Segall-Shapiro *et al.*[3]. Using an iFFL topology (**Fig. 1**), the authors design a repressor to bind to the promoter of the gene of interest. Next, this constitutively expressed repressor sequence is cloned upstream of the promoter of the gene of interest. Since copy number variations affect both the expression of the gene of interest and the repressor, an iFFL can be used to maintain output gene expression levels. For example, if copy number increases, the amount of repressor protein will also increase, which in turn will reduce the expression rate of the gene of interest.

Segall-Shapiro *et al.*[3] establish two requirements for a perfectly balanced iFFL-stabilized system. First, the dynamic range of the repressor must be wide. If this is the case, expression of the target gene will be proportional to the copy number ($c$) and inversely proportional to the repressor levels ($R$), approaching the power law $c/R^n$ where $n$ is the degree of cooperative binding of the repressor to its cognate promoter. If the repressor is not regulated, $R$ will be proportional to the copy number $c$, meaning that the expression levels of the target gene will depend on the copy number according to $c^{1-n}$. Second, if repression is non-cooperative (i.e., $n = 1$), the target gene expression level will be independent of copy number.

The authors show that both conditions are met by transcription-activator-like effector (TALE) repressors expressed in *Escherichia coli*, which function non-cooperatively as monomers and can be readily designed[9] to repress a cognate promoter by binding to a single DNA operator site. To validate their iFFL design, the authors examine expression of a target green fluorescent protein (GFP) gene. They compare *gfp* expression from control (not stabilized) and iFFL-stabilized promoters present at 1 to 100 copies per cell. When *gfp* was expressed from a control promoter on a plasmid, the amount of GFP varied proportionally with the copy number. When *gfp* was present on the chromosome, expression varied with its proximity to the chromosomal ORI. GFP expressed from iFFL-stabilized promoters, however, was measured at near-constant levels for all tested plasmid backbones and chromosome integration loci. Strikingly, not only could an iFFL-stabilized promoter enable copy number variability to be overcome, it also enabled near-constant *gfp* expression levels in different growth media and when the host cells were stressed by a mutation that globally affects RNA stability.

Next, the authors showed that iFFL-stabilized promoters can be used to control more than one gene in an operon. They validated their approach by porting a three-gene metabolic pathway for prodeoxyviolacein from a plasmid to the chromosome. Importantly, they showed that similar yields were obtained whether the pathway was present on a multicopy plasmid or integrated into the chromosome without any additional genetic tinkering.

Owing to their architecture, iFFL-stabilized promoters (**Fig. 1**) could be applied to overcome a wide set of perturbations that symmetrically affect the expression rate of the repressor and the gene of interest. For example, if a decrease in free ribosomes reduces the translation rate of a target gene, then a similar decrease in the TALE repressor translation rate will ease repression and increase the transcription rate of the target gene, thereby counterbalancing, to some extent, the reduction in translation.

One limitation of iFFLs is that they cannot overcome perturbations such as ribosomal traffic jams, anti-sense RNA inhibition, or enzymatic mRNA and protein degradation, which act asymmetrically on the TALE repressor and the target gene (**Fig. 1**). Asymmetric effects are likely to become more problematic as engineered networks and pathways grow and become more complex. Another limitation in longer pathways arises from retroactivity[10]. Specifically, if multiple iFFL-stabilized promoters are present in a gene pathway or network, the repressor binding sites would sequester, and in turn decrease, the pool of available TALE repressors. This is likely to impose a limit on the number of different promoters that can be simultaneously stabilized using a single TALE.

Balancing the expression of genes present at different copy numbers has been a long-standing goal for synthetic biologists and metabolic engineers alike. iFFL-stabilized promoters can be used to solve this conundrum and enable the design of pathways and networks that are robust to copy number variations and, to some extent, changes in growth conditions or other stressors. Incorporation of iFFLs may allow portability of designed pathways, not only between plasmids and chromosomes in the same species, but also perhaps among different species.

COMPETING INTERESTS
The authors declare no competing interests.

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