The continued reliance on agricultural and petrochemical-based methods of production for many high-value compounds threatens future generations with shortages of essential manufacturing materials, organic solvents, biofuels, and pharmaceuticals. The application of synthetic biology to metabolic engineering has worked to address this growing concern by transferring requisite enzymatic pathways from native organisms to standardized chasses and manipulating them to both decrease dependence on non-renewable inputs and increase overall production yield. Despite continued efforts to improve the tunability and consistency of reaction progress within large-scale bioreactors, traditional controller-based methods of optimization remain stymied by excessive variability characteristic of biological systems.

Intellectual Merit: Recent developments in the application of optogenetic tools to metabolic pathways have demonstrated potential in addressing the lack of tunability and irreversibility of traditional chemical-inducer based control schemes. Leveraging the implicit reversibility of optogenetic induction, Milias-Argeitis et al. (2016) developed an automated transcriptional control system to maintain a constant concentration of fluorescent protein as a proof of concept. While the proposed systems can be used to rapidly increase protein production, the tunability of the system is limited by the slow rate at which the proteins degrade. In the context of metabolic applications this delay could contribute to the non-optimal accumulation of toxic intermediates and decrease the applicability of feedback structures, reducing overall fermentation yield. The development of a reversible post-transcriptional control mechanism presents a novel, generalizable solution to this meaningful challenge.

Hypothesis: A reversible, post-translational system for the control of selective protein degradation can be created using existing optogenetic toolkits to rapidly and precisely decrease protein concentration.

Approach: A selective protein degradation tag is conjugated to the coding sequence of a target protein. The tag marks the target protein for degradation via ClpXP, a selective protease comprised of ClpX and ClpP subunits[2]. The reconstitution of these subunits is facilitated through heterodimerization of the cryptochrome Cry2 to the protein CIB1[3]. Sufficiently orthogonal to the green light (535nm) and red light (672nm) utilized for transcriptional control, instances of Cry2 and CIB1 reconstitute in the presence of blue light (470nm) and spontaneously disassociate in its absence[4].

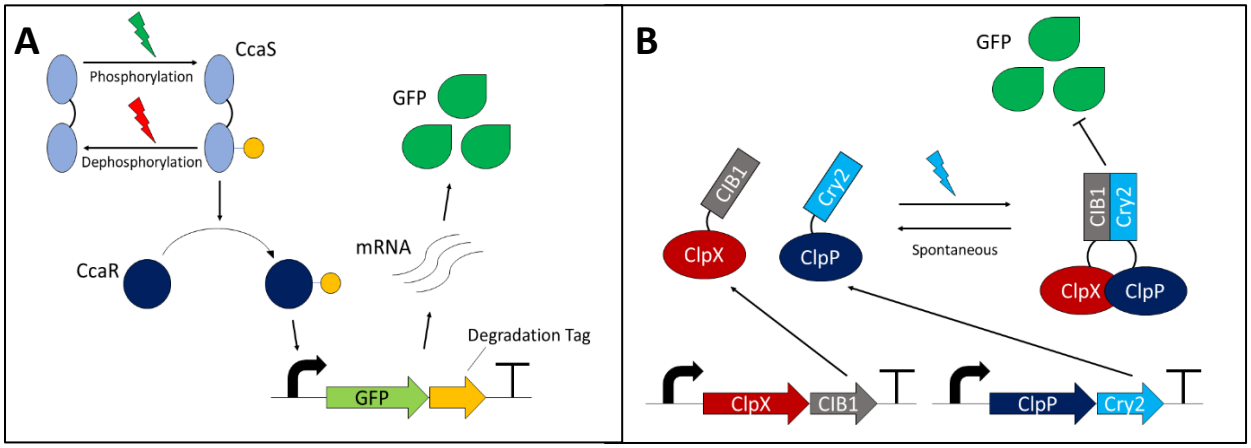

Fig. 1 Genetic circuit for proposed selective degradation scheme. **(A)** Representation of "slow response" transcriptional control used in Milias-Argeitis et al. (2016) updated with a protein degradation tag. **(B)** Representation of "fast response" post-transcriptional control featuring the reconstitution of the ClpXP protease in the presence of blue light to degrade tagged proteins.

Aim 1: Ensure conjugation of CIB1 tag to ClpX subunits has negligible impact on hexamer formation.

The ClpX subunit is itself composed of six $ClpX_a$ subunits. In the context of this project, each $ClpX_a$ subunit would be conjugated to a CIB1 dimerization domain and constitutively produced. Although a small protein, it is critical to ensure that the conjugation of CIB1 to $ClpX_a$ does not interfere with the formation of hexameric ClpX. To achieve this aim, a library of mutant $ClpX_a$ subunits with CIB1 conjugated at different locations will be generated via rational design and screened for their ability to recombine via western blot. Utilizing a non-conjugated ClpP subunit, the functionality of structurally-promising CIB1-$ClpX_a$ mutants will then be evaluated by their enzymatic capacity to degrade tagged fluorescent proteins.

Aim 2: Ensure ClpX/ClpP fusion is negligible in the absence of blue light and reversible following exposure to blue light.

Wild type ClpX and ClpP subunits independently recombine via the formation of hydrogen bonds between key looping peptides on their exteriors. For the system to selectively degrade tagged proteins of interest, it is necessary to minimize any spontaneous recombination and subsequent functionality of conjugated ClpXP in the absence of blue light. Simultaneously, the utility of this light-based system is contingent on the reversible nature of the optogenetic reactions. Once background ClpXP functionality is minimized, it is also possible that the ClpXP complex formed upon initial Cry2/CIB1-mediated binding interactions will remain cohesive and functional despite the dissociation of conjugated light domains. It is therefore necessary to rationally generate and screen mutations within the ClpX/ClpP binding domains capable of both minimizing subunit binding affinities and maintaining enzyme functionality in the presence of blue light.

Broader Impacts and Future Directions: Properly tuned to compatibly function with existing transcriptional control systems, a rapid, light-controlled protein degradation system would serve as a valuable tool in improving the sensitivity of optogenetic feedback systems in industrial fermentation processes. By effectively decreasing system lag, target concentrations of potentially toxic enzymes can be maintained more consistently despite excessive background noise. In the context of metabolic engineering this increase in control has the potential to improve both the speed and yield of fermentations. For those individuals relying on fermentation-based pharmaceuticals for the treatment of disease or fermentation-based biofuels for energy, even minor improvements in fermentation yield could decrease costs and improve accessibility to such essential compounds. In the future, the implementation of optogenetic protein controllers at each step in a metabolic process could serve to drastically improve the tunability and engineering capacity of large-scale fermentation processes.

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