Harnessing the central dogma for stringent multi-level control of gene expression

F. Veronica Greco[1], Claire S. Grierson[1,2] and Thomas E. Gorochowski[1,2,*]

[1] School of Biological Sciences, University of Bristol, Tyndall Avenue, Bristol BS8 1TQ, UK

[2] BrisSynBio, University of Bristol, Tyndall Avenue, Bristol BS8 1TQ, UK

* Correspondence should be addressed to T.E.G. (thomas.gorochowski@bristol.ac.uk)

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Abstract

Strictly controlled inducible gene expression is crucial when engineering biological systems where even tiny amounts of a protein have a large impact on function or host cell viability. In these cases, leaky protein production must be avoided at all costs. Here, we demonstrate how the central dogma offers a simple way to effectively address this challenge. By simultaneously regulating both transcriptional and translational levels, we show how basal expression of an inducible system can be reduced to virtually undetectable levels, with minimal impact on the maximum induced expression rate achieved. Using this approach, we create several stringent expression systems displaying >1000-fold change in their output after induction. Furthermore, we find that multi-level regulation is able to supress transcriptional noise and create a digital-like switch when transitioning between ‘on’ and ‘off’ states. This work provides foundational knowledge and a genetic toolkit of parts to create multi-level gene expression controllers for those working with toxic genes or requiring precise regulation and propagation of cellular signals. It also demonstrates the value of exploring more complex and diverse regulatory designs for synthetic biology.

Introduction

Since the development of the first inducible systems in the early 1980s (de Boer et al., 1983), the ability to dynamically control gene expression through the use of small molecules (Gallivan, 2007), light (Baumschlager et al., 2017; Castillo-Hair et al., 2019), and other signals (Sen et al., 2017) has revolutionized biotechnology. From controlling shifts between cell growth and protein production stages during large-scale fermentations (Sivashanmugam et al., 2009), to the detailed characterization of genetic parts and circuitry (Olson et al., 2014), the control of gene expression underpins a huge variety of applications. However, while switching expression of a gene 'on' or 'off' is conceptually simple, it is rare for genes to have such discrete states or ever be completely silenced. Stochastic effects (Elowitz et al., 2002; Raj and van Oudenaarden, 2008) and leaky expression are widespread and potentially important for adaptation in natural systems but can wreak havoc in engineered systems where genes are toxic to a host or responses are highly sensitive and easily triggered by mistake (Rosano and Ceccarelli, 2014; Süel et al., 2006).

Early systems for controlling gene expression relied on the repurposing of native regulatory components such as transcription factors. One of the most widely used is the P_{tac} system (de Boer et al., 1983). This consists of a constitutively expressed LacI repressor that can form dimers and tetramers to strongly bind operator sites within a P_{tac} promoter sequence and sterically block initiation of RNA polymerase (RNAP). LacI is sensitive to Isopropyl β-d-1-thiogalactopyranoside (IPTG) and at high concentrations, the DNA binding activity of LacI is abolished. This lifts repression of P_{tac} and leads to strong transcription of genes regulated by this promoter. While in most cases such systems offer strong repression, because such regulatory systems focus on a single step during protein synthesis (i.e. transcription), they are vulnerable to fluctuations in regulator production and the stochastic nature of biochemical reactions during gene expression (Raj and van Oudenaarden, 2008).

Over the past decade, synthetic biologists have developed more advanced methods to control gene expression. These include engineered regulators based on DNA binding proteins such as zinc fingers (Khalil et al., 2012), TALENs (Deng et al., 2014) and CRISPRi (Gilbert et al., 2014), RNA-RNA interactions (Bartoli et al., 2020; Chappell et al., 2017; Green et al., 2014), post-transcriptional/translational processes such as RNA and protein degradation (Cameron and Collins, 2014), as well as using directed evolution to optimize existing inducible systems (Meyer et al., 2019). This offers a wealth of options to more strictly regulate gene expression through the coupling of multiple forms of regulation (e.g. affecting both transcription and translation of a gene) to reduce unwanted expression and improve the robustness of a system to component failure. However, few examples of such multi-level regulation have been implemented to date (Westbrook and Lucks, 2017). This has resulted in

52 an unclear picture of how best stringent multi-level control can be achieved and the trade-offs
53 that exist between performance, regulatory complexity, and cellular burden when
54 designing these systems.

55 Here, we address this problem by systematically studying the combined use of
56 transcriptional and translational regulators to stringently control protein expression. Using a
57 combination of mathematical modelling and a combinatorial genetic assembly method, we are
58 able to design, build and test a variety of synthetic multi-level controllers (MLCs) and elucidate
59 the relative performance of each. These controllers all implement a coherent type 1 feed-
60 forward loop (C1-FFL) regulatory motif (**Figure 1A**) that is commonly found in natural genetic
61 systems and is known to enable more stringent control of an output but is rarely used when
62 designing new expression systems (Mangan and Alon, 2003). We show how MLCs offer
63 advantages for many applications spanning the stringent control of protein expression to the
64 accurate propagation of information in a cell (Beal, 2015; Nielsen et al., 2016) and
65 demonstrate how applying modern synthetic biology tools to even simple regulatory systems
66 can offer paths towards the precise and reliable control of biological systems.

67

68 Results

69 *Stringent control of gene expression by harnessing the central dogma*

70 In most synthetic genetic circuits, control of gene expression is achieved through the use of a
71 single type of regulation (**Figure 1A**), with control of transcription predominantly used. While
72 this type of single-level control (SLC; **Figure 1B**) is often sufficient for many applications, the
73 central dogma naturally lends itself to more stringent multi-level regulation where both
74 transcription and translation are controlled simultaneously by different types of regulator (e.g.
75 via transcription factors and RNA-based translational switches). Such multi-level control (MLC;
76 **Figure 1C**) can be generalised by a genetic design that consists of an *L1* gene encoding a
77 level 1 transcriptional regulator with cognate promoter P_{L1}, and an *L2* gene encoding a level 2
78 translational regulator. Both *L2* and the gene of interest (GOI) are separately transcribed by
79 P_{L1} promoters and the product of *L2* activates translation of the GOI transcript. This MLC
80 encapsulates a coherent type 1 feed forward loop (C1-FFL) in which both *L1* and *L2* are
81 necessary for production of the GOI.

82 To explore the possible benefits of this regulatory motif, we developed mathematical
83 models to capture how the rate of production of a GOI varied in response to differing
84 concentrations of an input inducer for both the SLC and MLC designs (**Supplementary Note**
85 **1**; **Supplementary Data 1**). We generated steady state response functions by simulating the
86 models using biologically realistic parameters (**Supplementary Table 1**) over a range of
87 different input IPTG concentrations. As expected, the output production rate displayed a

sigmoidal shape with both controllers reaching near identical maximum rates at high input IPTG concentrations (**Figure 1D**). The main difference was that the MLC design displayed a 50-fold lower output than the direct controller at low IPTG concentrations, leading to significantly reduced basal expression when no input was present (**Figure 1D**). This caused the MLC design to have both an increased dynamic range and fold-change between 'off' and 'on' states when compared to the SLC design.

We also simulated the output protein production rate for both models when exposed to a range of dynamic inputs. These included delta functions, as well as pulse and step inputs (**Figure 1E**). Simulations showed that both types of controller displayed virtually identical output responses for both the pulse and step inputs, with only a small reduction in output expression rate for the MLC that matched its lower basal expression level. However, significant differences were observed in the responses to the delta function input. While the SLC led to moderate sized pulses in output, the MLC design fully suppressed all output activity with only tiny fluctuations in the output expression rate observed. The behaviour of the MLC arose from the need for both *L1* and *L2* to be expressed to sufficiently high levels for expression of the GOI to be triggered. The short pulses of expression caused by the delta function input were insufficient to cause this switch and allowed the MLC to effectively filter out these transient events in its input.

The ability to filter out rapid fluctuations is particularly important for stringent control in systems where input promoters exhibit high levels of intrinsic noise. In such scenarios, protein levels can vary significantly across a population of cells (Elowitz et al., 2002) due to the often bursty nature of gene transcription. This is commonly seen for weak promoters where intrinsic noise dominates. Rather than the activity of a weak promoter being uniformly low, it instead displays short bursts of strong activity separated by long periods of inactivity (Elowitz et al., 2002; Golding et al., 2005). Across a population this averages out to a low overall expression level, but large variability is present between cells. As seen for the delta function inputs, such input profiles driving the SLC will lead to large fluctuations in the output. However, because intrinsic promoter noise is specific to an individual promoter and uncorrelated between multiple identical versions of a promoter within a construct, the MLC design which contains two copies of the input promoter P_{L1} should find that a burst of expression from one P_{L1} promoter is highly unlikely to occur at the same time as a burst from the other. Therefore, the MLC will suppress noise in the output.

To test this hypothesis, we generated accurate time-series promoter activity profiles based on a two-state model (Golding et al., 2005) where the mean length of time a promoter was in an 'on' active and 'off' silent state (Δt_{ON} and Δt_{OFF}, respectively) were $\langle \Delta t_{ON} \rangle$ = 6 min and $\langle \Delta t_{OFF} \rangle$ = 37 min. These values were taken from previous experimental measurements in *E. coli* (Golding et al., 2005). We also set the activity of the P_{L1} promoter when in an 'on' state to

125 a biologically realistic 0.25 RNAP/min. Independent time-series were generated for each P_{L1}
126 promoter in the MLC and only one of these was used for the SLC where only a single P_{L1}
127 promoter is present. These profiles were then fed into our existing dynamic models and the
128 responses of the systems simulated. We found that the output production rate for the SLC
129 saw large increases, especially where the input consisted of longer bursts of activity or several
130 bursts in short succession (**Figure 1F**). In comparison, the MLC fully supressed all output
131 production making it an excellent filter of intrinsic promoter noise.
132
133 ***A genetic template to explore multi-level gene regulation***
134 There are many ways that an MLC could be implemented biologically. Furthermore, when
135 implementing such a controller it is often necessary to switch the input that is used and internal
136 regulators such that multiple controllers can be used simultaneously within the same cell. To
137 meet these requirements, we developed an 8-part genetic template and toolkit of parts to allow
138 for the rapid combinatorial assembly of MLCs (**Figure 2A**). The design enables both single
139 and multi-level regulation, has the option to introduce protein tags for further post-translational
140 control of the GOI (e.g. through signalled degradation) and is structured to minimise the
141 chance for transcriptional readthrough to cause unwanted expression of the component parts.
142 The toolkit comprises of 8 types of part plasmid (pA–pH) and a backbone plasmid (pMLC-
143 BB1) in which the final MLC design is inserted (**Supplementary Data 2**). Assembly is
144 performed using a standard one-pot Golden Gate reaction with individual blocks designed to
145 use 4 bp overhangs with minimal cross reactivity to ensure the correct and efficient ligation of
146 parts (Woodruff et al., 2016). Furthermore, rapid screening of successful inserts is enabled by
147 the drop-out of an orange fluorescent protein (*ofp*) expression unit (Engler et al., 2009)
148 (**Supplementary Note 2**).
149 Using this toolkit, we aimed to compare the *in vivo* behaviours of different SLC and
150 MLC designs with a focus on the different mechanisms that could be used for *L2* control and
151 the affect these might have on overall performance. For the SLC design we chose the widely
152 used P_{tac} system introduced earlier (**Figure 2C**). To simplify comparisons, we also used the
153 P_{tac} system for *L1* control in all the MLC designs and combined it with three different RNA-
154 based *L2* regulators. These included a toehold switch (THS; **Figure 2D**) (Green et al., 2014,
155 2017), a small transcription activating RNA (STAR; **Figure 2E**) (Chappell et al., 2015), and a
156 dual control system (DC; **Figure 2F**) (Westbrook and Lucks, 2017).
157 The THS regulator encodes a structural component followed by a ribosome binding
158 site (RBS) that is used to drive translation of the GOI (**Figure 2D**). The structural region is
159 designed to form a strong hairpin loop that when transcribed hinders the ability for ribosomes
160 to bind the RBS, and thus inhibits translation. Translation is activated by expression of a
161 complementary small RNA (sRNA) trigger that hybridizes to a short unstructured region of the

THS which causes a breakdown in its secondary structure. This conformational change allows ribosomes to bind the RBS and translation of the GOI to proceed. THSs were selected because they offer strong repression of translation, can be designed computationally, and large libraries of designs exist with minimal crosstalk when used together (Green et al., 2014, 2017).

Unlike the THS, the STAR regulator works at a transcriptional level. The STAR's target is placed before an RBS in the 5' untranslated region (UTR) of the GOI (**Figure 2E**). This forms an intrinsic terminator when transcribed and inhibits GOI expression. Activation is achieved by expression of the STAR RNA, which interacts with the target, prevents terminator formation and thus allows for expression of the downstream GOI. Similar to THSs, STARs have been shown to offer strong repression and there exist large libraries of orthogonal variants (Chappell et al., 2015, 2017).

Finally, the DC regulator combines both transcriptional and translational control by modifying the pT181 attenuator (Westbrook and Lucks, 2017). The DC target is placed in the 5'-UTR of the GOI and encodes an intrinsic terminator that includes the RBS (**Figure 2F**). When transcribed, the intrinsic terminator not only halts transcription, but also represses translation by causing the RBS to form a strong RNA secondary structure making it inaccessible to the ribosome. Activation is achieved by expression of a STAR, which interacts with the target, both preventing terminator formation and causing a conformational change in the RNA structure that makes the RBS accessible for translation initiation. The DC regulator was chosen due to this combined regulatory action which has been shown to produce strong repression (Westbrook and Lucks, 2017). However, to date, only a single of these regulators has been created, limiting future applications.

DNA encoding parts for each of these regulatory systems was synthesised and our toolkit used to assemble the SLC and three MLC designs. Superfolder green fluorescent protein (*gfp*) was chosen as the GOI to allow for the measurement of output expression in single cells using flow cytometry.

Performance comparison of the controllers

To characterise the performances of the controllers, we transformed *Escherichia coli* cells with each construct and measured GFP fluorescence using flow cytometry for 'off' and 'on' input states. As P_{tac} was used as an input for all the designs, this corresponded to growing the cells in either 0 or 1 mM IPTG, respectively (**Methods**). Data from these experiments was then used to calculate the dynamic range and fold change in output GFP fluorescence (**Table 1**).

We found a clear separation between output states for all designs with little variation between biological replicates (**Figures 3A**). All MLCs (THS, STAR and DC) reached higher expression levels than the P_{tac} SLC, and the THS and DC designs achieved large >1000-fold

199 changes between output states. Notably, while the STAR design reached a much higher 'on'
200 state than the P_{tac} design, the STARs high levels of basal (leaky) expression when no input
201 was present resulted in a 43% lower fold change (**Table 1**).

202 A challenge when calculating these measures (especially fold change) is the ability to
203 accurately quantify very low level of output GFP fluorescence, which are near or identical to
204 the autofluorescence of the cells. To better understand this aspect, we measured the GFP
205 autofluorescence of untransformed *E. coli* cells, performing 11 biological replicates to estimate
206 a fluorescence distribution that could be used as an approximate detection limit. Overlaying
207 the average and standard deviation of the cell autofluorescence onto our results (**Figure 3A**,
208 dashed line and grey shaded region), we found that the 'off' states for the P_{tac}, THS and DC
209 designs all fell within this region and very close to the average suggesting they have virtually
210 no leaky expression at all.

211 While comparisons of average expression levels between 'on' and 'off' states are
212 useful, they are not able to capture the role of cell-to-cell variability inherent in all gene
213 expression (Raj and van Oudenaarden, 2008). Such variation is crucial when assessing the
214 performance of stringent expression systems because even though average output states
215 might be sufficiently separated to be distinguished, cell-to-cell variation across a population
216 can lead to overlaps in the output distributions. Cells falling in this overlap are impossible to
217 classify resulting in some cells with an undetermined state. Engineers have developed
218 measures to help characterise the strength and quality of a signal (i.e. the ability to distinguish
219 'on' and 'off' output states) with the Signal to Noise Ratio (SNR) commonly used in other fields
220 such as electronics. SNR has also recently been adapted for use when studying engineered
221 genetic systems making it easier to understand how the quality of signals in a circuit are
222 maintained or degraded as they pass through various genetic devices (Beal, 2015).

223 Using the flow cytometry distributions, we calculated the SNR for each controller in
224 decibel (dB) units (**Table 1**; **Methods**). We found that the P_{tac} SLC performed worst with a low
225 SNR of 0.2 dB, corresponding to a signal barely larger than the noise. This was evident for
226 the flow cytometry distributions where a sizable overlap in the 'on' and 'off' states was seen
227 (**Figure 3B**). All MLCs performed better with the THS achieving an SNR >10 dB. This
228 improved performance was also evident from the flow cytometry data with clear gaps of
229 varying sizes between the 'on' and 'off' output distributions (**Figure 3B**).

230

231 ***Burden of controllers on the host cell***

232 There is a growing awareness of the importance of considering the burden that engineered
233 genetic parts and circuits place on their host cell (Boo et al., 2019). The introduction of a
234 genetic construct that sequesters large quantities of shared cellular resources like ribosomes
235 or heavily impact core metabolic fluxes can lead to reduced growth rates and trigger stress

236 responses that impact the function of engineered genetic parts (Ceroni et al., 2015, 2018;
237 Gorochowski et al., 2014, 2016; Gyorgy et al., 2015; Weiße et al., 2015). When designing the
238 MLCs, we purposefully selected RNA-based regulators as they impose a small metabolic
239 burden on the cell (Kelly et al., 2018). However, to experimentally verify this was the case, we
240 generated growth curves for all SLC and MLC designs (**Supplementary Figure 1**). Because
241 the metabolic demands of the controllers would vary based on the concentration of inducer
242 present (because varying levels of sRNA or STAR are produced), cells were exposed to 4
243 different IPTG concentrations (0, 0.1, 1, 10 mM) spanning the 'off' and 'on' states of the
244 controllers.

245 From these growth curves, we estimated the doubling time during the exponential
246 growth phase (**Methods**). We found that the SLC and all MLCs displayed similar doubling
247 times of ~70 min (**Figure 3C**). Furthermore, we saw a slight decrease in the doubling times of
248 all controllers as the IPTG concentration increased. This trend is counterintuitive given that an
249 increasing IPTG concentrations will cause expression of the GOI and any *L2* regulators,
250 increasing the burden on the cell. However, it is known that IPTG can have unexpected effects
251 on cell physiology (Malakar and Venkatesh, 2012) and cause changes in plasmid stability
252 (Gomes et al., 2020), which could lead to reduced overall burden due to fewer copies of the
253 controller plasmid or more efficient utilisation of available nutrients by the cell.

254 We also measured the lag time after inoculation into fresh media before the cells
255 entered exponential growth (**Methods**). We found differences between many of the controllers
256 with a lag time of ~165 min for the P_{tac} and THS designs, a shorter lag time of 88 min for the
257 DC design, and a significantly longer lag time of 373 min for the STAR design (**Figure 3D**).
258 Closer inspection of the growth curves showed that the DC design had a consistently higher
259 initial cell density (optical density at 600 nm of 0.07 compared to 0.04 for the THS design),
260 which could account for the shorter lag phase (**Supplementary Figure 1**). For the STAR
261 design the elongated lag phase coincided with a consistently longer additional time of ~100
262 min to reach saturation of the culture.

263 To better understand if the extended lag phase of the STAR-based MLC was a general
264 feature to be expected when using this type of regulator, we rebuilt this construct using a
265 different STAR ($STAR_2$) that had an identical initial 72 bp sequence, but unique 10 bp
266 sequence at its 3'-end (**Supplementary Table 2**). As we would expect for such a similar
267 design, testing of the $STAR_2$ construct showed similar performance to the initial STAR design
268 with a good dynamic range and similar leaky expression in its output (**Table 1**;
269 **Supplementary Figure 2**; **Methods**). However, unlike the original, the $STAR_2$ design
270 displayed a lag phase (161 min) and doubling time (72 min) that closely matched the other
271 MLCs. This suggests that long lag times observed for the original STAR design were likely

272 due to some highly specific and uncharacterised off-target interactions with endogenous
273 cellular processes and not due to a general feature of the STAR's regulatory mechanism.
274
275 ***Digital-like transitions and suppression of weak input signals***
276 Our previous modelling of the MLCs showed that in addition to improved performance in 'on'
277 and 'off' states, the addition of the *L2* regulator also altered the response function, causing a
278 sharper transition from an 'off' to 'on' state due to the lower basal expression, and an ability to
279 supress low level noise in the input (**Figure 1D, E**).
280 To assess if these features were present, we generated response functions of the
281 controllers by growing the cells in varying concentrations of input inducer and measuring
282 steady state output GFP fluorescence. The sharpness of the transition is captured by the
283 cooperativity of Hill function fits to this data. We found that in comparison to the P_{tac} SLC, both
284 the THS and STAR MLCs saw more than a doubling in its value from 3.4 to more than 7, while
285 the DC design maintained an identical value (**Table 1**). High cooperativities correspond to a
286 very sharp step-like transition between 'on' and 'off' states that is clearly evident from the
287 response function curves (**Figure 4A**). The high non-linearity in the response functions of the
288 THS and STAR MLCs is potentially useful for information processing tasks. In particular,
289 implementing digital logic within cells requires clear 'on' and 'off' states and limited chance for
290 signals to reside at intermediate states. Sharp transitions in the response function ensure that
291 there is less room for an input to fall at an intermediate point during the transition, ensuring an
292 'on' or 'off' state is always given. Furthermore, a high non-linearity can also be exploited to
293 generate bimodality. For example, if a noisy input is positioned to span the transition point in
294 the response function, a population of cells will have large groups of cells in 'on' and 'off'
295 states, with much fewer in intermediate states because of the sharp transition and small
296 probability of falling in this small region.
297 To quantify the ability of each MLC to supress low level input noise, we further
298 analysed the response functions. A difficulty when comparing the response functions is the
299 large differences observed in the dynamic range of each design. Given that the promoter
300 driving transcription for each MLC is identical (P_{tac}), this discrepancy comes from differing *gfp*
301 translation rates controlled by the associated ribosome binding site. These do differ in
302 sequence and strength for each design and in some cases are specific and integral to the
303 RNA regulator's function. Therefore, to allow for comparisons, we normalised the output of
304 each MLC to its maximum output and used data from the P_{tac} SLC to estimate the input activity
305 of the P_{tac} promoter used in each controller. If no secondary regulation was present (as in the
306 SLC), then we would expect the normalised input and output to follow a straight line where
307 one equals the other (see Ptac design in **Figure 4B**). However, if the secondary regulation

308 supresses the input P_{tac} activity then a lower normalised output to input will be seen, and
309 conversely, an amplification of the input will lead to a higher normalised output to input.
310 Using this approach, we assessed the responses of each MLC and found that all
311 caused a suppression of low levels of input promoter activity, while an amplification of higher
312 input activities. This effect was most prominent for the THS and STAR designs, with both able
313 to ensure controller output is maintained below 1% even when the input promoter reaches
314 3.5% activity (**Figure 4B**, insert). These results confirm the findings of our modelling and
315 demonstrate the potential for using MLCs to filter out unwanted input activity in noisy
316 environments.
317

318 ## Discussion

319 In this work we have shown how multi-level control of gene expression offers a means to more
320 stringently regulate gene expression. By harnessing the multi-step process of transcription
321 and translation that underpins the central dogma of biology and simultaneously regulating both
322 processes in response to an input signal, we demonstrate through modelling (**Figure 1**) and
323 experiments (**Figures 2** and **3**) how inducible expression systems can be created with virtually
324 undetectable leaky expression when in an 'off' state, while also maintaining high expression
325 rates once induced. Furthermore, we have shown that multi-level regulation creates a more
326 digital-like switch when transitioning between 'off' and 'on' states and supresses low-level
327 transcriptional noise (**Figure 4**), both of which are valuable properties when developing
328 genetic systems for information processing or when highly toxic products or excitable systems
329 act as downstream products.
330 Our top MLC design, which makes use of a THS for *L2* regulation, achieved >2000-
331 fold change in output upon induction and displayed a 10 dB SNR (**Table 1**) making it one of
332 the most tightly controlled and high-performance induction systems built to date. Furthermore,
333 the flexibility of our modular genetic toolkit for assembling new multi-level controllers (**Figure**
334 **2**), and the availability of many other THSs, makes it easy to develop additional orthogonal
335 MLCs that could be used in parallel within the same cell. It is worth noting that the underlying
336 P_{tac} promoter that the THS MLC uses, achieved only a 93-fold change and 0.2 dB SNR when
337 used alone as an SLC. Therefore, employing the multi-level regulatory approach outlined in
338 this work could offer a means to greatly improve the performance of many existing low-
339 performance transcriptional sensors, without any need to modify the transcription factors or
340 promoter sequences making up these devices.
341 With the improvements we see when employing multi-level regulation, it is likely no
342 coincidence that small interfering RNAs (siRNAs) are also widely used by bacteria to refine
343 the regulation of many endogenous processes (Gottesman, 2005; Storz et al., 2004; Waters

344 and Storz, 2009). RNAs are perfectly tailored for this task, imposing a small metabolic burden
345 and offering a fast response. In this work, we selected synthetic RNA-based regulators that
346 function through RNA-RNA hybridisation alone. While this reduces our dependencies on other
347 cellular machinery and makes them easier to transfer between strains/organisms, it is known
348 that many endogenous siRNA regulators employ protein chaperones such as Hfq to increase
349 their binding affinity to targets and strengthen their regulatory effect (Soper et al., 2010). It
350 would be interesting to explore the use of synthetic regulators that make use of these
351 chaperones (Kelly et al., 2018) or exploit recent advances in the RNA part design (Chappell
352 et al., 2017) to see whether further improvements in performance are possible.

353 The stringent regulation of our controllers is achieved by incorporating a C1-FFL
354 regulatory motif that is known to be evolutionarily selected in many natural and engineered
355 systems (Milo et al., 2002) and can be used to implement many useful functionalities (Mangan
356 and Alon, 2003). More recent work has also demonstrated the importance of interconnections
357 and clustering of many motifs in coordinating more complex behaviours (Gorochowski et al.,
358 2018; S. Dunn et al., 2019). While this work focused on demonstrating that transcriptional and
359 translational regulation can fit neatly into a C1-FFL structure, an intriguing future direction
360 would be to explore how these higher-level structures (e.g. motif clusters or higher-level
361 network structures) might be implemented using the approaches outlined in this work to aid
362 the coordination of multiple interrelated processes in parallel.

363 This study started with the goal of more stringently controlling gene expression.
364 However, through the design of our MLCs it became evident that the more intricate regulatory
365 designs we built had many other benefits. Synthetic biology to date has often focused on
366 simplifying complexity and reducing systems to their minimal parts. Our findings indicate that
367 complementary studies exploring the complexification of synthetic regulatory systems might
368 also reap rewards allowing us to more efficiently exploit the capabilities of biology by
369 combining many diverse processes and parts in unison. The genetic toolkit presented here
370 offers a starting point for such studies focused on the fundamental processes of transcription
371 and translation.

372

373 ## Methods

374 ### *Strains, media and chemicals*

375 All cloning and characterization of genetic constructs was performed using *Escherichia coli*
376 strain DH10-β (Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- ϕ80dlacZΔM15
377 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC) (New England
378 Biolabs, C3019I). Cells were grown in DH10-β outgrowth medium (New England Biolabs,
379 B9035S) for transformation, LB broth (Sigma-Aldrich, L3522) for general propagation, and M9

380 minimal media supplemented with glucose (6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl,
381 0.5 g/L NaCl (Sigma-Aldrich, M6030), 0.34 g/L thiamine hydrochloride (Sigma T4625), 0.4%
382 D-glucose (Sigma-Aldrich, G7528), 0.2% casamino acids (Acros, AC61204-5000), 2 mM
383 $MgSO_4$ (Acros, 213115000), and 0.1 mM $CaCl_2$ (Sigma-Aldrich, C8106)) for characterization
384 experiments. Antibiotic selection was performed using 100 µg/mL ampicillin (Sigma-Aldrich,
385 A9518) and 50 µg/mL kanamycin (Sigma-Aldrich, K1637). Induction of the expression systems
386 was performed using varying concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG)
387 (Sigma-Aldrich, I6758).
388
389 ***Assembly of controllers***
390 All part plasmids were either directly synthesised (GeneArt, Thermo Fisher Scientific) or
391 assembled as complementary single-stranded DNA oligos annealed together. Controllers
392 consisting of 8-parts (pA–pH) plus a backbone (pMLC-BB1) were assembled using a standard
393 Golden-Gate cloning method (**Figure 2B**) (Engler et al., 2009). Briefly, for each assembly, we
394 started from the 18.5 ng of required part plasmids (pA–pH) and 18.5 ng of the backbone
395 (pMLC-BB1) to be added to a 5 µL Golden-Gate reaction. The standard manufacturer's
396 reaction conditions were used, but at a quarter of their normal volume (New England Biolabs,
397 E1601). 2 µL of this reaction mix was then used to transform 12.5 µL of chemically competent
398 DH10-β cells (New England Biolabs, C3019) for further experiments. All assembled constructs
399 were sequence verified by Sanger sequencing (Eurofins Genomics). Annotated sequences of
400 all part and backbone plasmids and assembled controllers are provided in GenBank format in
401 **Supplementary Data 2**.
402
403 ***Characterisation experiments***
404 Single colonies of cells transformed with an appropriate genetic construct were inoculated in
405 200 µL M9 media supplemented with glucose and kanamycin for selection in a 96-well
406 microtiter plate (Thermo Fisher Scientific, 249952). Cultures were grown for 14 hours in a
407 shaking incubator (Stuart, S1505) at 37 °C and 1250 rpm. Following this, the cultures were
408 diluted 3:40 (15 µL in 185 µL) in M9 media supplemented with glucose, kanamycin for
409 selection and IPTG for induction in a new 96-well microtiter plate and grown for a further 4
410 hours under the same conditions. Finally, the cultures were further diluted 1:10 (10 µL into 90
411 µL) in phosphate-buffered saline (PBS) (Gibco,18912-014) containing 2 mg/mL kanamycin to
412 halt protein translation. These samples were incubated at room temperature for 1 hour to allow
413 for full maturation of GFP before flow cytometry was performed.
414
415 ***Flow cytometry***

416 Measurements of GFP fluorescence in single cells was performed using an Acea Biosciences
417 NovoCyte 3000 flow cytometer equipped with a NovoSampler to allow for automated collection
418 of samples from a 96-well microtiter plate. Data collection was performed using the
419 NovoExpress software. Cells were excited using a 488 nm laser and GFP fluorescence
420 measurements taken using a 530 nm detector. At least 10^6 events were captured per sample.
421 In addition, to enable conversion of GFP fluorescence into calibrated MEFL units (Castillo-
422 Hair et al., 2016) a single well per plate contained 15 µL of 8-peak Rainbow Calibration
423 Particles (Spherotech, RCP-30-5A) diluted into 200 µL PBS. Automated gating of events and
424 conversion of GFP fluorescence into MEFL units was performed using the forward (FSC) and
425 side scatter (SSC) channels and the FlowCal Python package version 1.2.2 with default
426 parameters (Castillo-Hair et al., 2016). To correct for the GFP autofluorescence of cells, *E.*
427 *coli* DH10-β cells containing no genetic construct were grown in identical conditions. An
428 average measurement of GFP fluorescence in MEFL units from three biological replicates of
429 these cells was then subtracted from fluorescence measurements of cells containing our
430 genetic constructs to correct for cell autofluorescence.
431
432 ***Plate reader measurements***
433 Single colonies of cells transformed with an appropriate genetic construct were inoculated in
434 200 µL M9 media supplemented with glucose and kanamycin for selection in a 96-well
435 microtiter plate (Thermo Fisher Scientific, 249952). Cultures were grown for 4 hours in a
436 shaking incubator (Stuart, S1505) at 37 °C and 1250 rpm. Following this, the cultures were
437 diluted 3:40 (15 µL in 185 µL) in M9 media supplemented with glucose, kanamycin for
438 selection and IPTG for induction in a 96-well 190 µm clear base imaging microplate (4titude,
439 Vision Plate™, 4ti-0223). This spectrophotometric assay was performed using a BioTek
440 Synergy Neo2 plate reader at 37°C. Optical density at 600 nm (OD_{600}) was measured every
441 10 min over a 16-hour period. OD_{600} measurements were also taken from samples of M9
442 medium supplemented with glucose containing no cells to allow for quantification of media
443 autofluorescence. Shaking was automated for the all the length of the experiment. Data
444 collection was performed using the Gen5 version 3.04 software. For each time point, media
445 autofluorescence was subtracted from the sample measurement.
446
447 ***Signal to noise ratio***
448 The signal to noise ratio (SNR) in decibel (dB) units was calculated from the flow cytometry
449 GFP fluorescence distributions using the equation (Beal, 2015)
450 $$\mathrm{SNR_{dB}} = 20 \cdot \log_{10} \frac{|\log_{10}(\mu_{\mathrm{ON}}/\mu_{\mathrm{OFF}})|}{2 \cdot \log_{10}(\sigma)}. \quad (8)$$

Here, μ_{ON} and μ_{OFF} are the geometric means of distributions for the ON and OFF states, respectively, and σ is the geometric standard deviation of the distribution for the OFF state. OFF and ON states correspond to cells grown in 0 and 1 mM IPTG, respectively.

Data analysis and numerical simulation

Data analysis was performed using Python version 3.7.4 and the NumPy version 1.17.4, SciPy version 1.3.1, Pandas version 1.0.3, FlowCal version 1.2.2, and Matplotlib version 3.1.1 libraries. ODE models were simulated using the odeint function of the SciPy.integrate Python package version 1.1 with default parameters. Steady-state response functions of the controllers were calculated by fitting median GFP fluorescence values from the flow cytometry distributions for a range of input IPTG concentrations to the following Hill function

$$y = y_{\min} + (y_{\max} - y_{\min})\frac{x^n}{K^n + x^n}. \quad (9)$$

Here, y is the output GFP fluorescence in MEFL units, y_{min} and y_{max} are the minimum and maximum output GFP fluorescence in MEFL units, respectively, K is the input IPTG concentration at which the output is half-maximal, n is the Hill coefficient, and x is the input IPTG concentration. Fitting of the experimental data was performed using non-linear least squares and the curve_fit function from the SciPy.integrate package version 1.1. Genetic diagrams were generated using DNAplotlib version 1.0 (Bartoli et al., 2018; Der et al., 2017) and figures were composed using Omnigraffle version 7.15 and Affinity Designer version 1.8.3.

Data availability

Python scripts simulating the ODE models of the direct and multi-level controllers be found in **Supplementary Data 1**. Annotated sequences for all plasmids in GenBank format are available in **Supplementary Data 2**. All plasmids are available from Addgene.

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Author Contributions

T.E.G. conceived the study. V.G. performed all experiments. T.E.G. developed and simulated the mathematical models. V.G. and T.E.G. analysed the data. T.E.G. and C.S.G. supervised the work. All authors helped to write the manuscript.

Competing Interests

The authors declare no competing interests.

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605 ## Tables

606 **Table 1: Performance summary of the single- and multi-level controllers[a]**

Controller	Type[b]	Dynamic range[c] (10^3 MEFL)	Fold change[c]	Cooperativity[d], *n*	Signal to Noise Ratio (dB)
P_{tac}	SLC	0.9	93	3.4	0.2
THS	MLC	65.5	2166	7.3	10.1
STAR	MLC	4.6	53	9.3	4.7
$STAR_2$	MLC	3.4	37	7.7	4.5
DC	MLC	10.4	1030	3.4	7.1

607 a. All values are averages calculated from three biological replicates.
608 b. SLC refers to 'single-level controller' and MLC refers to 'multi-level controller'.
609 c. Calculated between 'on' and 'off' states for cells grown in 0 and 1 mM IPTG, respectively, and
610 given in calibrated molecules of equivalent fluorescein (MEFL) units.
611 d. From the Hill function fitting of the steady state response functions (**Figure 4A**; **Supplementary**
612 **Figure 2**).

Figures and captions

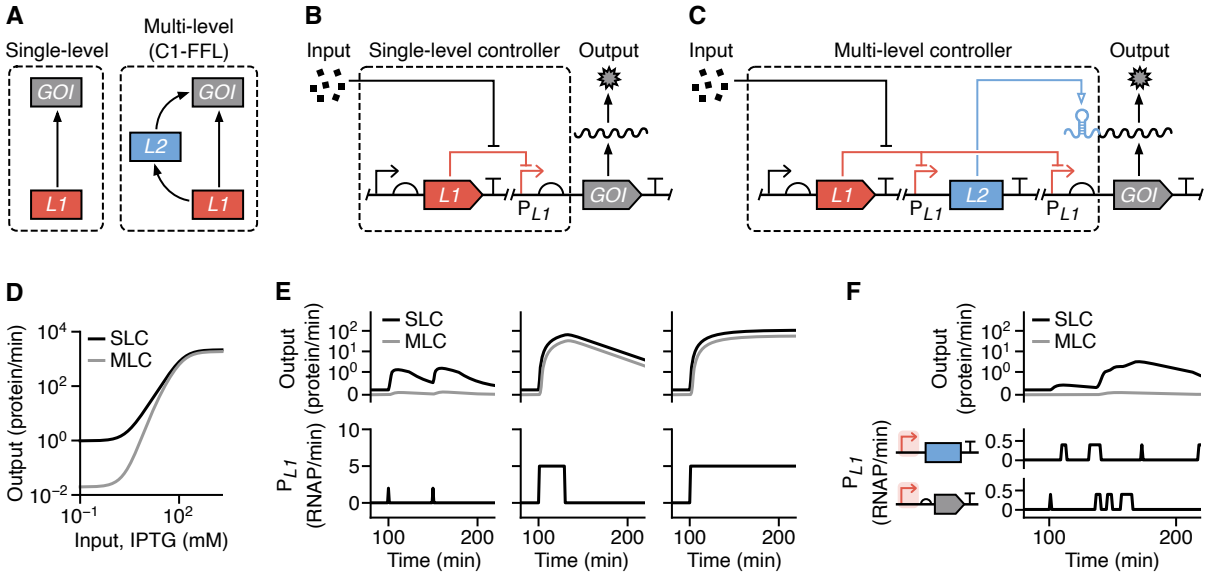

Figure 1: Stringent control of protein expression through multi-level gene regulation. (**A**) Two possible regulatory schemes to control the expression of a gene of interest (GOI): 1. control using a single regulator (*L1*), and 2. multi-level control using two separate regulators (*L1* and *L2*) connected in the form of a coherent type 1 feed-forward loop (C1-FFL). (**B**) Schematic of a genetic implementation of a single-level controller (SLC) that uses only transcriptional (red lines) regulation. An input (e.g. small molecule) modulates activity of the P_{L1} promoter and production of the GOI. (**C**) Schematic of a genetic implementation of a multi-level controller (MLC) that uses both transcriptional (red lines) and translational (blue line) regulation. An input (e.g. small molecule) modulates activity of the two P_{L1} promoters and an internal *L2* regulator activates the translation of GOI transcripts to finally produce the output protein. (**D**) Steady state response functions from mathematical models of the SLC and MLC. (**E**) Dynamic model simulations of the SLC and MLC and their response to different forms of temporal input (left to right): short pulses (P_{L1} activity = 2 RNAP/min for 1 min at 100 min and 150 min), square wave (P_{L1} activity = 5 RNAP/min from 100–130 min), and a step function (P_{L1} activity = 5 RNAP/min from 100 min onwards). The activity of both P_{L1} promoters in the MLC is considered identical. (**F**) Dynamic model simulations of the SLC and MLC showing suppression of intrinsic promoter noise by the MLC. The two identical P_{L1} promoters for the *L2* regulator and GOI are separately driven by independent and biologically realistic bursty transcriptional activity profiles (**Methods**).

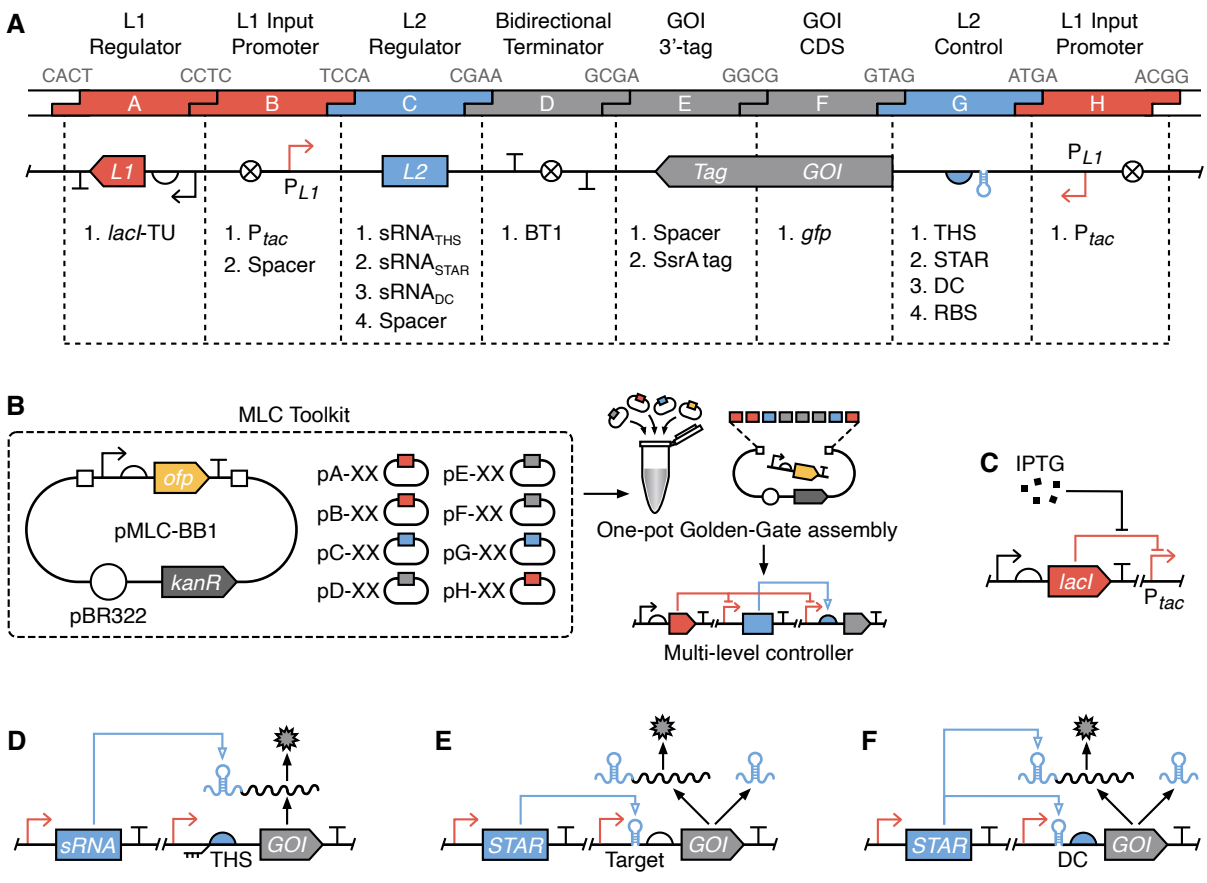

Figure 2: Combinatorial assembly of gene expression controllers. (**A**) Summary of the 8-part genetic template used to allow for systematic exploration of direct and multi-level gene regulation. The 4 bp overhangs used for Golden-Gate assembly are shown in grey at their respective junctions. Available genetic elements are listed below each corresponding part type (A–H). (**B**) The MLC toolkit contains a set of plasmids that can be combined using Golden-Gate assembly to create a variety of direct and multi-level controllers (**Supplementary Figure 3**). (**C**) The *lacI* transcription factor responsive to IPTG used for level 1 (*L1*) transcriptional regulatory control. (**D**) Toehold switch (THS) translational regulator used for level 2 (*L2*) control. (**E**) Small transcription activating RNA (STAR) transcriptional regulator used for *L2* control. (**F**) Dual control (DC) transcriptional and translational regulator used for *L2* control.

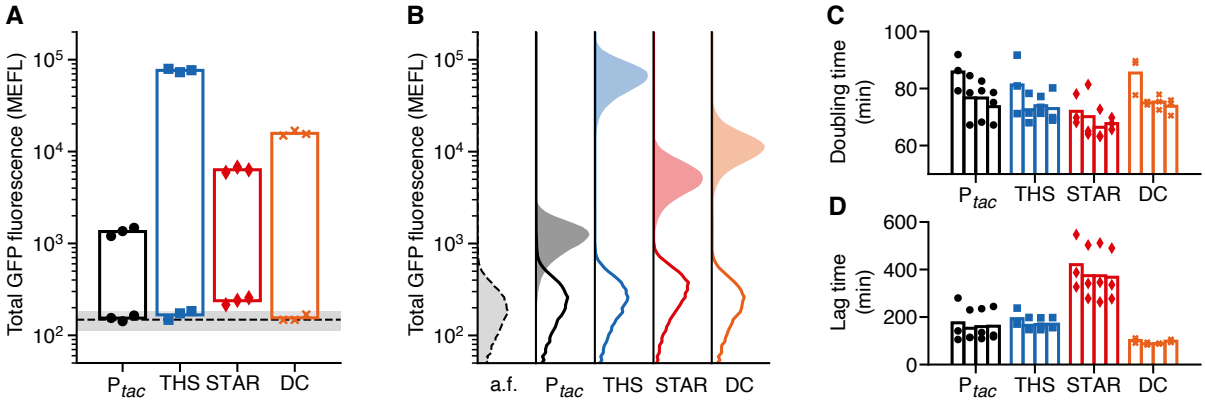

Figure 3: Performance comparison of single- and multi-level controllers. (**A**) Total GFP fluorescence for 'off' and 'on' input states (0 and 1 mM IPTG, respectively). Points show the three biological replicates for each controller and condition (black circles, P_{tac}; blue squares, THS; red diamonds, STAR; orange crosses, DC). Black dashed line denotes the mean fluorescence of cell autofluorescence (a.f.) controls containing no plasmid with grey shaded region showing ± 1 standard deviation of 11 biological replicates. Fluorescence given in calibrated molecules of equivalent fluorescein (MEFL) units. (**B**) Flow cytometry distributions of total GFP fluorescence for 'off' (line) and 'on' (shaded) input states. Cell autofluorescence (a.f.) controls containing no controller are shown by black dashed line and light grey filled distributions. (**C**) Doubling time of cells harbouring direct and multi-level controllers for varying concentrations of IPTG (bars left to right for each design: 0, 0.1, 1, 10 mM IPTG). (**D**) Lag time calculated as the time to reach an OD_{600} = 0.15 after inoculation of cells harbouring controllers for varying concentrations of IPTG (bars left to right for each design: 0, 0.1, 1, 10 mM IPTG).

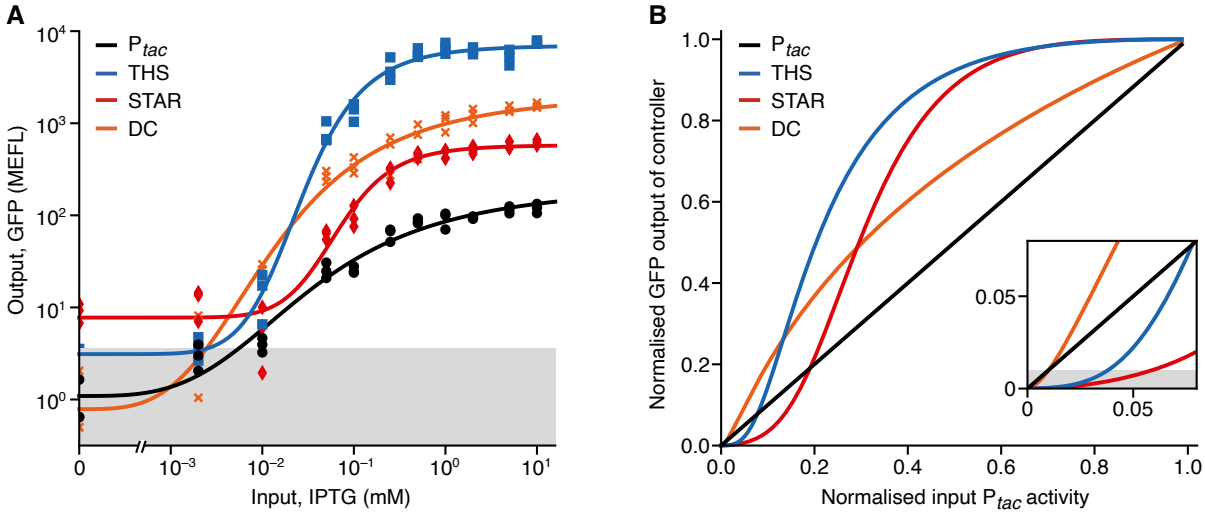

Figure 4: Response functions of single- and multi-level controllers. (**A**) Steady state response functions of the controllers showing output GFP fluorescence (corrected for cell autofluorescence) for varying input IPTG concentrations (0, 0.002, 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, 5, 10 mM). Points show the three biological replicates for each controller and condition (black circles, P_{tac}; blue squares, THS; red diamonds, STAR; orange crosses, DC). Grey shaded region shows the standard deviation of cellular GFP autofluorescence from 11 biological replicates. (**B**) Comparison of how normalised GFP output (as a fraction of the maximum GFP fluorescence) varies in response to changes in the normalised transcriptional activity of P_{tac} (as a fraction of its maximum activity). Multi-level regulation can lead to the suppression or amplification of the output GFP production rate compared to direct transcriptional regulation (i.e. a specific multi-level controller's line falls below or above the diagonal, respectively). Insert shows zoomed area and grey shaded region denotes a GFP output level of 1% for the controller.