understanding the regulation/deregulation of mitochondrial homeostasis is vital for uncovering the underlying mechanisms involved. While the cellular stress and apoptosis pathways triggered by various mitochondrial disturbances like hypoxia and oxidative stress are well-established (2), the homeostatic regulations of mitochondrial proteome and the deregulations in diseases like cancers remain largely unclear. Moreover, it is important to note that the connections between proteomic and metabolomic homeostasis of mitochondria remain elusive.

In eukaryotic cells, it is crucial to continuously monitor the mitochondrial integrity to effectively respond to environmental challenges and physiological signals. The mitochondrial unfolded protein response (UPRmt) is mechanism that monitors the mitochondrial proteome. It involves sensing the proteome disturbances and initiating transcriptional activation programs that lead to the production of mitochondrial chaperone proteins and proteases. These components integrate together to maintain proper protein folding and prevent protein aggregation within the mitochondria (3). UPRmt serves as a signaling pathway that facilitates communication between the mitochondria and the nucleus. While acute activation of UPRmt allows for adaptation to environmental stressors and physiological cues, chronic UPRmt signaling can be maladaptive and represents a potential therapeutic target for a wide range of disorders. Dysregulation of UPRmt pathways plays a significant role in inter-tissue signaling and has functional implications in congenital metabolic disorders (4), age-related diseases, cancers (5) and neurodegenerative disorders (6), etc.

Sirtuin 3 (SIRT3) is a mitochondrial deacetylase that is predominantly expressed in tissues with high metabolic demand, such as the brain. Its catalytic activity relies on NAD as a cofactor. SIRT3 plays a vital role in regulating various processes, including energy homeostasis, redox balance, mitochondrial quality control, mitochondrial unfolded protein response, biogenesis, dynamics, and

mitophagy. It achieves these functions by modifying the acetylation status of proteins within the mitochondria. When SIRT3 expression or activity is reduced, it leads to hyperacetylation of numerous mitochondrial proteins (7). In ERα negative cells, Timothy (8) discovered that under stress conditions in either the intermembrane space (IMS) or the matrix of the mitochondria, the expression of SIRT3 is increased. This elevated SIRT3 expression leads to the deacetylation of the transcription factor FOXO3a. The deacetylation of FOXO3a in response to mitochondrial stress was found to be dependent on SIRT3, although the mechanism is likely indirect due to the distinct subcellular localization of FOXO3a and SIRT3 (9). Furthermore, Timothy (8) also found that the deacetylated FOXO3a molecule relocates to the nucleus, where it stimulates the transcription of specific target genes, namely SOD2 and catalase (9). These genes are involved in antioxidant defense mechanisms. In these cells, both IMS-stress and matrix-stress activate the protein CHOP, along with its downstream targets hsp60 and hsp10, which are molecular chaperones involved in protein folding (10). Despite these knowledges, how SIRT3 functions in cancer cell to regulate/deregulate mitochondrial homeostasis remain unknown.

Chemical probes capable of detecting SIRT3 activity and dissecting its signaling partners will help elucidate SIRT3 regulation mitochondrial homeostasis. Prof. Zhang Liang's group has the expertise in proteomics and mitochondrial homeostasis and Prof. Sun Hongyan's group has the exquisite strength in developing chemical probes of enzyme. In this project, we will collaborate and develop activity-based probes of SIRT3 that are of dual purpose (Figure 1A). It will enable detecting SIRT3 activity with robust fluorescent emission and labeling SIRT3-interacting proteins in complex cellular environments. We will validate the diverse functions of the probes and investigate the regulation of SIRT3 in mitochondrial stress of cells. We will also collaborate with Prof. Yin Huiyong to investigate how mitochondrial proteomic homeostasis is connected to a metabolomic homeostasis. The outcome will ensure us to prepare a CRF grants with collogues in other universities (CUHK, HKU and HKUST).

(b) Research plan and methodology

Aim 1. To synthesize the dual-purpose probes of SIRT3 and validate the activities in vitro.

We will first synthesize the dual-purpose probes of SIRT3 based on two substrates FOXO3A and SOD2 (11). Our preliminary results have observed that purified SIRT3 could efficiently catalyze the deacetylation of Kac from the optimized peptides of FOXO3A and SOD2 (Figure 1B-C). To systhesize the probes, Kac peptides will be attached with an O-NBD group and a minimalist photo-cross-linker. Upon deacetylation, the resulted free amine undergoes spontaneous intramolecular exchange with O-NBD, forming N-NBD with fluorescent signals as the reporter. In parallel, the photo-cross-linker contains an aliphatic diazirine group and a terminal alkyne group that are used in combination for downstream applications, such as in-gel fluorescence scanning and pulldown-MS assays. We have successfully applied this system in investigating a series of deacetylation/deacylation enzymes and generated multiple publications (12-14). After synthesis, we will assess the reporter and crosslinking activities of the probe in vitro using purified SIRT3. The specificity will be evaluated using other SIRT/HDAC deacetylases as we performed before (12). We expect to obtain robust dual-purpose probes of SIRT3 for in vitro applications.

Aim 2. Applying the probes to investigate the regulation of SIRT3 in mitochondrial stress of cells.

In parallel to in vitro assays, we will assess the performance of the probes in cellular applications. Previous studies in Prof. Zhang Liang's group have established that hypertonic stress disrupts mitochondrial homeostasis in adipocytes (15) and cancer cells (Figure 2A, C and D). If not corrected, it can lead to metabolic deregulation and apoptosis. Mechanistically, we observed downregulation of SIRT3 (Figure 2B). It remains unclear how SIRT3 deacetylation, the other important pathway of mitochondrial homeostasis, is activated in the process. Therefore, in this aim, we will utilize the synthesized probes to investigate the function of SIRT3 during hypertonicity-induced mitochondrial stress. The fluorescent reporter function will reflect the spatiotemporal dynamics of SIRT3 activity in a continuous manner. The photo-cross-linker will enable labeling and enrichment of protein complexes during SIRT3 activation. Prof. Zhang Liang's group will perform quantitative MS analysis to decode the enriched proteins. Bioinformatic and network analysis will offer valuable insights to delineate the roles and mechanisms of SIRT3 in mitochondrial homeostasis.

Aim 3. Investigate the SIRT3 signaling in mitochondrial dysregulation of cancer cells.

We will functionally validate the identified spatiotemporal patterns and interacting proteins of SIRT3 activation in mitochondrial dysregulation of cancer cells. SIRT3 antagonists/silencing will be applied to investigate how they affect the protein complex assembly (evaluated by co-IP), mitochondrial function (evaluated by Seahorse assays), and cell apoptosis (evaluated by FACS). In parallel, SIRT3 interactors will be silenced or overexpressed to evaluate their functions. Once validated in hypertonic stress, we will seek additional collaborators to investigate SIRT3 signaling and mitochondrial dysregulation of cancer cells at single-cell levels using patient samples.

Our two teams will have regular meetings (every two weeks) to monitor the progress, discuss the results (troubleshooting if necessary), and arrange experiments in the next steps. This close collaboration will integrate our technologies to generate exciting results and clear direction for preparation of a CRF proposal.

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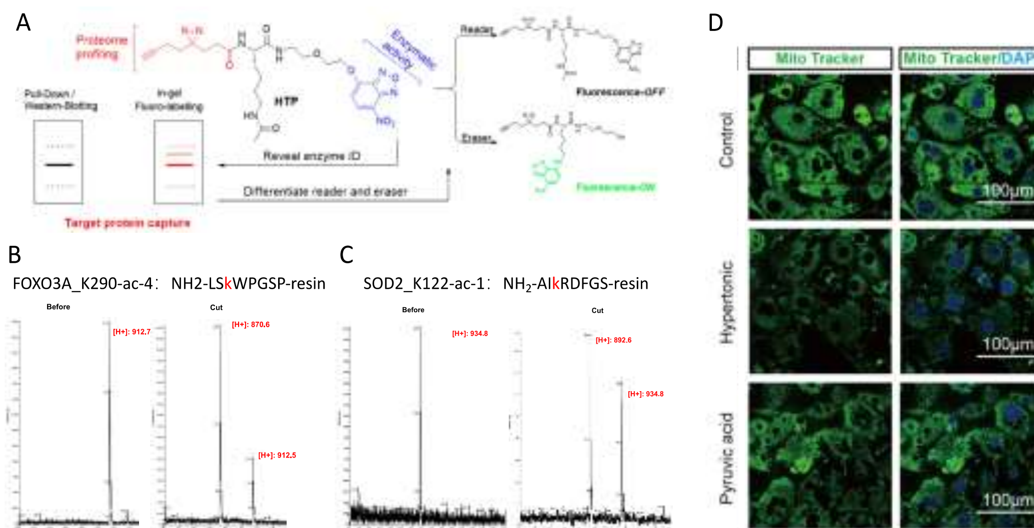

Figure 1. A. Schematic explanation of the probes for the dual-purpose applications: fluorescent reporter of deacetylation activity and photo-crosslink to capture interacting proteins. **B-C.** The Kac of the SIRT3 probes precusors can be effectively removed by purified SIRT3. The probes are based on SIRT3 substrates FOXOA3 (B) and SOD2 (C). **D.** Hypertonic treatment leads to mitochondrial stress, as visualized by the reduction of the signal of MitoTracker that indicates the mitochondria membrane potential. The stress can be relieved by reducing reagents such as pyruvic acid.

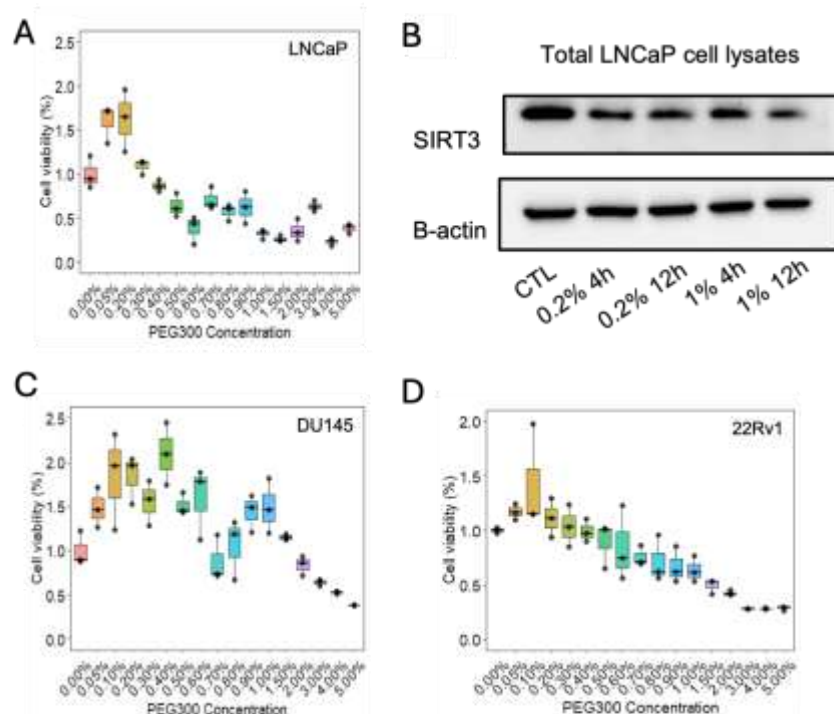

Figure 2. A. MTT assays showed that prostate cancer cell LNCaP have dynamic responses to hypertonic stress of mitochondria. **B.** The hypertonic stress of LNCaP cells leads to downregulation of SIRT3. **(C- D).** DU145 (C) and LNCaP (D) also display similar dynamic responses to hypertonic stress of mitochondria.