



### **Kinase-substrate analysis via diaPASEF phosphoproteomics**

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The *S. cerevisiae* (yeast) *ISR1* gene encodes a putative kinase with no specific function ascribed, however over expression of *ISR1* is lethal. To determine the function and substrates of this kinase, we conducted diaPASEF phosphoproteomics experiment in both *ISR1* knockout and *ISR1* expressing cells. From this analysis we quantified 8,879 phosphopeptides. Of these sites, we identify 4 sites of phosphorylation on Gfa1, one of which (S332) was up-regulated by nearly 200-fold in the presence of the *ISR1* kinase. Alanine mutations of this residue, as well as adjacent S/T residues, rescued the lethality associated with *ISR1* over expression. These results suggest that Gfa1 is a bonafide substrate of *ISR1*, and implicates a role for *ISR1* in the hexosamine biosynthesis pathway. In short, we demonstrate the utility of diaPASEF phosphoproteomics to rapidly and confidently determine the substrates and function for uncharacterized kinases.