Project 1

The hexokinase phosphorylation landscape: new regulation of an "old" enzyme

1- State of the art

Hexoses, particularly glucose, serve as primary carbon and energy sources for most organisms, influencing biosynthetic and regulatory pathways. Despite being the most abundant monosaccharide, extracellular glucose levels fluctuate, requiring cells to adapt for survival. Eukaryotic cells optimize metabolism in response to glucose availability through sensing systems and signal transduction pathways. Glucose catabolism involves multiple enzymatic processes, beginning with glycolysis—one of the most conserved biological pathways. The first glycolytic step is the irreversible phosphorylation of glucose into glucose-6-phosphate by hexokinases/glucokinases, a tightly regulated process ensuring efficient glucose metabolism.

Hexokinase dysfunction in mammals leads to several diseases, including hereditary diabetes, hyperinsulinemia, and the Warburg effect in cancer cells. Beyond glucose phosphorylation, hexokinases contribute to insulin secretion, apoptosis, and longevity. Similar to cancer cells, the yeast *Saccharomyces cerevisiae* favors glycolysis and fermentation over oxidative phosphorylation, even in oxygen presence, due to glucose repression. The yeast hexokinase Hxk2 is crucial in this process, fueling glycolysis and acting as a nuclear transcriptional regulator that represses genes for alternative carbon metabolism. Understanding the molecular mechanisms controlling hexokinase functions is essential for appreciating their physiological and pathological roles.

Among these mechanisms, **hexokinase phosphorylation is particularly important**. In *S. cerevisiae*, glucose-dependent phosphorylation of Hxk2 at serine 15 regulates its transcriptional role in glucose repression by modulating its nucleo-cytoplasmic distribution and interactions with transcription factors. Recent phosphoproteomic studies reveal that Hxk2 undergoes phosphorylation at multiple sites, suggesting intricate regulatory control. However, **the physiological effects of these modifications on hexokinase function and yeast metabolism remain largely unknown**.

This project aims to systematically characterize the role of hexokinase phosphorylation in cellular adaptation to glucose fluctuations. Yeast serves as an ideal model due to its evolutionary conservation and availability of genetic, molecular, and genomic tools for studying glucose signaling and metabolism.

2- Strategy

Unbiased identification of Hxk2 phosphorylation sites: to identified/confirmed Hxk2 phosphorylation sites a Hxk2 phospho-mapping strategy will be followed. After GFP-trap purification followed by proteolytic cleavage, Hxk2 phospho-peptides will be enriched by iMAC and/or TiO2 and sequenced by MS/MS analysis (collaboration with the IBCP proteomic facility, Lyon). The effect of carbon source on Hxk2 phosphorylation will be then addressed via the same strategy after glucose starvation of the cells. This might allow to identify differentially phosphorylated residues in Hxk2 in response to carbon source.

Functional characterization of Hxk2 phosphorylation sites: Each Hxk2 phosphorylated serine, threonine or tyrosine identified will then be mutated to alanine (non-phosphorylatable) an aspartic/glutamic acid (phosphomimetic residues) via a CRISPR/Cas9 approach recently developed in the lab. Each mutant will be then analyzed for hexose-kinase activity by enzymatic assays and glucose dependent growth. The influence of these phosphorylation events on Hxk2 regulatory function will be addressed by analyzing in each mutant 1/ the expression of glucose repressed genes (transcriptional reporter systems) and 2/ the ability of mutated Hxk2 to interact with the transcriptional repressor Mig1 (Co-IP) in response to carbon sources. This might allow to understand if Hxk2 phosphorylation at these different residues promotes or inhibits hexokinase functions. These mutants would be further characterized by a structural approach (in

collaboration with Lionel Ballut, IBCP) to correlate the effect of Hxk2 phosphorylation on its structure and on its activities.

Hxk2 phosphorylation and signaling pathways: It is important to identify the different kinases phosphorylating Hxk2 at the identified residues and what are the signals controlling their activity toward Hxk2. To isolate the kinases phosphorylating Hxk2 a targeted proximity-dependent biotynylation labelling strategy is currently developed in the lab. Our preliminary results indicate that several protein kinases might interact with Hxk2. Theses identified kinases will be tested for Hxk2 phosphorylation by *in vitro* kinases assays and by analyzing the effect of their belonging signaling networks on Hxk2 *in vivo* functions during glucose repression.

3 - Expected Results

By identifying new Hxk2 phosphorylation sites, the belonging kinases and the consequence on hexokinase functions this project will help to better understand the complex mechanisms of Hxk2 regulation necessary for yeast cells to adapt to carbon sources. Regarding the high degree of identity between yeast and mammalian hexokinases, the conservation of theses regulatory mechanisms might be later addressed in mammals, helping to better understand the role of hexokinase in physiological and pathological situation such as diabetes or during the Warburg effect in cancer cells.

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Proposed collaboration within ArchiFun network (not mandatory at this stage):

Proposed list of secondments (not mandatory, but recommended if known already):

Main ArchiFun theme involved:

Mechanisms of cancer onset

Physiology