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# 'Deadman' and 'Passcode' microbial kill switches for bacterial containment

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**Biocontainment systems that couple environmental sensing with circuit-based control of cell viability could be used to prevent escape of genetically modified microbes into the environment. Here we present two engineered safeguard systems known as the 'Deadman' and 'Passcode' kill switches. The Deadman kill switch uses unbalanced reciprocal transcriptional repression to couple a specific input signal with cell survival. The Passcode kill switch uses a similar two-layered transcription design and incorporates hybrid LacI-GalR family transcription factors to provide diverse and complex environmental inputs to control circuit function. These synthetic gene circuits efficiently kill *Escherichia coli* and can be readily reprogrammed to change their environmental inputs, regulatory architecture and killing mechanism.**



With the advent of synthetic biology, genetically modified microorganisms are being increasingly used for biomedical, industrial and environmental applications[1–6]. Deployment of these engineered microbes in large scales and open environments calls for the development of safe and secure means to restrain their proliferation. Pioneering biocontainment systems used metabolic auxotrophy in which target cells could only grow in the presence of an exogenously supplied metabolite[7,8], and the recent creation of an *E. coli* strain with an altered genetic code enabled production of synthetic auxotrophy strains that require an exogenous supply of non-natural amino acids for cell survival[9,10]. Traditional metabolic auxotrophy strains are hampered by the potential for inadvertent complementation by cross-feeding or by the presence of the metabolite in heterogenous environments, and synthetic auxotrophy systems rely on extensive genome-wide engineering that may be impractical for many industrial production and biotherapeutic microbes. Furthermore, they are intrinsically difficult to reprogram for different environmental conditions, potentially limiting their application. An alternative approach to biocontainment is to use gene circuits to maintain essential gene expression or block toxin gene expression under the assigned biocontainment conditions[7,11–14]. Upon loss of the biocontainment signal, the circuit blocks essential gene expression or induces toxin gene expression to kill the cell. These circuits offer the promise of complex environmental signal integration but are hindered by a relative lack of programmable environment sensors to enable their use under nonlaboratory conditions[15].

Here we design and construct two programmable biocontainment circuits in *E. coli*: a Deadman kill switch that uses a transcription-based monostable toggle design to provide rapid and robust target cell killing and a Passcode circuit that uses hybrid LacI-GalR family transcription factors (TFs) to construct complex environmental requirements for cell survival. We use a tripartite strategy of TF protein engineering to detect diverse signals, robust circuit design to provide signal processing and redundant toxin-induced and protease-mediated cell killing mechanisms. The resulting biocontainment systems are modular, flexible and extensible and should prove useful across many industrial and biotherapeutic applications.

## RESULTS

### Deadman circuit development

We developed the Deadman kill switch to serve as a passively activated biocontainment system for engineered microbes. Similar to pioneering biocontainment systems in *E. coli*[12] and *Pseudomonas putida*[16], the Deadman circuit uses a small molecule–binding TF to produce a 'survival' state in which repression of toxin production is linked to the presence of a specific environmental signal. Upon loss of the environmental signal, the circuit switches to the 'death' state, in which derepressed toxin production kills the cell. To increase the robustness of these biocontainment states, the Deadman circuit uses a genetic 'toggle switch' architecture in which reciprocal repression by the LacI and TetR transcription factors form transcription states that are maintained by the circuit's linked feedback loops[17,18] (**Supplementary Results**, **Supplementary Fig. 1**). To create a circuit in which the death state is dominant in the absence of the survival signal, we altered the ribosome binding site (RBS) strengths of LacI and TetR to favor TetR expression in a single-copy plasmid (**Supplementary Fig. 2** and Online Methods). In the resulting monostable circuit, the presence of the TetR inhibitor anhydrotetracycline (ATc) is required to maintain the circuit in the subordinate $LacI^+$ survival state (**Supplementary Fig. 3**). Incorporation of toxin genes into the $TetR^+$ state creates a kill switch where the presence of ATc is required to block toxin expression and cell death.

We included additional palindromic LacI operator sites in the toxin gene promoter to minimize leaky toxin expression[19] and introduced a transcriptional terminator upstream of the promoter to insulate the gene from spurious transcription (**Supplementary Fig. 4**). To accelerate the circuit's switching dynamics, we fused to the C terminus of LacI a degradation tag that is specifically recognized by *mf*-Lon[20], a heterologous protease under control of a LacI-dependent promoter (**Supplementary Fig. 5a**). Upon removal of ATc, TetR repression of *lacI* allows expression of *mf*-Lon, which targets LacI for degradation to create a positive feedback loop that accelerates the switch to the $TetR^+$ state (**Supplementary Fig. 5b**). Notably, single-cell analysis of these circuits by flow cytometry showed a monomodal distribution of cells

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in the LacI+ and TetR+ state, demonstrating stable circuit expression across the cell population (**Supplementary Fig. 5c**).

## Deadman kill switch characterization

To identify an efficient mechanism to kill the host cells upon circuit activation, we tested several toxins that directly damage the host cell's DNA or RNA. We chose to test the endonuclease EcoRI[21], DNA gyrase inhibitor CcdB[22] and endoribonuclease MazF[23] genes because they are well characterized, are native to *E. coli* and provide a range of killing mechanisms. The toxin genes were independently incorporated into the Deadman circuit, and a range of RBS strengths were tested for each toxin to optimize cell death upon circuit activation[24] (**Supplementary Fig. 6**). Upon removal of ATc, the toxins produced 3–5 logs of killing within 6 h, as measured by colony-forming units (CFUs) (**Fig. 1a**). To increase the robustness of the circuit and provide an independent method of circuit-dependent cell death, we used *mf*-Lon protease to not only degrade LacI but also target essential proteins for degradation (**Fig. 1b**). We attached the *mf*-Lon degradation tag pdt#1 to the 3′ end of five essential genes whose protein products are particularly sensitive to *mf*-Lon degradation[20], and we then measured cell viability following removal of ATc (**Fig. 1b**). Among the tested essential gene targets, the peptidoglycan biosynthesis gene *murC* provided the strongest and fastest cell death phenotype (survival ratio $< 1 \times 10^{-4}$ within 6 h).

To determine whether the toxin- and *mf*-Lon–mediated killing mechanisms produce synergistic effects, we created Deadman circuits containing each of the toxins in combination with the *mf*-Lon–MurC targeting module (**Fig. 1c**). In each instance, the combinatorial approach provided more effective biocontainment and, in particular, coordinated EcoRI expression and *mf*-Lon–mediated MurC degradation resulted in cell killing below the limit of detection (survival ratio $< 1 \times 10^{-7}$) 6 h after removal of ATc (**Fig. 1c**). Furthermore, the Deadman circuit's design provides an additional fail-safe mechanism that bypasses the circuit's sensor system to directly activate toxin expression to cause cell death. Direct derepression of the subordinate TF, in this case derepression of LacI with isopropyl β-D-1-thiogalactopyranoside (IPTG), activates toxin production and cell death irrespective of the presence of the programmed survival signal (**Fig. 2**).

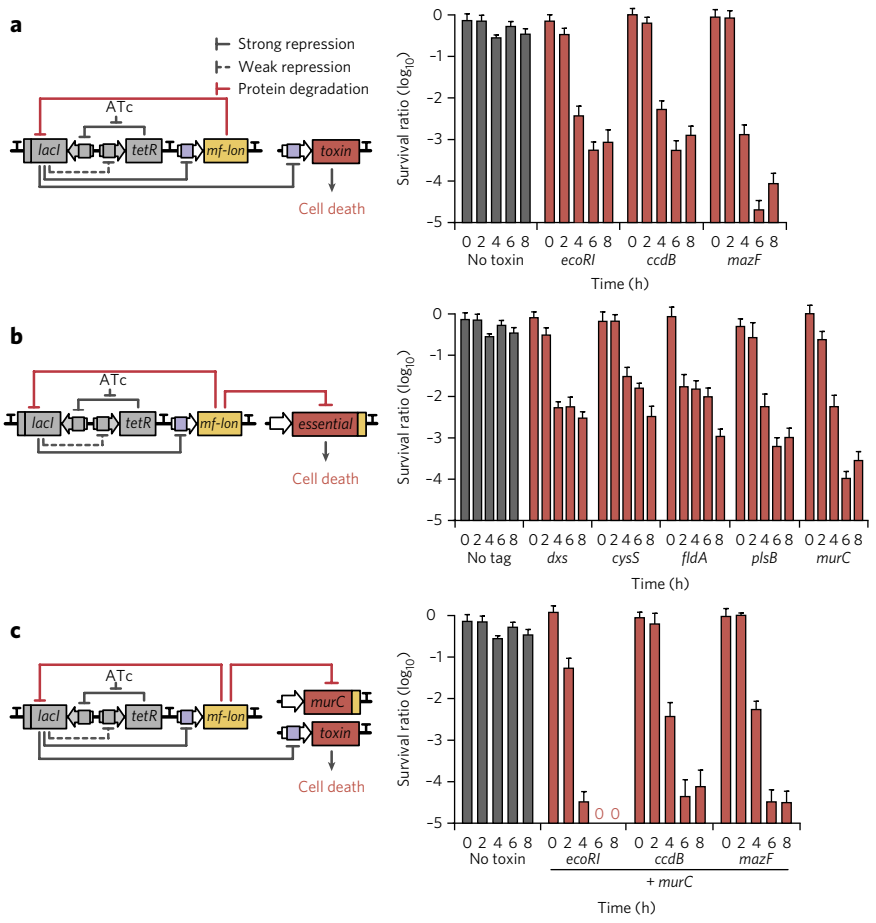


**Figure 1 | Deadman kill switch.** (**a**) Deadman circuit control of toxin gene expression. Cell viability was measured by CFU count following removal of the survival signal (ATc) and is displayed as a ratio of cells without ATc to cells with ATc at each time point. (**b**) Deadman circuit control of targeted essential protein degradation. Inclusion of the *mf*-Lon–specific pdt#1 tag on the specified essential gene causes *mf*-Lon–mediated degradation of the essential protein upon Deadman circuit activation. (**c**) Combined control of toxin expression and targeted essential protein degradation increases Deadman-induced cell death. In particular, targeted MurC degradation and EcoRI expression reduced cell viability to below the limit of detection ($<1 \times 10^{-7}$) after 6 h (indicated by a '0'). All data points represent mean ± s.d. of three biological replicates.

## Hybrid transcription factor design

To extend the versatility and modularity of this system, we built a second circuit, called the Passcode circuit, that uses hybrid LacI-GalR family TFs to expand the range and complexity of environmental signals used to define biocontainment conditions. This survival 'passcode' can be easily reprogrammed to restrict cell growth to a new environment or to limit knowledge of the growth conditions to authorized personnel. To build hybrid TFs, we first identified the boundaries of the environmental sensing modules (ESMs) and DNA recognition modules (DRMs) found in LacI-GalR family members (**Supplementary Figs. 7–11**). As in previous studies[25,26], we generated hybrid TFs that use the small-molecule input defined by the hybrid's ESM to regulate the promoter defined by the hybrid's DRM (**Fig. 3a** and **Supplementary Fig. 12**).

To construct the hybrid TFs, we used the cellobiose-responsive from *Thermobifida fusca* and the galactose-responsive CelR, GalR and IPTG-responsive LacI from *E. coli*. We fused the ESMs from CelR and GalR to the DRM of LacI to generate the hybrid TFs CelR-LacI and GalR-LacI. To test their functionality, we used these hybrid TFs or native LacI to control GFP expression from a promoter containing *lacO* operator sites recognized by the LacI DRM. The hybrid TFs allowed strong GFP expression upon exposure to the small-molecule input defined by their ESM and showed almost no response to the other inputs (**Supplementary Figs. 11b** and **12b**). Furthermore, we fused the LacI, GalR and CelR ESMs to the DRM of ScrR from *Klebsiella pneumoniae* and used the resulting hybrid TFs to regulate a promoter containing *scrO* operator sites. As predicted from their design, these hybrid TFs only respond to the input defined by their ESM (**Supplementary Figs. 11b** and **12c**), although it is notable that the GalR ESM shows distinct inhibition by high levels of IPTG, as previously reported[27] (**Supplementary Fig. 13**). The DRMs used in these hybrid TFs provided similar specificity, as they regulated promoters containing their cognate operator sites but not other LacI family operator sites (**Supplementary Fig. 14**). As in

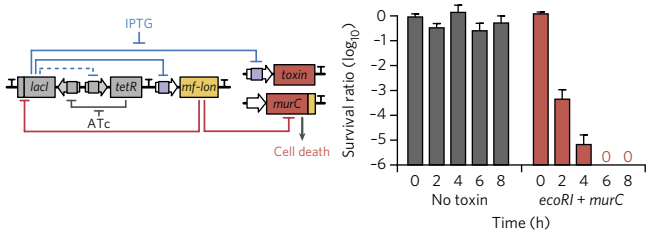


**Figure 2 | The fail-safe mechanism for Deadman circuit activation.** To demonstrate active control over host cell viability, cells grown under survival conditions (with ATc) were exposed to 1 mM IPTG to directly induce EcoRI and *mf*-Lon expression. Cell viability was measured by CFU count and is displayed as a ratio of cell survival with and without IPTG at each time point. Data points represent the mean ± s.d. of three biological replicates.

previous work[27], we found that coexpression of hybrid TFs containing the same DRM could be used to regulate a single promoter, creating an AND logic gate function (**Supplementary Fig. 15**).

## Development of the Passcode kill switches

We used these hybrid TFs to create a series of Passcode circuits that contain a single transcriptional architecture but respond to distinct combinations of environmental inputs to control gene expression and cell survival. The Passcode circuits contain the toxin output module under control of a TF (hybrid C) whose expression is controlled by an AND gate formed by two TFs (hybrid A and hybrid B) (**Fig. 3a**). This serial arrangement, made possible by the orthogonality of the hybrid DRMs and ESMs, creates the condition that both of the inducers recognized by hybrid A and hybrid B (inputs a and b, respectively) must be present to allow expression of hybrid C to repress toxin expression. Loss of input a or input b or the presence of input c allows toxin expression, causing cell death.

To test the functionality and modularity of this circuit architecture, we created three versions of the Passcode circuit that respond to different combinations of input signals to control output expression (**Fig. 3a**). For example, in one Passcode circuit (**Fig. 3b**), we used GalR-LacI (A) and CelR-LacI (B) to control expression of LacI-ScrR (C), which in turn represses toxin expression. In this circuit, loss of galactose (input a) or cellobiose (input b) allows GalR-LacI or CelR-LacI to bind the *lacO* operator, blocking LacI-ScrR expression and thereby enabling toxin expression and causing cell death. Any exposure to IPTG (input c) releases LacI-ScrR repression of toxin expression, thereby killing the cell as well. Notably, the passcode combinations for cell survival and cell death can be reprogrammed simply by rearranging the ESMs of the three TFs to rewire the connections between the environmental sensing and transcriptional regulation.

These Passcode circuits were first evaluated with GFP as the output module in all eight combinations of the three environmental inputs. All three circuits allowed high-level GFP expression in all conditions except that designated by the desired three-input combination (**Supplementary Fig. 16**), and single-cell fluorescence showed a monomodal population distribution under all conditions (**Supplementary Fig. 16c**). GFP was then replaced with the EcoRI and *mf*-Lon–MurC toxin modules described for the Deadman switch above (**Fig. 3a**), and toxin expression levels were optimized by testing a range of calculated RBS strengths[24] (**Supplementary Fig. 17**). Hybrid C, which directly controls toxin expression in the circuit, was also engineered in the same manner to optimize circuit performance (Online Methods). Each kill switch circuit was tested in *E. coli* using eight combinations of input signals, and cell survival was measured by CFU count at multiple time points (**Supplementary Fig. 18**). Only circuits that received the proper survival code allowed the host cells to survive (**Fig. 3b**). Furthermore, inclusion of both the EcoRI and *mf*-Lon toxin modules in the Passcode circuit caused the cell survival ratio to drop below $1 \times 10^{-6}$ for all nonpasscode conditions.

## Long-term circuit stability

To measure the long-term stability and robustness of the Passcode and Deadman kill switches, we passaged cells containing the circuits for 4 d under survival conditions and periodically tested subsets of cells for circuit function under nonpermissive conditions. Both the Deadman and Passcode circuits showed reduced killing efficiency

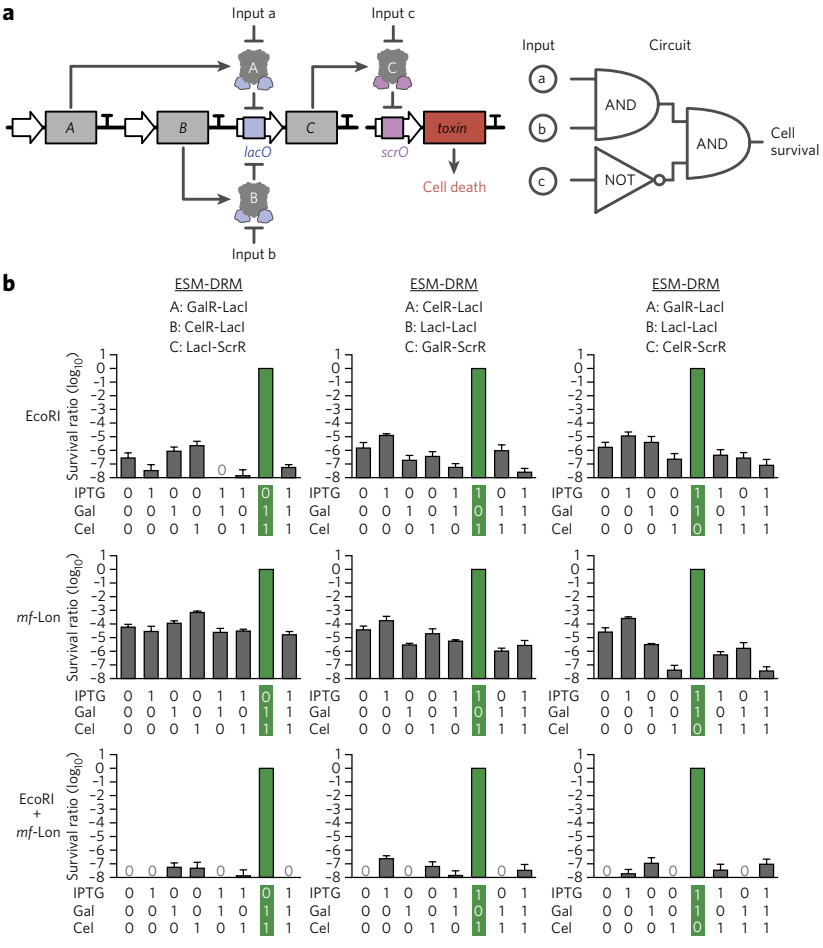


**Figure 3 | Passcode kill switch.** (**a**) Passcode circuit schematic and logic gate behavior. Cell survival requires the continued presence of inputs a and b and the absence of input c. The loss of input a or b or the addition of input c causes the passcode circuit to activate toxin expression, leading to cell death. (**b**) Three versions of the Passcode kill switch were used to control expression of *ecoRI*, *mf*-Lon–mediated MurC degradation (*mf*-Lon) or both *ecoRI* and *mf*-Lon. Cells containing each circuit were placed in each of eight possible combinations of the three input molecules, and cell viability was measured by CFU count after 8 h. In each condition, cell survival is displayed as a ratio of cells in that condition to cells in the 'survival' condition highlighted in green. Cell survival below the limit of detection ($<1 \times 10^{-7}$) is indicated by a '0'. All data points represent mean ± s.d. of three biological replicates.

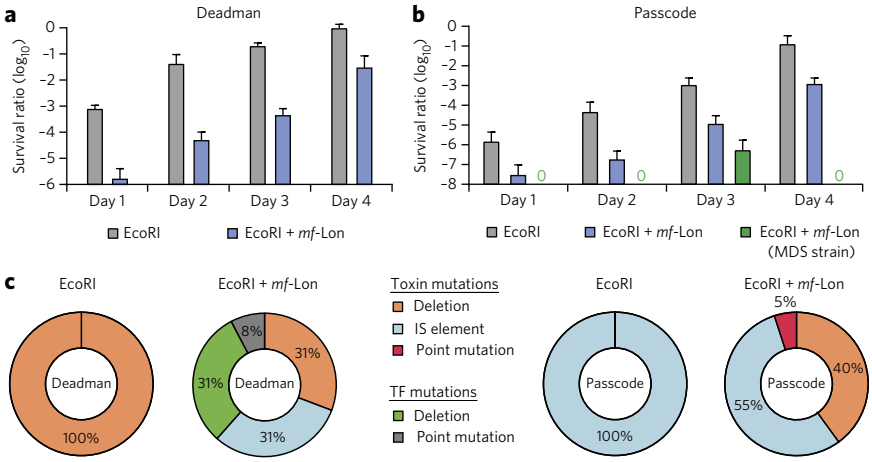


**Figure 4 | Long-term circuit stability.** (**a**,**b**) Cells with Deadman or Passcode circuits containing one toxin (EcoRI) or two toxins (EcoRI and *mf*-Lon) were passaged under survival conditions for 4 d, and subpopulations of cells were periodically switched to nonpermissive media (Deadman: no ATc; Passcode: no inducer) for 8 h. The survival ratio is the ratio of cells that survive in the death state to those in the survival state. Data points represent the mean ± s.d. of six biological replicates. The passcode circuit was also passaged in *E. coli* MDS42pdu *ΔrecA* (MDS strain), which lacks recombinogenic and mobile genomic elements[34]. Deadman and Passcode circuits that do not contain toxin modules displayed increased stability throughout the 4-d experiment (**Supplementary Figs. 20** and **21**). (**c**) Cells containing Deadman and Passcode circuits that survived exposure to their respective death states were isolated, and the entire circuit and toxin (or toxins) were sequenced to identify the inactivating mutations. Toxin gene disruption by genome-encoded IS elements and large deletions were the predominant cause of circuit inactivation. In the two-toxin Deadman circuit, inactivating TetR mutations allowed continued LacI expression and repression of toxin genes in nonbiocontainment conditions (**Fig. 1**).

over time, and sequence analysis of cells that escaped biocontainment predominantly showed inactivating mutations in the toxin genes (**Fig. 4** and **Supplementary Figs. 19–21**). Noted exceptions were independent TetR mutations in the two-toxin Deadman circuit where TetR inactivation repressed toxin expression even in the absence of the ATc survival signal. It is notable, however, that these 'escapees' are still sensitive to IPTG-mediated fail-safe circuit activation, as described above (**Fig. 2**). Genome-encoded insertion-sequence (IS) elements[28], particularly IS1 and IS5, caused a large percentage of inactivating mutations in the one-toxin and two-toxin Passcode circuits. Deletion of these IS elements and other genome repair mechanisms in *E. coli* reduced the Passcode escapee rate by 3–5 logs after 4 d, demonstrating that increased stability of the host genome will augment the functionality of these biocontainment systems (**Fig. 4b** and **Supplementary Fig. 19**). As the toxin genes were the main target for circuit inactivation, inclusion of additional redundant killing systems into each circuit should further reduce the escapee rate.

## DISCUSSION

The Deadman and Passcode switches provide robust information processing circuits to couple environmental signals with conditional survival of the microbial host. The Deadman kill switch described above is based on a monostable circuit that passively activates toxin gene expression in the absence of the small-molecule input ATc. As ATc is not normally found in nature, engineered cells that escape biocontainment will trigger cell death to prevent the spread of the organism or its genetic content into the surrounding ecosystem. Unlike auxotrophy-based biocontainment, where the environmental signal is an intrinsic feature of the system[9,10], the environmental sensing and cell killing systems are decoupled in the Deadman switch. This circuit relies on two main elements for functionality: (i) the orthogonality of the TFs to create a toggle switch and (ii) their relative activity under induced expression. As such, the Deadman circuit is highly modular, and the environmental signal detected by the circuit may be altered by replacing TetR with a wide range of transcription factors[29]. In addition, the Deadman circuit has fail-safe mechanism that activates toxin production and cell death in the presence of IPTG, enabling exogenous control over the microbe's survival even as the cell uses the circuit to monitor its environment.

Like the Deadman switch, the Passcode circuits are based on a two-layered transcriptional repression design. To build hybrid TFs, we identified the conserved boundaries of the ESMs and DRMs within the LacI-GalR family members LacI, GalR, CelR and ScrR. The resulting environmental sensing and DNA binding modules provide independent control of the sensory input and regulatory output of each hybrid TF. Previous pioneering work used the boundary between the conserved regulatory domain and hinge-helix (HH) motif to create hybrid TFs[25,26], but some of these hybrids required additional protein engineering and mutagenesis to become functional. Here we identify a discrete boundary between the conserved HH and helix-turn-helix (HTH) motifs to create independent environmental sensory and DNA-binding domains that can be efficiently combined without further protein engineering. The ESM and DRM boundaries defined in this study may be used to incorporate sensing modules from many of the ~29,000 LacI-GalR family members[30] that detect diverse environmental signals. The modularity provided by these hybrid TFs markedly expands the number and range of environmental signals that can be used to control biocontainment systems such as the Deadman and Passcode circuits described here.

These hybrid TFs may also be used to functionalize other synthetic circuits, including the Deadman switch, to respond to different environmental signals. Moreover, the regular use of LacI and TetR in other bacteria[31,32] suggests that these circuits may be readily transferred to other microbes, including industrial production strains. Replacement of the antibiotic resistance cassettes in these plasmids with well-characterized selection systems that use toxin-antitoxin modules or auxotrophy complementation should also enable their use in biotherapeutic applications[4,33].

In summary, we have established two circuit-based microbial kill switches that constrict host cell survival to an environment defined by specific input signals. Unlike existing biocontainment systems with fixed survival conditions that are difficult to modify, the Deadman and Passcode kill switches are inherently customizable, both in the environmental conditions that control circuit activation and in the output modules that control cell fate. In addition to its use as a biocontainment system, the Passcode circuit may find particular utility as a tool for intellectual property protection, where unauthorized growth of strains without the appropriate passcode molecules would induce cell death. With the proper choice of toxins, such as the endonuclease EcoRI described here, the Passcode circuit could be used to not only kill the host cell but also degrade its genome and accompanying plasmids to deter attempts at reverse-engineering the strain of interest. The use of hybrid TFs that respond to proprietary small-molecule inputs may further secure the strain against theft, even if its genome is sequenced.

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## METHODS

Methods and any associated references are available in the online version of the paper.

**Accession codes.** GenBank: DNA sequences have been submitted under accession numbers KT893253, KT893254, KT893255, KT893256, KT893257, KT895272, KT895273, KT895274, KT895275, KT895276 and KT895277.

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## Author contributions

C.T.Y.C., J.W.L., D.E.C., C.J.B. and J.J.C. designed the study, analyzed data and wrote the paper. C.T.Y.C. and J.W.L. performed the experiments.

## Competing financial interests

The authors declare competing financial interests: details accompany the online version of the paper.

## Additional information

Any supplementary information, chemical compound information and source data are available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to J.J.C.

## ONLINE METHODS

**Analysis of protein sequences and crystal structures.** ClustalW2 (ref. 35) was used for protein sequence alignment of GalS, GalR, AscG, RbsR, PurR, GntR, LacI and MalI from *E. coli*; CelR from *T. fusca*; ScrR from *Vibrio alginolyticus* (ScrR-V); and ScrR from *K. pneumonia* (ScrR-K). Protein crystal structure analysis was performed with PyMol 1.5.x using Protein Data Bank (PDB) entries 1EFA, 1LBG, 1LBI, 1LBH, 1QPZ and 1TLF[36–40].

**Strains.** *E. coli* MG1655*ΔlacI* was used to perform functional analysis of hybrid TFs as shown in **Supplementary Figures 10–13**. In this strain, transcription from the *pLtetO-1* promoter driving TF expression is constitutive because it does not contain *tetR*. *E. coli* MG1655Pro, which produces high levels of LacI and TetR[11], was used in hybrid TF analysis when LacI regulation of *pLlacO-1* was a desired feature (**Supplementary Fig. 14**). In these assays, the TetR inhibitor anhydrotetracycline (ATc; 100 ng/ml) was included in the medium to ensure TF expression from the *pLtetO* promoter. *E. coli* MG1655*ΔlacI* was the parental strain for all circuit characterization and was created through P1 phage transduction of *lacI::kanR* from the Keio collection[41] into *E. coli* MG1655 (ATCC 47076). Flp recombinase, expressed on pCP20, was used to remove the *kanR* cassette[42]. To construct *E. coli* strains containing *mf*-Lon recognition tags on the essential genes *dxs*, *cysS*, *fldA*, *plsB* or *murC*, the pdt#1 *mf*-Lon recognition tag from each corresponding gene in the EPD library[20] was transferred to MG1655*ΔlacI* by P1 phage transduction, and the *kanR* cassette was removed as above. P1 phage transduction was used to convert *E. coli* MDS42pdu[34] (Scarab Genomics) for use in the Passcode switch analysis. Specifically, *lacI::kanR* and *recA::kanR* deletions from the Keio collection[41] and *murC-pdt#1* from the EPD library[20] were independently transferred to MDS42pdu by P1 phage transduction, and the accompanying kanamycin cassettes were removed by FlpE-mediated excision using pECA102 (ref. 20).

**Cell growth and medium.** Luria-Bertani (LB) medium was used for all experiments, and the following antibiotics and inducers were included when appropriate: ampicillin (50 μg/ml), chloramphenicol (10 μg/ml), kanamycin (50 μg/ml), ATc (100 ng/ml), IPTG (1 mM), galactose (20 mM) and cellobiose (5 mM). For the Deadman switch, single colonies grown on LB agar plates containing ATc were inoculated into liquid cultures containing ATc for growth overnight at 37 °C with shaking. Similarly, cells harboring each of the three Passcode switches were picked from plates with the survival combination of inputs and inoculated into their respective survival liquid medium. Overnight cultures were inoculated 1:20,000 into 96-well plates and grown at 37 °C and 900 r.p.m. for further tests.

**Plasmid construction.** All plasmids were constructed using conventional molecular cloning protocols[43] and Gibson Assembly[44]. *E. coli* NEB Turbo (New England BioLabs) was used for cloning purposes, and all primers were purchased from IDT. To create the Deadman switch pDM1 (GenBank accession number KT893253), genetic elements from the toggle pECJ3 (ref. 20) were cloned into the conditionally amplified single-copy plasmid pBAC/oriV[45], and the *lacI* and *tetR* RBS strengths were modified as described below. To provide increased control over the promoter controlling mCherry expression, the T1 terminator from *rnpB* (Registry of Standard Biological Parts BBa_J61048) was inserted upstream (**Supplementary Fig. 4a**), and three palindromic *lac* operator sites[19] were inserted around the −35 and −10 region of the promoter (pDM2, GenBank accession number KT893254). The *Mesoplasma florum* protease gene *mf-lon* was cloned under the control of this LacI-regulated promoter (pDM2L; GenBank accession number KT893255). The resulting plasmid served as the base Deadman circuit, mCherry was cloned to yield pDM3 and *ecoRI*, *ccdB* and *mazF* were cloned to make the toxin variants (**Supplementary Table 1**).

Hybrid TF genes (*lacI-galR LG36-LG46*, *galR-lacI*, *celR-lacI*, *lacI-scrR*, *galR-scrR*, and *celR-scrR*) were constructed by overlap extension PCR to fuse the environmental sensing modules (ESMs) and the DNA recognition modules (DRMs) of the designated genes. The hybrid TFs were cloned into pTR, a derivative of pKE2_MCS[17] containing the *pLtetO-1* promoter and T0 terminator from pZA11 (ref. 46), using restriction sites BamHI and BsrGI. Transcription from the *pLtetO-1* promoter driving TF expression is constitutive because the *E. coli* strains used in this study did not contain *tetR*. Reporter plasmids (pREPORT) were constructed from the plasmid pZA12 (ref. 46), with genes encoding mCherry or GFP inserted downstream of the *pLlacO* promoter using KpnI and HindIII. To test hybrid TFs that contain the ScrR DRM, *pLlacO-1* was replaced with *pLscrO-1* or *pLscrO-2* using Gibson Assembly method[44]. For implementation of both LacI/*pLlacO-1* and GalR-ScrR/*pLscrO* inducible expression systems in the same cells (**Supplementary Fig. 14**), the *pLlacO-1-mCherry-T1* cassette was subcloned into pTR using NheI and SalI.

The Passcode circuit was developed using a two-plasmid system. Plasmid pPasscode (GenBank accession numbers KT895272, KT895273 and KT895274), derived from pKE2_MCS[17], was constructed to contain the hybrid TF circuit, and pToxin (GenBank accession numbers KT895275, KT895276 and KT895277), derived from pZA12 (ref. 46), was constructed to contain the toxin output module under control of the *pLscrO* promoter. For pPasscode, three promoter-hybrid TF-terminator fragments were used to construct each hybrid TF circuit version, as listed in **Supplementary Table 1**. For version 1 of pPasscode (pPasscode1), in which LacI-ScrR is used as hybrid C, the promoter *pLscrO-2* was used to control the expression of toxin gene (or genes) in pToxin. For pPasscode2, which contains GalR-ScrR, the promoter *pLscrO-1* was used for toxin control in pToxin. For pPasscode3, which contains CelR-ScrR, the promoter *pLscrO-1* was used to control the expression of *mf*-Lon, and the promoter *pLscrO-2* was used to control the expression of *ecoRI*. For Passcode circuits that contain two toxin gene systems, the DNA fragments *pLscrO-mf-lon-terminator* and *pLscrO-ecoRI-terminator* were incorporated into pToxin using Gibson Assembly (**Supplementary Table 1**). For Passcode circuit characterization, pPasscode was first transformed into the desired *E. coli* strain and grown in medium containing the passcode combination of the three inputs (IPTG, galactose and cellobiose). Plasmid pToxin, which contains the toxin gene (or genes), was then transformed into the cells to complete the Passcode circuit.

**RBS strength optimization for monostable toggle construction.** An RBS calculator algorithm[24] was used to identify RBS variants that produce a range of LacI and TetR expressions (**Supplementary Table 1**). Cells containing each toggle RBS variant were grown overnight in the presence of ATc, transferred to medium without ATc and then measured for mCherry expression by flow cytometry after 6 h. Toggle variant 5, which showed the largest change in mCherry fluorescence upon loss of ATc, was chosen for use in the Deadman circuit (**Supplementary Fig. 2**). To quantify the relative LacI and TetR expression levels, mCherry was fused to the C terminus of LacI or TetR to yield pBAC-LC and pBAC-TC, respectively (GenBank accession numbers KT893256 and KT893257). RBS variants for LacI and TetR were then cloned into pBAC-LC and pBAC-TC, respectively, and a SpectraMax M5 microplate reader (Molecular Devices) was used to measure mCherry fluorescence with excitation and emission wavelengths of 587 nm and 610 nm, respectively, with an emission filter cutoff at 610 nm. mCherry fluorescence was normalized to cell growth ($OD_{600}$).

**RBS strength optimization for toxin expression.** To optimize cell death dynamics upon Deadman or Passcode circuit activation, a range of predicted RBS strength variants[24] was generated for each toxin (**Supplementary Table 1**). For the Deadman kill switches (**Supplementary Fig. 6**), RBS variants and the corresponding toxin genes *ecoRI*, *ccdB* and *mazF* were cloned into pDM2L using Gibson Assembly (**Supplementary Table 1**). Overnight cultures were grown in the presence of ATc and then transferred into medium with ATc (survival condition) or with IPTG (induced death condition). A SpectraMax M5 microplate reader (Molecular Devices) was used to measure cell growth ($OD_{600}$) every 15 min for 15 h, and the cell growth ratios of the induced death state to the survival state were calculated at 15 h.

For Passcode kill switches, RBS variants (**Supplementary Table 1**) and the corresponding toxin genes *ecoRI* and *mf-lon* were cloned into pREPORT to replace *gfp* and tested for optimal expression under regulation by the hybrid TFs LacI-ScrR, GalR-ScrR and CelR-ScrR. Plasmids containing each RBS-toxin variant were transformed into cells constitutively expressing LacI-ScrR, GalR-ScrR or CelR-ScrR; grown overnight without inducers; and then transferred into medium with or without the appropriate

inducer (1 mM IPTG, 20 mM galactose or 5 mM cellobiose for cells containing LacI-ScrR, GalR-ScrR or CelR-ScrR, respectively). Cell growth analysis was performed as described for the Deadman circuit above, and the cell growth ratio was calculated at 12 h. Representative data are shown in **Supplementary Figure 17**.

**RBS strength optimization for ScrR ESM-containing TFs.** A range of RBS variants was tested to optimize the expression of ScrR ESM-containing TFs (TF 'C' in **Fig. 3a**) in the Passcode circuits (**Supplementary Table 1**). Cells with the Passcode circuit harboring RBS variants were transformed with the indicated pToxin plasmid, grown overnight under survival conditions (see **Supplementary Fig. 16** for the appropriate inducers for each circuit) and then transferred to medium with all eight combinations of the three inducers (IPTG, galactose and cellobiose). Performance of each circuit was determined by CFU count after 8 h of exposure, as described below.

**Survival assays.** Colony forming unit (CFU) cell viability assays were used to measure functionality of the Deadman and Passcode circuits. Overnight cultures were grown under the survival conditions (Deadman: with ATc; Passcode: with survival passcode inputs) and were transferred into fresh LB medium with or without the survival signal (or signals). For the Passcode circuit, all eight combinations of the three inputs were tested (± IPTG, ± galactose and ± cellobiose). Samples were collected every 2 h, serially diluted in PBS over a 7-log range and spotted (5 µl) onto LB agar plates with the appropriate survival signal (or signals). CFU and survival ratios were calculated as previously reported[11]: CFU/ml = (number of colonies) × (dilution factor)/0.005 ml, survival ratio ($\log_{10}$) = log {(CFU/ml without the survival signal)/(CFU/ml with the survival signal)}.

**Long-term growth analysis.** Cells containing the Deadman and Passcode kill switches were passaged under survival conditions for 4 days (Deadman: 100 ng/mL ATc; Passcode: unique inducer for each Passcode circuit; see **Supplementary Fig. 16**). Sub-populations of these cells were diluted 1:20,000 into media with or without the survival signal(s) (Deadman: no ATc; Passcode: no inducers), and samples were collected at 8 h after inoculation, serially diluted in PBS over a 7-log range, and spotted (5 µL) onto LB agar plates with the appropriate survival signal(s). CFU and survival ratios were calculated as described above.

**Escapee genetic analysis.** Cells containing independent Deadman and Passcode circuit transformants ($n = 20$ for each circuit) were grown under survival conditions (Deadman: 100 ng/ml ATc; Passcode: unique inducer for each Passcode circuit as described in **Supplementary Fig. 16**). The cells were then transferred to media without the survival signal(s) for 8 h and then placed on LB agar plates containing the appropriate survival signal(s). Deadman circuits were isolated from surviving cells by amplification with Phusion high-fidelity DNA polymerase (NEB), Passcode circuits were isolated by plasmid DNA purification, and the circuits were then sequenced by Quintara Biosciences (Boston, MA).

**Flow cytometry assay.** Cells containing Passcode circuits were grown as described for each experiment and at the appropriate time were fixed in 2% paraformaldehyde in PBS and then diluted 1:10 in PBS for analysis. mCherry and GFP fluorescence measurements were performed using a BD FACSAriaII (BD Biosciences) or a BD LSRFortessa flow cytometer (BD Biosciences). Flow cytometry data were gated by forward and side scatter to eliminate multicell aggregates, and the geometric mean of mCherry and GFP fluorescence distributions were calculated using FlowJo software (Treestar). At least 10,000 events were collected for each measurement.


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