Phosphoproteomics links glycogen synthase kinase-3 to RNA splicing

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Protein kinases play essential biological roles by phosphorylating a diverse range of signaling molecules, but deciphering their direct physiological targets remains a challenge. A new study by Shinde et al. uses phosphoproteomics to identify glycogen synthase kinase-3 (GSK-3) substrates in mouse embryonic stem cells (mESCs), providing a broad profile of GSK-3 activity and defining a new role for this central kinase in regulating RNA splicing.

A central question in cell biology research is how cell fate decisions are made in response to internal and external cues under physiological and pathological conditions. Cells frequently use protein post-translational modifications such as phosphorylation, ubiquitination, glycosylation, methylation, and acetylation as signal transducers that integrate information gained from environmental stimuli (1) and to activate intracellular signaling cascades that lead to spatially and temporally controlled gene expression. Protein phosphorylation has been extensively studied owing to its broad implications in virtually every cellular process in cells, including cell division, immunity, and cell differentiation (1). However, the identification of genuine substrates for a given kinase in specific cellular context is incredibly challenging: Not only is protein phosphorylation in biological processes highly context-dependent, but the extent to which a particular substrate is phosphorylated is influenced by substrate specificity, availability, and competition in kinase-substrate interaction networks. In this Editors’ Pick, Shinde et al. (2) shed new light on the substrates of one important kinase in their systematic analysis of GSK-3-dependent protein phosphorylation in mESCs using quantitative mass spectrometry. Their data provide a global picture of GSK-3 function that prompts new thinking about substrate motifs, identifies new substrates, and points to unexpected functional space ripe for further investigation.

Like many kinases, GSK-3 participates in several signaling pathways by phosphorylating serine and threonine residues of important signal transduction proteins (e.g. Wnt/β-catenin, insulin/AKT, Hedgehog). GSK-3 dysfunction causes early embryonic lethality (3, 4), and its aberrant hyperactivity is linked to a plethora of human diseases, including obesity, diabetes mellitus, inflammation, and tumorigenesis (5), raising interest in this particular kinase. To date, >100 proteins have been reported to be phosphorylated by GSK-3. GSK-3 substrates generally bear a consensus (S/T)XXX(S/T) sequence and tend to be phosphorylated by other “priming” protein kinases at the C-terminal S/T prior to being phosphorylated by GSK-3 at the first S/T (6). However, because most of these substrates were identified in vitro where absent priming kinases might lead to incomplete profiles or through pharmacological inhibition where off-target inhibition might complicate analysis, whether they are bona fide substrates for GSK-3 in cells requires further investigation (7). Complicating matters further is the fact that GSK has two isoforms, GSK-3a and GSK-3b (5), which are largely but not entirely redundant.

To address this issue, Shinde et al. (2) employed mESCs as a model cell type, as the double knock-out (DKO) of both isoforms can be tolerated (i.e. does not cause any defect in mESC self-renewal). Stable isotope labeling of amino acids in culture-coupled MS analysis identified 89 phosphorylated sites in 65 proteins that displayed reduced phosphorylation in DKO mESCs as compared with WT cells. Interestingly, the majority of GSK-3-dependent phosphosites identified in this study have no (S/T)XXX(S/T) consensus sequence, a departure from the canonical view of GSK-3 substrates. This discrepancy could be due to experimental limitations of prior studies, or, as the authors note, it could reflect the capability of their genetic approach to report on indirect targets. Gene ontology analysis of the putative GSK-3 substrates revealed a significant enrichment in unexpected functional pathways (Fig. 1), whereas retrieval of interacting gene/protein STRING analysis showed that these GSK-3 substrates constitute an interaction network that is associated with alternative splicing (e.g. RBM8A, SRSF9), transcriptional regulation (e.g. c-Jun, PHF6), and cell division (e.g. CDK1, SEPT9).

Although earlier work had suggested roles for GSK-3 related to RNA splicing (8, 9), the newly defined interaction network may imply a larger role in regulating splicing than previously anticipated. To further understand GSK-3’s activity in phosphorylating splicing factors in mESCs, Shinde et al. (2) first performed an in vitro phosphorylation assay, followed by MS analysis to determine phosphosites of proteins involved in RNA splicing. Notably, the authors found that GSK-3b directly phos-
phosphorylated multiple splicing factors (e.g. RBM8A, SRSF9, and PSF) at serine and/or threonine residues. Next, the authors sought to elucidate whether GSK-3 regulates RNA splicing in mESCs, which had not been investigated. To this end, they carried out deep sequencing of poly(A)-selected RNA from WT and DKO mESCs and found a substantial difference in the splicing modes of 188 genes in DKO mESCs as compared with WT cells, including changes in the generation of cassette exons, intron retention, and alternative first exon usage. Because alternative splicing could affect gene expression, the authors further tested whether GSK-3-mediated RNA splicing activities affect mRNA level. However, they found only six of 188 genes showed a consistent change in both RNA splicing and mRNA expression in DKO mESCs, indicating that alternative splicing regulated by GSK-3 in mESCs does not result in dramatic alteration in mature transcripts.

The Wnt-signaling pathway has been shown to promote mESC self-renewal, somatic reprogramming, and mesendodermal differentiation (10). In mESCs, GSK-3 serves as a negative regulator of the Wnt signaling pathway through phosphorylating and targeting β-catenin for ubiquitin-dependent degradation. Inhibition of GSK-3 facilitates the establishment and maintenance of mESC pluripotency. It would be interesting to examine whether the function of GSK-3 in mESC is mediated, at least in part, through phosphorylating the substrates identified in this study. Specifically, it will be interesting to discover whether GSK-3-mediated regulation of alternative splicing is a prerequisite for ESC differentiation toward the mesendodermal lineage and how the two functionally redundant isoforms, GSK-3α and GSK-3β, cooperatively act in this context.

In summary, the study by Shinde et al. (2) not only reinforces the previously established GSK-3 substrates but also significantly expands the list of GSK-3 substrates to functional pathways such as RNA splicing. The use of WT and DKO mESCs for identification of GSK-3 substrates eliminates potential non-specific effects of small molecule inhibitors, which had been frequently utilized in previous studies. The new GSK-3 substrates provide a platform for the further investigation of myriad GSK-3 functions in development and disease pathogenesis (e.g. leukemia and diabetes). Furthermore, although additional studies are still needed to distinguish direct versus indirect targets of GSK-3, results from this study provide unambiguous candidates for GSK-3-dependent phosphorylation, which may serve as benchmarks for the development of new and more specific GSK-3 inhibitors for therapeutic strategies.

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