Final Report

Global analysis of two-component signal transduction systems in *Caulobacter crescentus*

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Overview

Our team of investigators from MIT (Michael Laub) and Stanford (Harley McAdams and Lucy Shapiro) conducted a multi-faceted, systematic experimental analysis of the 106 *Caulobacter* two-component signal transduction system proteins (62 histidine kinases and 44 response regulators) to understand how they coordinate cell cycle progression, metabolism, and response to environmental changes. These two-component signaling proteins were characterized at the genetic, biochemical, and genomic levels. The results generated by our laboratories have provided numerous insights into how *Caulobacter* cells sense and respond to a myriad of signals. As nearly all bacteria use two-component signaling for cell regulation, the results from this project help to deepen our general understanding of bacterial signal transduction. The tools and approaches developed can be applied to other bacteria. In particular, work from the Laub laboratory now enables the systematic, rational rewiring of two-component signaling proteins, a major advance that stands to impact synthetic biology and the development of biosensors and other designer molecular circuits.

Below we summarize the results from our work. Each section lists publications and publicly-available resources which result from the work described.

1) Skerker, J.M., Perchuk, B.S., Siryaporn, A., Lubin, E., Ashenberg, O., Goulian, M., Laub, M.T. (2008) "Rewiring the specificity of two-component signal transduction systems" Cell, vol. 133, p. 1043-1054.

Genome sequencing projects have revealed that most organisms contain large expansions of a small number of signaling families. Understanding how cells coordinate the activity of multiple, highly-related signaling pathways to prevent unwanted cross-talk remains a major challenge. Our paper tackled this problem in the context of two-component signal transduction systems, the predominant family of signaling proteins in bacteria. For two-component signaling, the specificity of a kinase-substrate interaction is based on molecular recognition, but the amino acids responsible for the specificity of this recognition process had, prior to this paper, remained elusive and refractory to structure-based approaches. To identify key specificity-determining residues, we examined patterns of amino-acid coevolution in large multiple sequence alignments of cognate kinase-substrate pairs. We then showed that a subset of these coevolving residues is sufficient, when mutated, to completely switch the specificity of a histidine kinase, both *in vitro* and *in vivo*. Our results shed significant new light on the molecular basis of substrate discrimination and the fidelity of signal transduction by an important class of bacterial receptor kinases. Our approach also now enables the rational rewiring of two-component pathways in synthetic signaling circuits. While synthetic biology to date has been focused mainly on the design of transcriptional networks, our work helps pave the way to construction of novel, signal transduction-based circuits. Finally, our success in using analyses of amino-acid coevolution to understand two-component signaling specificity suggests this computational approach will prove useful as a complement, or alternative, to structural studies of other signaling pathways or protein-protein interactions.

2) Biondi, E.G., Reisinger, S.J., Skerker, J.M., Arif, M., Perchuk, B.S., Ryan, K.R., Laub, M.T. (2006) "Regulation of the bacterial cell cycle by an integrated genetic circuit" Nature, vol. 444, p. 899-904.

Although the cell cycle of eukaryotes is understood in molecular detail, the bacterial cell cycle has remained poorly understood. In this paper my lab used a combination of genetic, biochemical, and cell biological assays to map, for the first time, an integrated genetic circuit that can account for cell cycle oscillations in *Caulobacter crescentus*. The *Caulobacter* cell cycle is driven by the periodic rise and fall in activity of the master regulator CtrA. Tight control of CtrA activity results mainly from the combined action of regulated phosphorylation and proteolysis. While some of the factors responsible for temporal control of CtrA activity were known prior to this paper, the connectivity of these factors was largely unknown and at least one key factor remained unidentified. We tackled these challenges by first identifying a long-sought, critical component (called ChpT) of the pathway that controls phosphorylation of CtrA. Then, using phosphotransfer profiling, a new technique developed in my lab (see Skerker et al 2005 below), we demonstrated that ChpT directly phosphorylates both CtrA as well as a second factor, CpdR, which inhibits CtrA proteolysis. ChpT thus serves to

simultaneously phosphorylate and stabilize CtrA. Importantly, we identified the key negative feedback loop involving CtrA that drives cell cycle progression; we showed that CtrA directly stimulates the synthesis of a regulator called DivK which then feeds back to inhibit CtrA by down-regulating the ChpT pathway. Bypassing this negative feedback loop severely disrupts cell cycle progression. In sum, our data led to a coherent model of cell cycle oscillations in *Caulobacter*, incorporating nearly all major regulatory factors described to date and establishing their connectivity. Notably, the topology of the *Caulobacter* circuitry unveiled bears a striking resemblance to that employed by eukaryotes suggesting that evolution may favor this circuit architecture, regardless of the molecules used to implement it.

3) Biondi, E. G., Skerker, J. M., Arif, M., Prasol, M. S., Perchuk, B. S., Laub, M. T. (2006) "A phosphorelay system controls stalk biogenesis during cell cycle progression in *Caulobacter crescentus*" Molecular Microbiology, vol. 59, p. 386-401.

Cell cycle progression in *Caulobacter crescentus* is accompanied by a series of precisely timed morphogenetic events culminating in an asymmetric cell division and the production of two distinct daughter cells, a swarmer cell and a stalked cell. The stalk plays a key role in nutrient acquisition and hence is crucial to the growth and survival of *Caulobacter* in its oligotrophic niche in the wild. Prior to this paper, very little was known about how the timing of stalk biogenesis was regulated and coordinated with the cell cycle. Our work remedied this deficiency by mapping a complex regulatory circuit involving both a transcriptional cascade and a four-tiered phosphorelay that collaborate to control the timing of morphogenesis in *Caulobacter*. We showed that the master cell cycle regulator, CtrA, initiates a transcriptional cascade that leads to synthesis of a newly identified transcription factor, StaR, which in turn triggers stalk biogenesis. Importantly, we also showed that one member of the transcriptional cascade is subject to further regulation by a complex phosphorelay, a common architecture of two-component signaling proteins. The phosphorelay we identified appears to integrate spatial information and ensure that the transcriptional cascade identified is only active in stalked cells. Our work thus produced a satisfying framework for understanding how a critical morphogenetic event is coordinated both in time and in space. The work for this paper further demonstrated the power of combining genetics, biochemistry, and genomics to rapidly map regulatory pathways in *Caulobacter*. This paper also included the further development of our novel technique, called phosphotransfer profiling, for rapidly mapping multicomponent phosphorelays, a technical advance that has since been adopted by other labs studying phosphorylation-based regulation in bacteria.

4) Skerker, J.M., Prasol, M.S., Perchuk, B.S., Biondi, E.G., Laub, M.T. (2005) "Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: a system-level analysis" PLoS Biology, vol. 3, p. 334-353.

Two-component signal transduction systems, comprised of histidine kinases and their cognate response regulators, are the primary means by which bacteria sense and respond to changes in their environment. In this paper, we systematically deleted each of the 106 two-component signaling genes from the *Caulobacter crescentus* genome and showed that 39 of them are essential or important for growth, cell cycle progression, or morphogenesis. Most importantly though, this paper represented a major advance as it introduced a new technique, called phosphotransfer profiling, for the rapid mapping of histidine kinase-response regulator pairs. The technique involves a systematic examination of phosphotransfer *in vitro* from a histidine kinase to a comprehensive set of response regulators. Significantly, we demonstrated that histidine kinases exhibit a large kinetic preference *in vitro* for phosphotransfer to their *in vivo* cognate substrate. This indicated that phosphotransfer profiling could be employed to rapidly map the cognate substrate(s) for any histidine kinase. In this paper, we demonstrated the power of this approach by mapping a new, highly-conserved pathway that is essential for growth in *Caulobacter*. We have subsequently mapped several other critical signaling pathways in *Caulobacter* and the technique has been adopted by other laboratories.

The success of phosphotransfer profiling has two other major implications. First, it suggests there is minimal cross-talk between two-component pathways. Previous reports speculated that histidine kinases were promiscuous with a potential for extensive cross-talk, but our work refuted this notion. Second, our results indicate that the specificity of a two-component pathway is dictated mainly by molecular recognition and does not rely on mechanisms such as scaffolds. This finding established two-component pathways as an excellent system for exploring the molecular basis of transient protein-protein interactions and the coevolution of signaling systems (see Skerker et al 2008 above).

5) Collier, J., and L. Shapiro, 2007 Spatial complexity and control of a bacterial cell cycle. Curr Opin Biotechnol 18: 333-340.

6) Goley, E. E., A. A. Iniesta and L. Shapiro, 2007 Cell cycle regulation in Caulobacter: location, location, location. Journal of Cell Science 120: 3501-3507.

These two papers each reviewed aspects of the importance role that three-dimensional organization of the cell plays in cellular regulation. In particular, protein localization, especially of signal transduction proteins, serves to coordinate cell division, chromosome replication, and cell differentiation.

7) Crosson, S., P. T. McGrath, C. Stephens, H. H. McAdams and L. Shapiro, 2005 Conserved modular design of an oxygen sensory/signaling network with species-specific output. Proc Natl Acad Sci U S A 102: 8018-8023.

This paper reports on the modular organization of an oxygen sensory/signaling network in *Caulobacter*. The network consists of the sensor histidine kinase FixL, it's cognate response regulator FixJ, the transcriptional regulator FixK, and the kinase inhibitor FixT. The core circuit topology of the Fix network is conserved between the *Rhizobia* and *C.*

crescentus. However, in the rhizobial bacteria these proteins form a network that regulates transcription of genes required for symbiotic nitrogen fixation, anaerobic and microaerobic respiration, and hydrogen metabolism under hypoxic conditions. Thus, the Fix network is a conserved sensory/signaling module whose transcriptional output has been adapted to the unique physiology of *C. crescentus* and the nitrogen-fixing rhizobia.

8) McGrath, P. T., A. A. Iniesta, K. R. Ryan, L. Shapiro and H. H. McAdams, 2006 A dynamically localized protease complex and a polar specificity factor control a cell cycle master regulator. Cell 124: 535-547.

9) Iniesta, A. A., P. T. McGrath, A. Reisenauer, H. H. McAdams and L. Shapiro, 2006 A phospho-signaling pathway controls the localization and activity of a protease complex critical for bacterial cell cycle progression. Proc Natl Acad Sci U S A.

10) Iniesta , A. A., and L. Shapiro, 2008 A regulatory feedback loop uses dynamic polar localization and proteolysis to control the Caulobacter cell cycle. Proc Natl Acad Sci U S A: 105; 16602–16607.

These three papers develop a remarkable story of a complex phospho-signaling cascade that acts to simultaneously control the phosphorylation state and the stability of the CtrA master cell cycle regulatory protein. The hybrid histidine kinase CckA phosphorylates the histidine phosphotransferase ChpT, which in turn phosphorylates both CtrA and CpdR. The unphosphorylated state of CpdR localizes the ClpXP protease.to the cell pole. Polar localized ClpXP then proteolyses CtrA. The simultaneous control of phosphorylation and degradation of CtrA provides for rapid removal and re-synthesis of the activated form of CtrA at critical times in the *Caulobacter* cell cycle.

11) Laub, M. T., L. Shapiro and H. H. McAdams, 2007 Systems biology of *Caulobacter*. Annual Review Genetics 41: 429-441.

This review focuses on the top-level regulatory circuitry of the *Caulobacter* cell cycle control system. Genetic components that control the *Caulobacter* cell cycle regulatory system interact with each other and with the panoply of subsystems that implement the cell cycle. Two-component signaling pathways are a central element of the *Caulobacter* cell's control system. *Caulobacter* has 106 two-component signaling genes (62 histidine kinases and 44 response regulators). The sophistication of the genetic regulatory circuits and the elegant integration of temporally controlled transcription and protein synthesis with spatially dynamic phosphosignaling and proteolysis pathways, and epigenetic regulatory mechanisms, form a remarkably robust living system.

12) Shen, X., J. Collier, D. Dill, L. Shapiro, M. Horowitz et al., 2008 Architecture and inherent robustness of a bacterial cell-cycle control system. Proc Natl Acad Sci U S A 105: 11340-11345.

This paper describes a hybrid simulation of the coupled *Caulobacter* cell cycle control system that includes asymmetric cell division and responses to external starvation signals. The model includes the role of the phospho-signaling pathway that controls CtrA

phosphorylation state and stability. The simulation model was shown to replicate mRNA and protein concentration patterns and to be consistent with observed mutant phenotypes. An asynchronous sequential digital circuit model equivalent to this validated simulation model was created. Then formal analysis of this equivalence digital circuit showed that the *Caulobacter* cell cycle control circuitry is robust to intrinsic stochastic variations in reaction rates and nutrient supply.