Insulation Mechanisms of *in vivo* Biomolecular Circuits

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Information transmission in signaling pathways can be substantially altered by 'retroactivity', a biological back-action from the downstream targets, which effectively applies a `load' (1,2,3). It has been theoretically shown that a system can be insulated from retroactivity by using high gain negative feedback. It was hypothesizes, in turn, that this may be implemented by phosphorylation cycles that have sufficiently high amounts of phosphatase (1).

Here, we experimentally demonstrate this hypothesis by assembling a phosphorylation cycle in *E. coli,* in which the amount of phosphatase can be tuned. In our *in vivo* system, the cycle protein is the transcriptional activator NRI, expressed from a constitutive promoter. The kinase (NRII(L16R)) and the phosphatase (NRII(H139N)) are inducible with aTc and IPTG, respectively. Once phosphorylated, NRI~P activates transcription of the reporter sf-*gfp* gene. To study the effect of the load, we placed *glnA* target sites for NRI~P on a high-copy number plasmid. The unphosphorylated NRI protein also binds to the target sites, without any transcriptional activation. Our experimental/modeling study shows that the binding of both unphosphorylated NRI and NRI~P to the DNA sites leads to non-intuitive changes in the dose-response curve of NRI~P (sf-GFP) to the NRII kinase in a system with low phosphatase (low feedback gain). In particular, DNA load increases the steady state values of NRI~P at low levels of kinase while it decreases it at high levels of kinase. Remarkably, induction of a fixed amount of phosphatase quenches the effect of the load on the dose-response curve, effectively insulating the cycle from retroactivity by downstream DNA targets.

Hence, phosphorylation cycles can be insulated from retroactivity and can behave modularly. This justifies further why they are ubiquitous in natural signaling pathways and suggests that these cycles could be potentially used in engineered biomolecular circuits as insulation devices to enforce modular composition.

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(2) Jiang P, Ventura AC, Sontag ED, Merajver SD, Ninfa AJ, Del Vecchio D (2011) Load-induced modulation of signal transduction networks. *Sci Signal* **4:** ra67.
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