(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 May 2017 (18.05.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/083501 A1

(51) International Patent Classification:
C12N 5/00 (2006.01) *C12N 15/00* (2006.01)
C12N 1/00 (2006.01) *C07H 21/04* (2006.01)

(21) International Application Number:
PCT/US2016/061297

(22) International Filing Date:
10 November 2016 (10.11.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/253,954 11 November 2015 (11.11.2015) US

(71) Applicant: **REGENTS OF THE UNIVERSITY OF MINNESOTA** [US/US]; 600 Mcnamara Alumni Center, 200 Oak Street Se, Minneapolis, MN 55455-2020 (US).

(72) Inventors; and
(71) Applicants : **MASELKO, Maciej** [US/US]; 1800 Larpenteur Ave W., #15, Falcon Heights, MN 55113 (US). **SMANSKI, Mike** [US/US]; 1791 Simpson St., Falcon Heights, MN 55113 (US).

(74) Agent: **GRAM, Christopher, D.**; Mueting, Raasch & Gebhardt, P.A., P.O. Box 581336, Minneapolis, MN 55458-1336 (US).

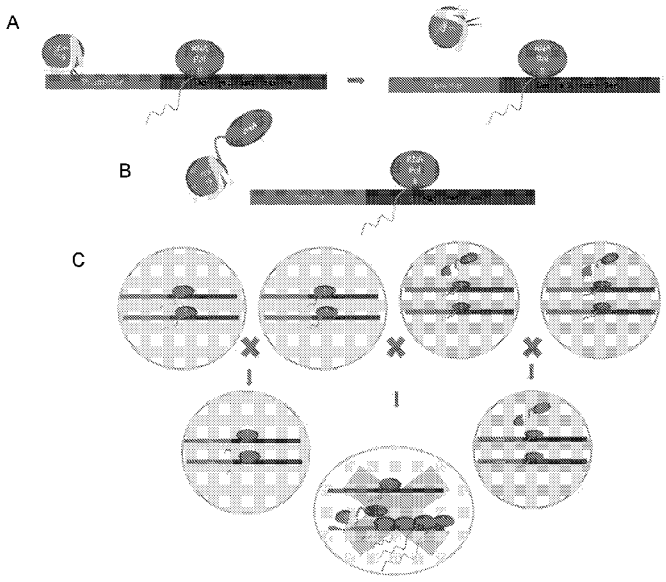
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: BIOCONTAINMENT/BIOCONTROL SYSTEM AND METHODS

FIG. 1



(57) Abstract: This disclosure describes, in one aspect, a cell that includes a biocontainment system. Generally, the biocontainment system includes a coding region whose overexpression decreases growth of the cell, a transcription regulatory region that includes a silent mutation and is operably linked upstream of the coding region, and a polynucleotide that encodes a programmable transcription activator engineered to bind to the transcription regulatory region in the absence of the silent mutation. Thus, in the absence of the silent mutation, the programmable transcription activator induces overexpression of the coding region; in the presence of the silent mutation, the programmable transcription activator does not initiate overexpression of the coding region.

WO 2017/083501 A1

BIOCONTAINMENT/BIOCONTROL SYSTEM AND METHODS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application No. 62/253,954, filed November 11, 2015, which is incorporated herein by reference.

SEQUENCE LISTING

This application contains a Sequence Listing electronically submitted via EFS-Web to the United States Patent and Trademark Office as an ASCII text file entitled "2016-11-10-SequenceListing_ST25.txt" having a size of 4 kilobytes and created on November 10, 2016. The information contained in the Sequence Listing is incorporated by reference herein.

SUMMARY

This disclosure describes, in one aspect, a cell that includes a biocontainment system. Generally, the biocontainment system includes a coding region whose overexpression decreases growth of the cell, a transcription regulatory region that includes a silent mutation and is operably linked upstream of the coding region, and a polynucleotide that encodes a programmable transcription activator engineered to bind to the transcription regulatory region in the absence of the silent mutation. Thus, in the absence of the silent mutation, the programmable transcription activator induces overexpression of the coding region; in the presence of the silent mutation, the programmable transcription activator does not initiate overexpression of the coding region.

Accordingly, an organism that is homozygous for the biocontainment system grows normally. In contrast, an organism that becomes heterozygous for the biocontainment system—whether as a result of sexual reproduction with another variety of the organism or by some spontaneous genetic event—exhibits retarded growth and/or death so that any hybrids can be efficiently culled from the population.

In some embodiments, the cell can be a single-celled organism. In other embodiments, the cell can be a germ cell of a multicellular organism.

In some embodiments, the programmable transcription activator can include dCas9 fused to an activation domain.

In some embodiments, the coding region can encode a cytoskeletal polypeptide, an ER-Golgi vesicle polypeptide, an mRNA processing polypeptide, an electron transport polypeptide, a nuclear trafficking polypeptide, a chromosome segregation polypeptide, a spindle pole duplication polypeptide, or an oxidative stress polypeptide.

In some embodiments, overexpression of the coding region can be lethal to the cell.

In some embodiments, the cell can include a second biocontainment system.

In another aspect, this disclosure describes a method of limiting hybridization of a genetically-modified organism with a genetically dissimilar variant. Generally, the method includes providing an organism genetically modified to include any embodiment of the biocontainment system summarized above so that a cross between the genetically-modified organism and the genetically dissimilar variant organism results in progeny that exhibit a phenotype that is distinct from the genetically-modified organism.

In some embodiments, the genetically dissimilar variant can include a wild-type organism. In other embodiments, the genetically dissimilar variants can include a different genetic modification compared to the genetically-modified organism having the biocontainment system.

In some embodiments, the phenotype exhibited by the progeny can include lethality.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Schematic diagram showing a general overview of synthetic incompatibility. (A) Silent mutations are introduced upstream of expression-sensitive coding regions. (B) A transcriptional activator is engineered to bind to the parental wild-type sequence. (C) A cross

between a wild-type organism (one that contains the parental promoter sequence) and an organism engineered as shown in (A) and (B) will result in overexpression of the parental allele.

FIG. 2. Schematic diagram showing illustrating synthetic incompatibility in detail. (A) Macromolecular components that constitute programmable transcription factors (above), and schematic illustration showing lethal expression from a wild-type promoter but not a refactored promoter (below). (B) Illustration of hybrid lethality upon mating of wild-type (right cell) and SI (left cell) parents. Macromolecular components are labeled in (A), dark DNA signifies WT promoter, and light DNA signifies refactored promoter. Skull and crossbones indicates a non-viable genotype as lethal expression is initiated from the wild-type promoter. (C) Possible applications for engineered speciation.

FIG. 3. Targeting dCas9-VP64 with an MS2-VP64 co-activator, a system referred to as DVM, to Act1/g4 severely stunts growth. This image was taken 14 days post transformation. The top left plate shows colony growth from yeast transformed with a control vector that does not target dCas9 to any location. The top right shows a negative control were yeast were mock transformed with water. The bottom plate has colonies from yeast transformed with dCas9-VP64 targeting Act1/g4, a location upstream of the Actin transcriptional start site. Very small pin-point colonies can be seen which did not grow beyond this size.

FIG. 4. Yeast were allowed to mate overnight in rich media and then plated on media lacking uracil and leucine to select for diploids. (A) The Mate A plate shows colonies from a cross between two strains which both have mutated Act1/g4 loci. dCas9-VP64 has no genomic target. (B) The Mate B plate demonstrates compatibility between one strain which has a mutated Act1/g4 locus and one with the wild type version. Neither expresses dCas9-VP64. (C) The Mate C plate shows the results of a cross between a strain with a wt Act1/g4 locus and a strain with a mutated Act1/g4 locus expressing dCas9-VP64 targeting the wt Act1/g4. (D) The Mate D plate shows colonies from a cross between two strains with wt Act1/g4 loci.

FIG. 5. Engineering speciation by synthetic incompatibility. (A) Growth curves of yeast expressing DVM targeted to promoter regions of SI candidate genes. Random sgRNA control shown in red. Best *ACT1* targeting sgRNA in light blue. All others in grey. (n=2, +/- SD, error bars omitted for grey lines for clarity) (B) (Left) Diagram of mutated and wild-type *ACT1* promoter-GFP constructs. (Right) GFP expression ratios with or without DVM and/or *ACT1* promoter specific sgRNA. (n=3, +/- SD). (C) (Left) Schematic representation of SI components

present in haploid strain crosses and (Right) the resulting diploid colonies. (D) Live cell imaging time lapse of diploid cells from crossing RFP$^+$ *MATα* with GFP$^+$ *Mata* cells in a compatible (Top) and incompatible (Bottom) mating. Green arrows indicate cells which swell and lyse.

FIG. 6. Plasmid maps of plasmids used herein.

FIG. 7. PCR Verification of Genomic Modifications (A) Results from PCR analysis of *Lys2* locus in YMM124 and CEN.PK wild-type control. (B) Results from PCR analysis of *Lys2* locus in YMM134, YMM155, and CEN.PK wild-type control. (C) Results from PCR analysis of *Leu2* locus in YMM134, YMM155, and CEN.PK wild-type control.

FIG. 8. Determining *ACT1* mutation's effect on growth rate. (A) Growth curves shown from strains with a wild-type (CEN.PK) and mutated (YMM127) actin promoters (n=3 independent cultures, mean +/- SEM). (B) Comparison of doubling time between CEN.PK and YMM127 ($p > .05$, two tailed t-test).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

This disclosure describes a biocontainment/biocontrol system, cells and organisms that include such systems, and methods involving the construction and use of such systems. As used herein, the term "biocontainment/biocontrol system" and variations thereof refer to a genetic system that decreases the likelihood and/or extent to which a genetically-modified organism can sexually reproduce with a genetically dissimilar variant—whether wild-type or genetically modified in another way. In use, the system can decrease the likelihood and/or extent to which a genetic modification in, for example, a genetically-modified crop variety can spread into other variants. The system also can decrease the likelihood and/or extent to which a genetic modification can be diluted in a genetically-modified variety by the re-introduction of a wild-type genotype into a population of the genetically-modified variety.

Genetic recombination due to sexual reproduction is a source of tremendous genetic variation and provides a mechanism for generating organisms with novel combinations of traits. Humans have exploited this process, often unknowingly, for the domestication of plants and animals. Unwanted genetic recombination also takes place between domesticated and wild varieties of plants and animals. This is of particular concern in agricultural systems where seed production or Organic (non-GMO) certification is concerned since unwanted crosses diminish

the value of a crop. Farmers usually prevent conflicts via spatial and temporal separation which often requires co-ordination with neighboring farms.

More recently, synthetic biology applications in which plants are engineered to make pharmaceuticals or other industrial compounds require effective biocontainment strategies in order to prevent contamination of the food supply. Determining sufficient spatial separation necessary for such protection is complicated and considers factors such as, for example, pollen size, duration of pollen viability, the presence of wild relatives, and typical meteorological conditions. For example, as of 2015 the U.S. Animal and Plant Health Inspection Service (APHIS), which regulates the environmental release of genetically modified plants, requires that corn engineered for the production of pharmaceutical or industrial compounds must be separated from all other corn by at least 1 mile despite 0.25 mile being sufficient to achieve 99.9% purity. This creates a substantial burden for companies wishing to use plant-based production systems.

Biocontainment approaches other than physical separation have been investigated, but each has at least one major drawback that prevents wide-spread adoption. Cleistogamy, in which flowers never open and therefore must self-pollinate is not applicable to all species. Maternal inheritance of transgenes by plastid engineering is likewise not applicable to all species and pollen-mediated transfer of plastids at low frequencies may be common in many other species. Excising a transgene from a pollen-expressed recombinase may be highly efficient, but control of recombinase activity can interfere with normal propagation. Total sterility requires asexual propagation and is not practical for many species. Using exogenous chemical inputs to regulate lethal genes requires changes to normal cultivation techniques and genome reprogramming to confer dependence on synthetic compounds. Furthermore, with the exceptions of cleistogamy and asexual propagation, these methods only prevent outward gene-flow. Unwanted flow of genes into genetically engineered plants, however, can result in bioncontainment failure in subsequent generations.

This disclosure describes a novel biocontainment approach in which a programmable transcriptional activator (e.g., dCas9-VP64) monitors for the presence of a binding site upstream of an expression-sensitive coding region—i.e., any portion of the genome whose overexpression results in death or a severely deleterious phenotype. Thus, overexpression of an expression-sensitive coding region can reduce the reproductive fitness of the organism in which the expression-sensitive coding region is overexpressed and, in certain embodiments, even prevent

the organism from reproducing. The upstream binding site is mutated in the engineered organism (FIG. 1A and FIG. 2A) that express the transcriptional activator (FIG. 1B and FIG. 2A). The mutation in the upstream binding site negates binding of the programmable transcription activator so that expression of the coding region is at a normal, nonlethal level.

Sexual crossing with the wild-type organisms activates the system (FIG. 1C and FIG. 2B). The programmable transcription activator is able to bind to the non-mutated, wild-type upstream binding site contributed by the wild-type parent, causing the lethal overexpression of the expression-sensitive gene. The engineered strain can effectively be considered a distinct species from the wild-type since it is no longer sexually compatible with the wild-type. This synthetic incompatibility approach does not require any changes in culture techniques or additional chemical inputs to maintain biocontainment. Furthermore, multiple orthogonal circuits can be introduced so that the same basic strategy can be used simultaneously in the same organismal background.

Beyond transgene containment, synthetic incompatibility also can be used for biological insect control via an alternative to the widespread sterile insect technique (SIT). SIT involves the release of sterile male insects (e.g., mosquitoes) which then find females to mate with. This can be an effective control strategy since the females of many insects often mate only once per lifetime or clutch of eggs. A drawback to SIT is that the males are typically sterilized via irradiation, which can cause behavioral changes that make them significantly less successful at finding a mate than non-irradiated males. Therefore, many more irradiated males have to be released for successful population control. Another drawback is that this technique is not applicable to several important insect species since the dose necessary for sterilization is not sufficiently below the lethal dose. Synthetic incompatibility is an appealing alternative since it requires no irradiation. Thus, synthetic incompatibility can produce more competitive males and may be applied to a larger number of insects.

The utility of synthetic incompatibility was established in the model organism *Saccharomyces cerevisiae* since it is easy to genetically manipulate, can be propagated as either a haploid or diploid, and has similar molecular biology to higher organisms. The approach involved first identify genes that can be sufficiently overexpressed by the programmable transcription factor dCas9-VP64 to cause a strong defect in growth.

Six target genes (Table 1) were initially chosen to be targeted for overexpression based on an "inviable" phenotype reported in the Saccharomyces Genome Database. Transcriptional start sites (TSS) were retrieved using the IGV genome browser (Broad Institute, Cambridge, MA) and previously mapped start sites. sgRNAs were designed to bind unique sequences upstream of NGG protospacer adjacent motif (PAM) sites in an approximately 250 bp window upstream of predicted transcriptional start sites of candidate coding regions. Four target sites were selected for each gene.

Table 1. *S. cerevisiae* overexpression target genes

Gene	Core Function
TUB2	Cytoskeletal
ACT1	Cytoskeletal
ABP1	Regulation of actin cytoskeleton
YIP3	ER-Golgi vesicle transport
SWT1	mRNA quality control
COX1	Electron transport chain

S. cerevisiae was transformed with a plasmid that expressed dCas9-VP64, a sgRNA to guide dCas9-VP64 to its target, and KlURA3 for selection on agar plates lacking uracil. Of the 24 plasmids tested, none resulted in a noticeable reduction in growth compared to controls, perhaps because dCas9-VP64 did not sufficiently activate transcription from the selected target loci. Thus, a yeast strain was engineered to express MS2-VP64, which recognizes hairpin structures present in the sgRNA and can boost gene expression. The yeast strain expressing MS2-VP64 (YMM-1) was then transformed with the same set of plasmids, which resulted in stunted growth for several of the targets (Table 2, FIG. 2A). The most striking phenotype resulted from targeting one of the sites upstream of Actin (ACT1/g4). This resulted in no growth after one week and only very slight colonies present after two weeks (FIG. 3). A target site on the bottom strand 190 nucleotides upstream of the *ACT1* transcriptional start site resulted in the strongest growth defect with no visible growth after 10 days (FIG. 5A). The nine PAM distal nucleotides are predicted to be Forkhead transcription factor binding sites.

Table 2: Growth characteristics of yeast strain YMM-1 transformed with sgRNA directed to the indicated target

Target/sgRNA	Day 7 Growth (transformation #1)
TUB2/g1	Slightly stunted

TUB2/g2	Slightly stunted
TUB2/g3	Stunted Growth
TUB2/g4	Stunted Growth
ACT1/g1	Stunted Growth
ACT1/g2	Larger Colonies than positive control
ACT1/g3	Stunted Growth
ACT1/g4	No growth.
ABP1/g1	Severely Stunted, Barely Visibly Colonies
ABP1/g2	Stunted Growth
ABP1/g3	Slightly stunted
ABP1/g4	Similar to positive control
YIP3/g1	Stunted Growth
YIP3/g2	Slightly stunted
YIP3/g3	Stunted Growth
YIP3/g4	Severely Stunted, Barely Visibly Colonies
SWT1/g1	Stunted Growth
SWT1/g2	Similar to positive control
SWT1/g3	Stunted Growth
SWT1/g4	Similar to positive control
COX9/g1	Similar to positive control
COX9/g2	Stunted Growth
COX9/g3	Similar to positive control
COX9/g4	Similar to positive control
No sgRNA (+) Control	Plenty of colonies
No DNA (-) Control	No growth

To generate a synthetically incompatible strain, Cas9 was used to introduce a mutation by non-homologous end joining in the *ACT1* promoter. The mutated promoter differs from wild-type by a single cytosine deletion 3 bp upstream of the PAM site. There is no observable growth phenotype resulting from the mutated *ACT1* promoter (FIG. 8). Transcription from the mutated promoter was characterized by expressing TurboGFP (Evdokimov et al., 2006. *EMBO Rep.* 7:1006–1012) under the control of the wild-type or mutated *ACT1* promoters in the presence and absence of DVM (FIG. 5B). There was a slight increase in TurboGFP expression from the

mutated promoter in the absence of DVM. However, no change was found with a non-targeting sgRNA. TurboGFP expression was 1.8-fold higher from the wild-type *ACT1* promoter than from the mutated promoter when DVM was guided by an sgRNA targeting the wild-type promoter. Together, these results indicate that the mutation in the *ACT1* promoter does not substantially change native expression but prevents targeted transcriptional activation by DVM guided to the wild-type sequence. The DVM targeted to the wild-type *ACT1* promoter sequence was then chromosomally integrated into the strain containing the mutated *ACT1* promoter to complete constructions of the synthetically incompatible strain (FIG. 2B).

Four additional genes were screened (Table 3), identifying additional targets that stunt growth (Table 4). In order to generate a system that produced lethality, targeting dCas9-VP64 to two targets at once was investigated. Several of the combinations eliminated all growth except for the presence of a handful of colonies that appeared normal (Table 5).

Table 3. Additional *S. cerevisiae* overexpression target genes

Gene	Core Function
BEM3	Cytoskeletal regulation
CSE1	Nuclear trafficking and chromosome segregation
DSK2	Spindle pole duplication
MGE1	Oxidative stress response

Table 4. Growth characteristics of yeast strain YMM-1 transformed with sgRNA directed to the indicated target

Target/sgRNA	Day 7 Growth
BEM3/g1	Similar to + Control
BEM3/g2	Similar to + Control
BEM3/g3	Similar to + Control
CSE1/g1	Slightly Stunted
CSE1/g2	Similar to + Control
CSE1/g3	Similar to + Control
CSE1/g4	Similar to + Control
DSK2/g1	Slightly Stunted
DSK2/g2	Similar to + Control
DSK2/g3	Slightly Stunted

DSK2/g4	Similar to + Control
MGE1/g1	Similar to + Control
MGE1/g2	Similar to + Control
MGE1/g3	Similar to + Control
MGE1/g4	Similar to + Control
No gRNA (+) Control	Plenty of colonies
No DNA (-) Control	No growth

Table 5. Growth characteristics of yeast strain YMM-1 transformed with sgRNA directed to the indicated target combinations

Targets/gRNA	Day 7 growth
ACT1/g4 & ABP1/g1	A couple normal colonies. Many severely stunted colonies.
ACT1/g4 & YIP3/g4	A couple dozen large colonies and many more stunted colonies.
ACT1/g4 & DSK2/g2	A couple small colonies. Otherwise like negative control.
ACT1/g4 & MGE1/g3	Handful of large colonies. Many severely stunted colonies.
Act1/g4	One large colony. Otherwise like negative control.
No gRNA (+) control	Lots of large colonies
No Plasmid (-) control	No growth.

The synthetic incompatibility strategy described herein creates a severe penalty to genetic crossing with the wild type. The Act1/g4 target site was mutated in the YMM-1 yeast strain that expresses MS2-VP64; also, dCas9-VP64 targeted to the wild-type Act1/g4 locus was stably integrated into the genome. This did not result in an apparent growth defect (Table 5), indicating that the Act1/g4 mutation in the YMM-1 strain prevents binding of the dCas9-VP64.

Next, the genetic compatibility between the synthetically incompatible strain and a strain with the wild-type *ACT1* promoter was examined. *S. cerevisiae* has haploid mating types *MAT*a and *MAT*α, and can be propagated as a haploid of either mating type or as a diploid after mating. Two different a-mating type strains and two different α-mating type strains were mated together (Table 6) and plated on media lacking both uracil and leucine to select for diploids (FIG. 2C and FIG. 4).

Table 6. Yeast strain information and mating key.

	Mat-a, ΔAct1/g4, Leucine Auxotroph, DVM	Mat-a, wt Act1/g4, Leucine Auxotroph
Mat-α, ΔAct1/g4, Uracil Auxotroph	Mate A	Mate B
Mat-α, wt Act1/g4, Uracil Auxotroph	Mate C	Mate D

A genetic cross between a strain with the wild-type Act1/g4 locus and a strain with a mutated Act1/g4 locus expressing MS2-VP64 and dCas9-VP64 targeted to the wild type results in genetic incompatibility (FIG. 4C). When both carry mutated Act1/g4 loci, however, they are genetically compatible (FIG. 4A). Crossing a strain with the mutated Act1/g4 locus with a strain carrying the wild type version in the absence of dCas9-VP64 does not inhibit growth. Together, this demonstrates that the block to sexual reproduction is due to activity of MS2-VP64 and dCas9-VP64 at the wild type Act1/g4 locus.

Mating a *MAT*a strain with the SI genotype but a random sequence sgRNA to a *MAT*α strain also containing the mutated *ACT1* promoter resulted in numerous diploid colonies (FIG. 5C, i; FIG. 4A). This shows that expression of the DVM machinery or a mutation in the *ACT1* promoter does not prevent sexual reproduction. This same *MAT*a strain was also successfully mated to a *MAT*α strain carrying the wild-type *ACT1* promoter (FIG. 2C, ii), as the random sequence sgRNA does not induce lethal overexpression of *ACT1*. The *MAT*a strain was crossed with a complete synthetically incompatible genotype to a *MAT*α strain with the mutated *ACT1* promoter (FIG. 2c, iii). However, when the SI *MAT*a strain was mated with a *MAT*α strain with wild-type *ACT1* promoter, diploid colonies were seen only in low frequencies (FIG. 2C, iv; FIG. 4C). This failed mating reflects the genetic incompatibility of the synthetically incompatible genotype with wild-type.

In order to understand the engineered genetic incompatibility on a cellular level, mating experiments were performed and diploid cells were monitored using live cell imaging (FIG. 5D). Diploid yeast resulting from a permissive mating (e.g., FIG. 5C, ii) are able to proliferate and produce a microcolony after 20 hours (FIG. 5D, top). Diploids arising from the non-permissive mating of wild-type *ACT1* promoter yeast with the synthetically incompatible strain undergo a limited number of divisions before swelling and eventually lysing (FIG. 5D, bottom). These results are consistent with uncontrolled cytoskeletal growth.

Thus, in one aspect, this disclosure describes a biocontainment/biocontrol system so that the progeny of an organism that possesses the system crossed with a wild-type organism exhibit reduced growth compared to a homozygous wild type organism. Generally, the system involved introducing a genetic barrier to sexual reproduction of a synthetically incompatible (SI) organism with a comparable wild-type organism of the same species. The system involves the use of a programmable transcriptional activator capable of lethal overexpression of one or more endogenous expression-sensitive coding regions. Lethality in the engineered synthetically incompatible strain is prevented by refactoring the target locus, allowing the programmable activator to be expressed in the synthetically incompatible strain. This activator serves as a sentinel for undesired—e.g., synthetically incompatible × wild-type—mating events. Hybridization between the synthetically incompatible strain and an organism containing the transcriptional activator's target sequence results in lethal expression of the expression-sensitive coding region (FIG. 2B).

The biocontainment/biocontrol system can be introduced into a single-celled organism such as, for example, a yeast such as *Saccharomyces cerevisiae*. In other cases, the biocontainment/biocontrol system can be introduced into the cells of a multi-cellular organism such as, for example, a plant or an animal. Exemplary plants into which the biocontainment/biocontrol system may be introduced can include, for example, a field crop (e.g., tobacco, corn, soybean, rice, etc.), a tree (e.g., poplar, rubber tree, etc.), or turfgrass (e.g. creeping bentgrass). Exemplary animals into which the biocontainment/biocontrol system may be introduced can include, for example, an insect (e.g., mosquito, tstetse fly, spotted-wing drosophila, olive fly, gypsy moth, codling moth, deer tick, etc.), a fish (e.g., salmon, carp, sea lamprey, etc.), a mammal (e.g., swine, a mouse, a rat, etc.), an amphibian (e.g., a cane toad, a bullfrog, etc.), a reptile (e.g., brown tree snake, etc.), or a crustacean (e.g., rusty crayfish, etc.).

Thus, when described herein in the context of the biocontainment/biocontrol system being present in a cell, that description encompasses, unless the context dictates otherwise, a single-celled organism, a germ cell of a multicellular organism, or a somatic cell of a multi-cellular organism.

Generally, the biocontainment/biocontrol system includes a genetically-modified cell that includes a coding region whose overexpression decreases growth of the organism, a transcription regulatory region operably linked upstream of the coding region and having a silent mutation,

and a polynucleotide that encodes a programmable transcription activator. The programmable transcription activator can be engineered to bind to the transcription regulatory region in the absence of the silent mutation, thereby initiating overexpression of the coding region in the absence of the silent mutation. Thus, in the absence of the silent mutation—i.e., if the organism is crossed with a wild type organism—the transcription activator initiates overexpression of the coding region and limits growth and/or viability of the organism. In the presence of the silent mutation—i.e., when the organism is crossed with another organism having the same biocontainment system—the transcription activator does not initiate overexpression of the coding region and the progeny organisms remain viable.

As used herein, the term "overexpression" refers to a level of transcription of the coding region that is greater than that of a suitable wild-type control. The overexpression of the coding region that occurs when the organism is crossed with a wild-type organism results in altered growth of the organism so that one can identify organisms that are progeny of a cross with a wild type organism. Altered growth can include reduced growth compared to a comparable wild-type organism or can include increased growth compared to a wild-type organism that results is reduced fitness (e.g., a deformity that results in death). Overexpression can refer tp ectopic expression, where genes are expressed in tissues where they are normally silent. Alternatively, or additionally, overexpression can refer to dysregulated expression, where the dynamic expression levels over time are perturbed such as, for example, a coding region that oscillates between an on-state and an off-state in wild-type that is constitutively in the on-state in the mutant.

In some cases, the result of cross between an organism having the biocontainment system and a wild-type organism can result in progeny that do not grow and/or are non-viable. In other cases, the result of cross between an organism having the biocontainment system and a wild-type organism can result in progeny that grow more slowly than organisms homozygous for the biocontainment system and are therefore readily identifiable and may be culled from the population. In still other embodiments, the result of cross between an organism having the biocontainment system and a wild-type organism can result in progeny that grow more rapidly than organisms homozygous for the biocontainment system, but the more rapid growth results in reduced fitness compared to the organisms homozygous for the biocontainment system.

As used herein, a "silent mutation" is a mutation in the DNA of the organism that does not significantly alter the phenotype of the organism outside of its effects within the context of the biocontainment system.

As used herein, the term "programmable transcription activator" refers to a transcription activator whose DNA binding specificity can be programmed. In the context of the biocontainment system described herein, the transcriptional activator is programmed to survey the genome of a cell for the wild-type transcription regulatory sequence that controls transcription of the target coding region, but does not bind to a variant of the transcription regulatory sequence that includes the silent mutation. While described herein in the context of an exemplary embodiment in which the programmable transcription activator is dCas9 fused to the activator domain VP64 and co-expressed with dCas9-VP64, other programmable transcription activators may be used in the biocontainment system. Exemplary alternative programmable transcription activators include, for example, fusions of dCas9, Cas9 (if combined with a short guide RNA), nuclease inactive CPF1, and TALEs to VP64, VP16, VPR, p65, Rta, EDLL, Gal4, TAD, SunTag or any combination thereof. In the case of RNA guided transcriptional regulators (e.g, dCas9-VP64), activation may be boosted by including aptamers in the RNA sequence which allow for the recruitment of aptamer binding protein such as, for example, transcription factor-fusions such as MS2/MCP, PCP, or COM fused to VP64, VP16, VPR, p65, Rta, and EDLL, Gal4, TAD or any combination thereof.

The coding region that is the target for overexpression can be any coding region whose overexpression is detrimental to growth of the organism to a degree sufficient to allow for easy identification of a hybrid cross between an organism having the biocontainment system and a comparable wild-type. In some cases, overexpression of the coding region can result a cross between an organism having the biocontainment system and a comparable wild-type being lethal—e.g., the progeny of the cross do not grow or are otherwise non-viable.

In some cases, the coding region encodes a cytoskeletal polypeptide, an ER-Golgi vesicle polypeptide, an mRNA processing polypeptide, an electron transport polypeptide, a nuclear trafficking polypeptide, a chromosome segregation polypeptide, a spindle pole duplication polypeptide, an oxidative stress polypeptide, a cell-signaling polypeptide, a pro-apoptotic polypeptide or a developmental morphogen polypeptide.

In some cases, an organism may be engineered to include a second biocontainment system involving the programmed overexpression of a second coding region in the absence of a second silent mutation in the transcriptional regulatory region of the second coding region. The second biocontainment system can include a second programmable transcription activator. The second programmable transcription activator may be the same as the first programmable transcription activator in all respects other than the transcription regulatory sequence it is programmed to survey. In other cases, the second transcription activator may include different components that the programmable transcription activator of the first biocontainment system.

EXAMPLES

Plasmids and Primers

Plasmid maps are shown in FIG. 6 and described in Table 8. Primer sequences are provided in Table 9.

Table 8. Plasmids

Plasmid	Description	Reference	GenBank
pMM2-20-1	Deleterious activation screening plasmid	this study	KX981587
pMM2-20-2 to 20-XX	pMM2-20-1 with spacers 1-XXX	this study	
pMM1-30A	sgRNA 2.0 cassette with a BsmbI site for oligos. BsaI releasable.	this study	KX981578
pMM1-32A	iCas9 vector. Contains destination for sgRNA cassettes.	this study	KX981579
pMM1-40A	MS2-VP64 integration cassette template.	this study	KX981582
pMM1-34Y	Integrating dCas9-VP64 cassette. sgRNA cassette destination.	this study	KX981581
pMM2-4A	Template for integrating dCas9-VP64. ACT1 sgRNA.	this study	
pMM2-22-2	Template for integrating dCas9-VP64. Random sgRNA.	this study	
pMM2-17-1	WT pACT1 driven TurboGFP	this study	KX981585
pMM2-17-2	Mutated pACT1 driven TurboGFP	this study	KX981586
pMM2-10-9	TurboRFP expression plasmid.	this study	KX981583
pMM2-10-10	TurboGFP expression plasmid	this study	KX981584
pCRCT	iCas9 source vector. Addgene #60621	1	
pICSL80004	TurboRFP source vector. Addgene #50325	2	
pICSL80005	TurboGFP source vector. Addgene #50322	2	
pCORE-UK	KlURA3 and KanMX4 source vector. Addgene #72238	3	
M-SPn-VP64	dCas9-VP64 source[a]. Addgene #48674	4	
pESC-Leu	Yeast replicative vector backbone source.	Agilent	
MS2-P65-HSF1_GFP	MS2 source plasmid. Addgene #61423	5	

1 Bao et al., 2014. *ACS Synth Biol* 4(5):585-594.
2 Engler et al., 2014. *ACS Synth Biol* 3(11):839–843.
3 Storici F and Resnick MA, 2003. *Genet Eng* 25:189–207.
4 Esvelt et al., 2013. *Nat Methods* 10(11):1116-1121.

5 Konerman et al., 2014. *Nature* 517(7536)583-588.

Table 9. Primers

Primer	Sequence	SEQ ID NO:
pMM2-20-16		
MM_WHI3_3F	GATCAGAGCAGATATCCAATAGTT	1
MM_WHI3_3R	AAACAACTATTGGATATCTGCTCT	2
pMM2-20-4		
MM_WHI3_4F	GATCGAAAGGGAAAGGAACTTCTT	3
MM_WHI3_4R	AAACAAGAAGTTCCTTTCCCTTTC	4
pMM2-20-28		
MM_Rando_F	GATCactgtataagactcttcaca	5
MM_Rando_F	AAACtgtgaagagtcttatacagt	6
Chromosomal Integration Primers		
MM_TA_LYS_uF	GGCATCGCACAGTTTTAGCGAGGAAAACTCTTCAATAG TTTTGCCAGCGGCATAGCTTCAAAATGTTTCTAC	7
MM_TA_LYS_uR	AATTCATATTTAATTATTGTACATGGACATATCATACG TAATGCTCAACCgggttaattaaggcgc	8
MM_d64_Leu2_F2	TTATAGAATTGTGTAGAATTGCAGATTCCCTTTTATGG ATTCCTAAATCCTCTTTGAAAAGATAATGTATGATTAT G	9
MM_d64_Leu2_R	TGAATTTCATTTATAAAGTTTATGTACAAATATCATAA AAAAAGAGAATCctcacataatgaaagagagag	10
Primers to Identify Genomic Modifications		
MM_TA_CPCR_F	GTTACGTCTATATTCATTGAAACTGA	11
MM_Kan_CPCR_R	AACCAAGCATGTCAAGGTC	12
MM_TA_WT_CPCR_R	ACTCTATATATCAATGCAGCC	13
MM_WT_Leu2_CPCR_F	TGGCCTCTTCAAGATTATGGA	14
MM_DV_Leu2_CPCR_F	tattgaaacttgttgaaacgT	15
MM_DV_Leu2_CPCR_R	CTGTATTCCTTTACATCCTCC	16

MM_Actg4_CPCR_F	CTACATTCTTCCTTATCGGATCC	17
MM_Actg4_CPCR_R	AGGAAGAATACAAGAGAGAGGA	18

Strains and Media

Detailed information for all yeast strains are provided in Table 10 and FIG. 7.

Table 10. Yeast strains

Name	Genotype	Description
YMM1[a]/YMM124[b]	*MAT*a, *lys2ΔMS2-VP64 KanMX4*	Used for screening growth defects caused by DVM
YMM31[a]/YMM125[b]	*MAT□ LEU2*	Wild-type *ACT1* promoter strain used for mating experiments
YMM27[a]	*MAT*a, KlURA3	Uracil prototroph
YMM134[b]	*MATa ACT1-Δ1 lys2ΔMS2-VP64 KanMX4 leu2ΔdCas9-VP64 Random sgRNA KlURA3*	Mutated *ACT1* promoter strain carrying DVM guided by random sgRNA
YMM139[b]	*MAT□ LEU2* pMM2-10-9 (*TurboRFP TRP1*)	Fluorescent wild-type *ACT1* promoter strain used for live-cell imaging
YMM30[a]/YMM141[b]	*MAT□ ACT1-Δ1 LEU2*	Mutated *ACT1* promoter strain used for mating experiments
YMM35[a]/YMM155[b]	*MATa ACT1-Δ1 lys2ΔMS2-VP64 KanMX4 leu2ΔdCas9-VP64 ACT1 sgRNA KlURA3*	Synthetic incompatible strain
YMM156[b]	*MATa ACT1-Δ1 lys2ΔMS2-VP64 KanMX4 leu2ΔdCas9-VP64 ACT1 sgRNA KlURA3* pMM2-10-10 (*TurboGFP TRP1*)	TurboGFP positive synthetic incompatible strain
YMM157[b]	*MATa ACT1-Δ1 lys2ΔMS2-VP64 KanMX4 leu2ΔdCas9-VP64 ACT1 sgRNA KlURA3* pMM2-10-10 (*TurboGFP TRP1*)	TurboGFP positive mutated *ACT1* promoter strain carrying DVM guided by random sgRNA
YMM158[b]	*MATa* pMM2-17-1 (*pACT1- TurboGFP LEU2)*	No DVM strain with wild-type *ACT1* promoter driving TurboGFP
YMM159[b]	*MATa* pMM2-17-2 (*pACT1-Δ1-TurboGFP LEU2)*	No DVM strain with mutated *ACT1* promoter driving TurboGFP
YMM160[b]	*MATa ACT1-Δ1 lys2ΔMS2-VP64 KanMX4 leu2ΔdCas9-VP64 Random sgRNA KlURA3* pMM2-17-1 (*pACT1- TurboGFP LEU2)*	Random guide DVM strain with wild-type *ACT1* promoter driving TurboGFP
YMM161[b]	*MATa ACT1-Δ1 lys2ΔMS2-VP64 KanMX4 leu2ΔdCas9-VP64 Random sgRNA KlURA3* pMM2-17-2 (*pACT1-Δ1-TurboGFP LEU2)*	Random guide DVM strain with mutated *ACT1* promoter driving TurboGFP
YMM162[b]	*MATa ACT1-Δ1 lys2ΔMS2-VP64 KanMX4 leu2ΔdCas9-VP64 ACT1 sgRNA KlURA3* pMM2-17-1 (*pACT1- TurboGFP LEU2)*	Synthetic incompatible strain with wild-type *ACT1* promoter driving TurboGFP

YMM163[b]	*MATa ACT1-Δ1 lys2ΔMS2-VP64 KanMX4 leu2ΔdCas9-VP64 ACT1 sgRNA KlURA3* pMM2-17-2 (*pACT1-Δ1-TurboGFP LEU2)*	Synthetic incompatible strain with mutated *ACT1* promoter driving TurboGFP

[a] Strain derived from the S288C (YNN216) background: *ura3-52 lys2-801*^{amber} *ade2-101*^{ochre}

[b] Strain derived from the CEN.PK background: *ura3-52 trp1-289 leu2-3_112 his3 Δ1 MAL2-8C SUC2*

Yeast transformations were performed using the Lithium-acetate method (Gietz et al., 2006. *Methods Mol. Biol.* 313:107–120). Chemically competent *E. coli* STBL3 (Thermo Fisher Scientific, Waltham, MA) was used for all plasmid cloning and propagation in LB media (MP) supplemented with appropriate antibiotics. All yeast strains were in the CEN.PK *MAT*a or *MAT*α (van Dijken et al., 2000. *Enzyme Microb. Technol.* 26:706–714) background. Yeast were grown at 28-30°C on plates or in liquid culture with 250 rpm agitation. Yeast were cultured in YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose), 2X YPD, or synthetic dropout (SD) media (1.7 g/L yeast nitrogenous base, 5 g/L ammonium sulfate, yeast synthetic dropout media supplements (Sigma-Aldrich, St. Louis, MO), 20 g/L dextrose). G418 sulfate resistant yeast were selected on YPD agar with 400 μg/ml G418 Sulfate. Counterselection for KlURA3 was performed using 1 g/L 5-floroorotic acid.

Insertion of the MS2-VP64 cassette in the *Lys2* locus was verified by PCR using primers MM_TA_CPCR_F and MM_Kan_CPCR_R which detect the presence of the transgene in the *Lys2* locus and MM_TA_CPCR_F and MM_TA_WT_CPCR_R which screen for the wild-type locus. (FIG. 7A). Insertion of the sgRNA and dCas9-VP64 cassette into *Leu2* locus was verified by PCR using MM_DV_Leu2_CPCR_F and MM_DV_Leu2_CPCR_R which detect the presence of the transgene and MM_WT_Leu2_CPCR_F and MM_DV_Leu2_CPCR_R which detect the wild-type locus (FIG. 7B). Mutations in the *Act1* promoter were detected by PCR amplifying a portion of the promoter using primers MM_Actg4_CPCR_F and MM_Actg4_CPCR_R. The gel purified amplicon was then Sanger sequenced using the MM_Actg4_CPCR_F primer.

Screening Candidate Coding Regions

The screening of target coding regions was performed by transforming yeast strain YMM124 (Table 10) with pMM2-20-1 backbone vectors (Table 8) expressing sgRNA to candidate coding regions (Table 11).

Table 11. Target coding regions

Gene	Function	Overexpression Phenotype [1]
ACT1	Actin. Cytoskeletal protein [2].	Inviable [3]
ABP1	Actin Binding Protein. Cytoskeletal regulation [4].	Inviable [3]
COX9	Subunit of cytochrome c oxidase [5].	Inviable [6]
SWT1	Endoribonuclease involved in mRNA quality control [7].	Inviable [8]
TUB2	β-Tubulin. Cytoskeletal protein [9].	Inviable [3]
WHI3	Regulator of cell cycle and cell size [10].	Inviable [10]
YIP3	Vesicular transport protein [11].	Inviable [12]

1 Cherry et al., 2012. *Nucleic Acids Res.*, vol. 40, no. Database issue, pp. D700-705.
2 Gallwitz D and Seidel R, 1980. *Nucleic Acids Res* 8(5):1043–1059
3 Liu et al., 1992. *Genetics* 132(3):665-673.
4 Drubin et al., 1988. *J Cell Biol* 107(6):2551-2561.
5 Wright et al., 1986. *J Biol Chem* 261(36):17183-17191.
6 Sopko et al., 2006. *Mol Cell* 21(3):319–330.
7 Röther et al., 2006. *J Biol Chem* 281(48):36518–36525.
8 Skružný et al., 2009. *PLoS Biol* 7(1):e1000008.
9 Neff et al., 1983. *Cell* 33(1):211–219.
10 Nash et al., 2001. *Genetics* 157(4):1469–1480.
11 Otte et al., 2001. *J Cell Biol* 152(3):503–518.
12 Geng et al., 2005. *Eukaryot Cell* 4(7):1166–1174.

Transformations were plated onto SD-Ura in 6-well plates and incubated at 30°C. To calculate growth rates of colonies on petri dishes, colonies were scanned as they grew using Epson Perfection V19 scanners in two hour intervals for 256 hours (Guillier et al., 2006. *J. Microbiol. Methods* 65:324–334; Baryshnikova et al., 2010. *Nat. Methods* 7:1017–1024). Image analysis was used to track the areas of colonies as they grew. This entailed converting RGB scans into HSV colorspace, selecting the V channel, performing a background subtraction, smoothing, and using a threshold to identify biomass. The V channel was selected because it had the highest contrast with the background. The background was the first image in a time-lapse, before any colonies appeared. Images were smoothed twice with a fine-grain Gaussian filter (sd = 1 pixel, filter width = 7 pixels) to remove noise. A single threshold was used for all images for consistency.

Colony centers were identified by applying regional peak detection to a z-projection through time using the thresholded images. When colonies merged, these peaks were used to find the dividing line between colonies: the peaks were used as seeds in a watershed on a distance-transformed image. Once colony boundaries were identified, the number of "on" pixels within a boundary at each moment in time was counted as the colony's area. Colonies that fell along the edge of the petri dish, that merged with colonies along the edge, or that had an ambiguous

number of peaks within a large merged region were not included in the analysis. To calculate growth rates, the area-over-time data were log-transformed and fit into a line in a 12-hour moving window. The maximum slope in each time series was recorded as that colony's growth rate. The growth rates were analyzed by one-way ANOVA followed by Bonferroni's post-test comparing each condition to the random sgRNA control.

Plate Based Mate Assay

Haploid *MATa* yeast strain YMM134 and YMM155 were mated to *MATα* strains YMM125 and YMM141 by combining overnight cultures in YPD to an OD_{600} of 0.1 each in 1 ml YPD. The cultures were then incubated at 30°C for four hours, washed once with water and 30 μL were plated onto SD-Ura/Leu dropout media.

Flow Cytometry

Flow cytometry was performed using yeast strains YMM158 through YMM163. YMM158, YMM160, and YMM162 expressed TurboGFP driven by the wild-type *ACT1* promoter from plasmid pMM2-17-1. YMM159, YMM161, and YMM163 contained pMM2-17-2 and expressed TurboGPF from a mutated *ACT1* promoter. Overnight cultures grown in 2 mL SD-Complete media were diluted to an $OD_{600} = 0.5$ and grown for an additional four hours. Cells were collected by centrifugation, washed with DPBS, resuspended in DPBS and placed on ice protected from light prior to analysis. Flow cytometry was performed using a LSRFortessa H0081 cytometer. At least 30,000 TurboGFP positive singlet events were collected per sample. The geometric means of GFP fluorescence intensity were compared using one-way ANOVA followed by Tukey's post-test for pairwise comparisons.

Live-Cell Imaging

Yeast strain YMM139 was mated separately with YMM156 and YMM157 in SD-Trp dropout media for 2 hours, pelleted, and resuspended in SD-Ura/Leu/Trp. Mated yeast were loaded onto a CellASIC ONIX diploid yeast plate and supplemented with SD-Ura/Leu/Trp. Cells were imaged using a Nikon Ti-E Deconvolution Microscope System every six minutes for 20 hours.

In the preceding description and following claims, the term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements; the terms "comprises," "comprising," and variations thereof are to be construed as open ended—i.e., additional elements or steps are optional and may or may not be present; unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one; and the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

In the preceding description, particular embodiments may be described in isolation for clarity. Unless otherwise expressly specified that the features of a particular embodiment are incompatible with the features of another embodiment, certain embodiments can include a combination of compatible features described herein in connection with one or more embodiments.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously. The particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference in their entirety. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are

approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. A cell comprising a biocontainment system that comprises:

a coding region whose overexpression alters growth of the cell;

a transcription regulatory region operably linked upstream of the coding region, and comprising a silent mutation; and

a polynucleotide that encodes a programmable transcription activator engineered to bind to the transcription regulatory region in the absence of the silent mutation, thereby overexpressing the coding region in the absence of the silent mutation, but does not initiate overexpression of the coding region when the transcription regulatory region comprises the silent mutation.

2. The cell of claim 1 wherein the cell is a single-celled organism.

3. The cell of claim 1 wherein the cell is a germ cell of a multicellular organism.

4. The cell of any preceding claim wherein the programmable transcription activator comprises dCas9 fused to an activation domain.

5. The cell of any preceding claim wherein the coding region encodes a cytoskeletal polypeptide, an ER-Golgi vesicle polypeptide, an mRNA processing polypeptide, an electron transport polypeptide, a nuclear trafficking polypeptide, a chromosome segregation polypeptide, a spindle pole duplication polypeptide, or an oxidative stress polypeptide.

6. The cell of any preceding claim wherein overexpression of the coding region is lethal to the cell.

7. The cell of any preceding claim further comprising a second biocontainment system comprising:

a second coding region whose overexpression decreases growth of the cell;

a second transcription regulatory region operably linked upstream of the second coding region, and comprising a second silent mutation;

a polynucleotide that encodes a second programmable transcription activator engineered to bind to the second transcription regulatory region in the absence of the second silent mutation, thereby overexpressing the second coding region in the absence of the second silent mutation, but does not initiate overexpression of the second coding region when the second transcription regulatory region comprises the second silent mutation.

8. The cell of claim 7 wherein the second coding region encodes a cytoskeletal polypeptide, an ER-Golgi vesicle polypeptide, an mRNA processing polypeptide, an electron transport polypeptide, a nuclear trafficking polypeptide, a chromosome segregation polypeptide, a spindle pole duplication polypeptide, or an oxidative stress polypeptide.

9. The cell of any preceding claim wherein the altered cell growth comprises a decrease in growth rate of a cell heterozygous for the biocontainment system compared to a suitable control.

10. The cell of any preceding claim wherein the altered cell growth comprises an increase in growth rate of a cell heterozygous for the biocontainment system, the increase in growth rate decreasing fitness of the cell compared to a suitable control.

11. The method of claim 9 or claim 10 wherein the suitable control comprises a wild-type cell.

12. The method of claim 9 or claim 10 wherein the suitable control comprises a cell homozygous for the biocontainment system.

13. A method of limiting hybridization of a genetically-modified organism with a genetically dissimilar variant, the method comprising:

providing an organism genetically modified to include the biocontainment system of any preceding claim, wherein a cross between the genetically-modified organism and the genetically dissimilar variant organism results in progeny that exhibit a phenotype that is distinct from the genetically-modified organism.

14. The method of claim 13 wherein the genetically dissimilar variant comprises a wild-type organism.

15. The method of claim 13 wherein the genetically dissimilar variants comprises a different genetic modification compared to the genetically-modified organism having the biocontainment system.

16. The method of any one of claims 13-15 wherein the phenotype exhibited by the progeny comprises lethality.

FIG. 1

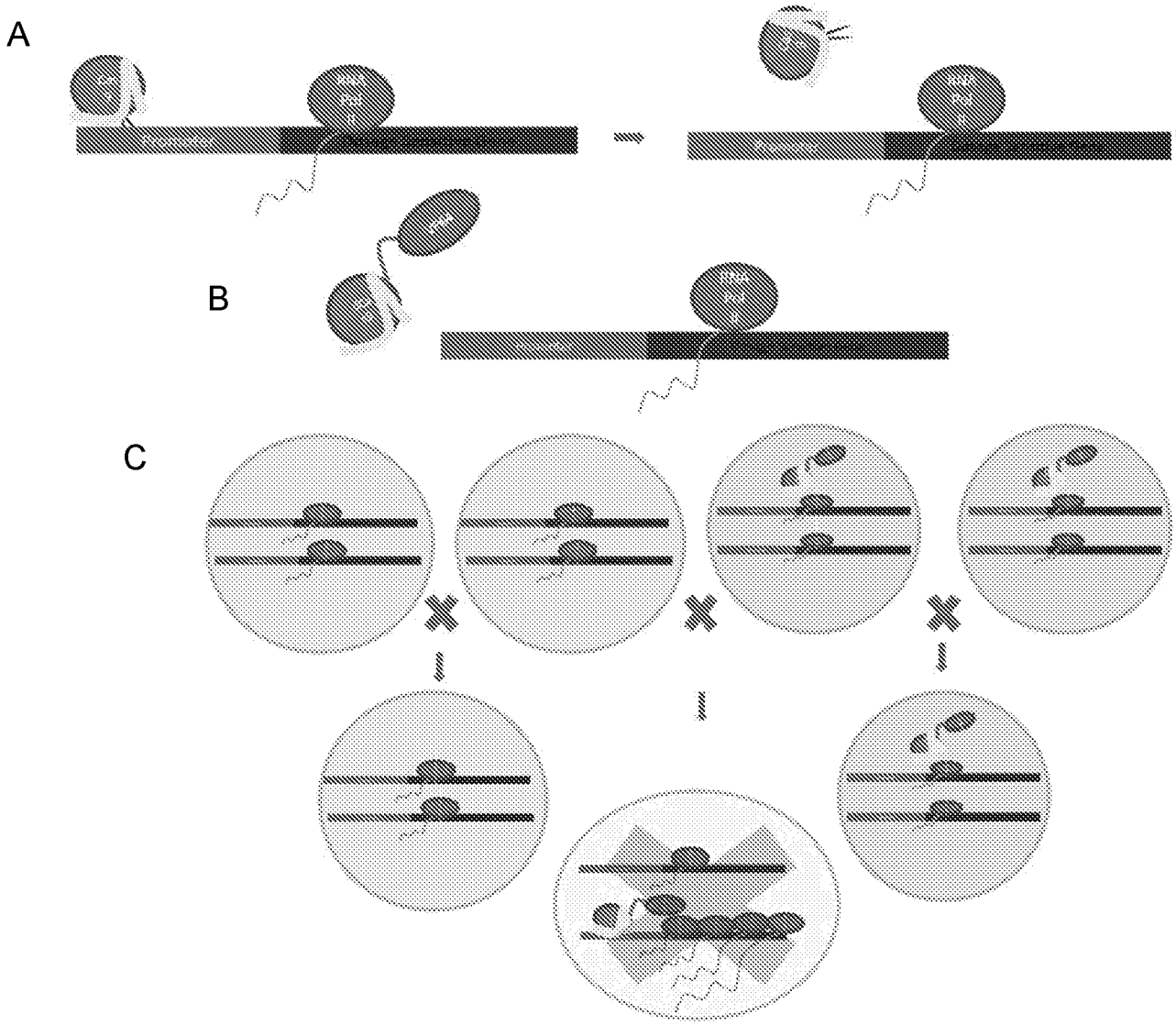


FIG. 2

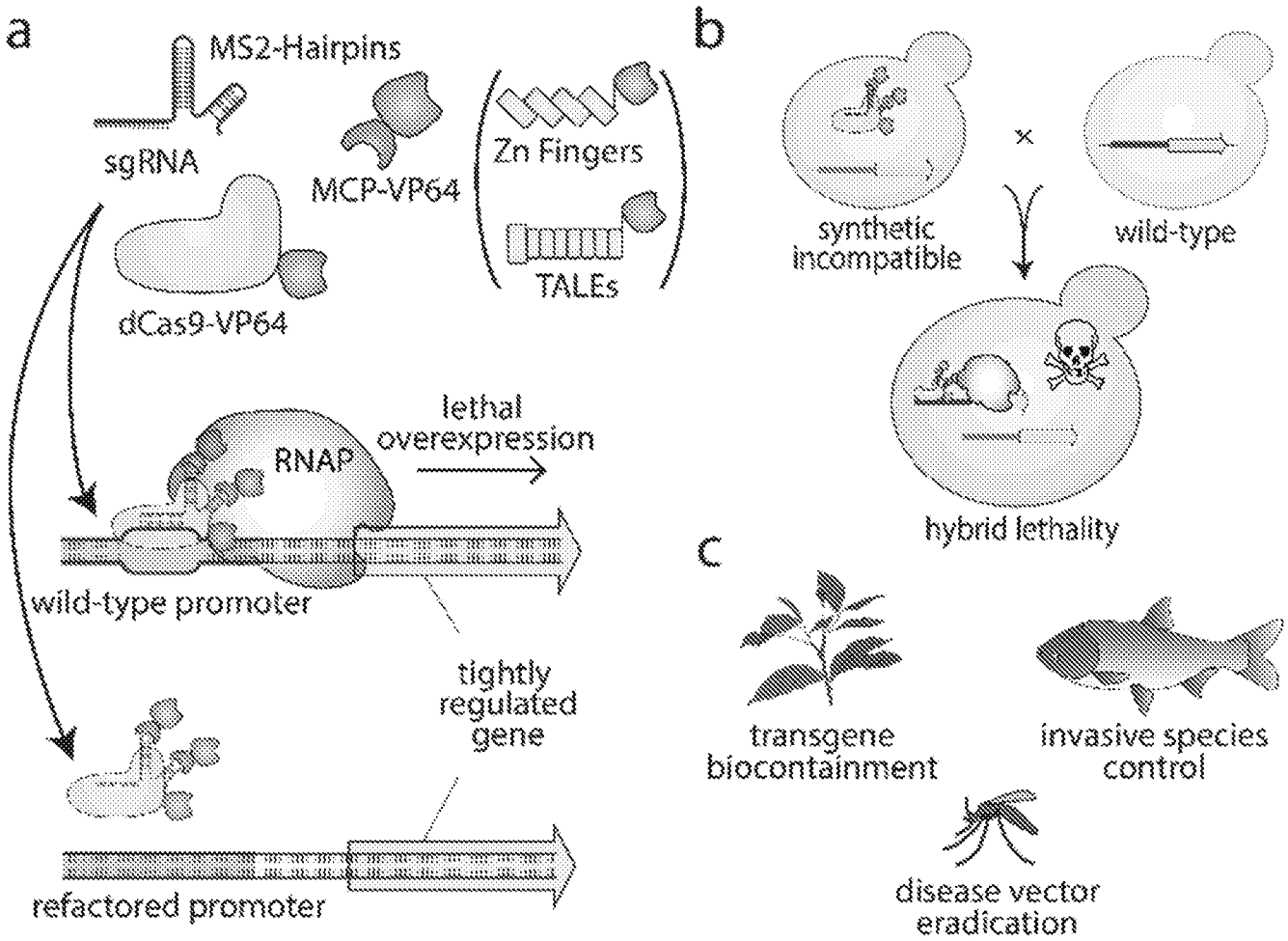

a
MS2-Hairpins
sgRNA
MCP-VP64
Zn Fingers
TALEs
dCas9-VP64
RNAP
lethal overexpression
wild-type promoter
tightly regulated gene
refactored promoter
b
synthetic incompatible
×
wild-type
hybrid lethality
c
transgene biocontainment
invasive species control
disease vector eradication

FIG. 3

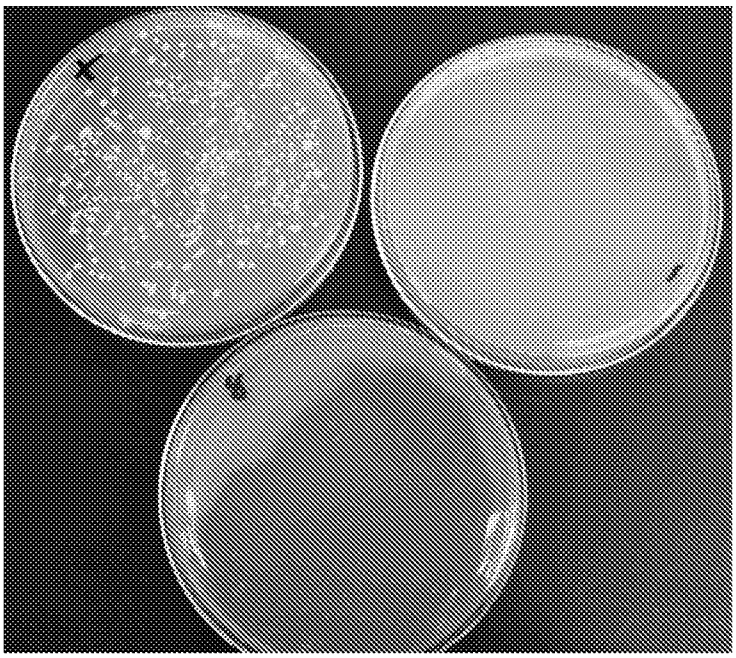


FIG. 4

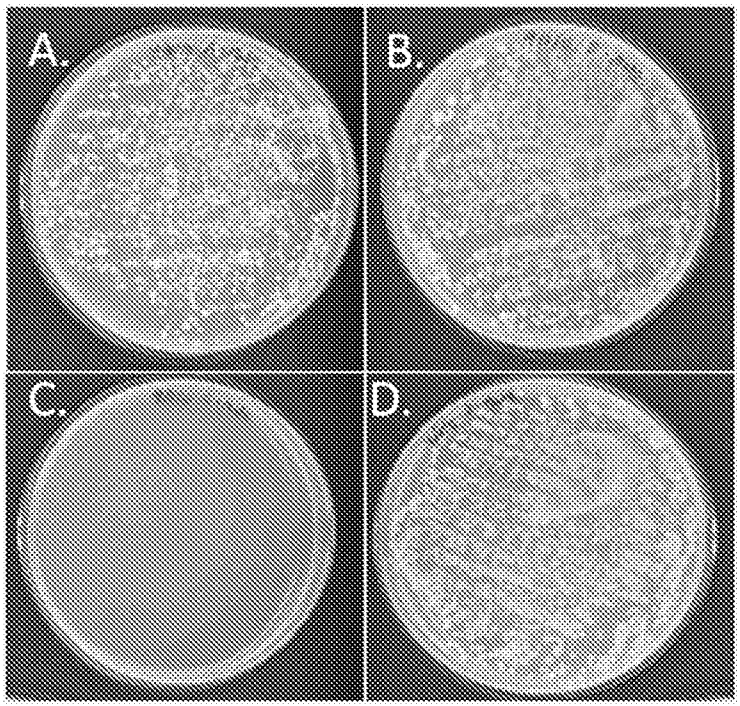


FIG. 5

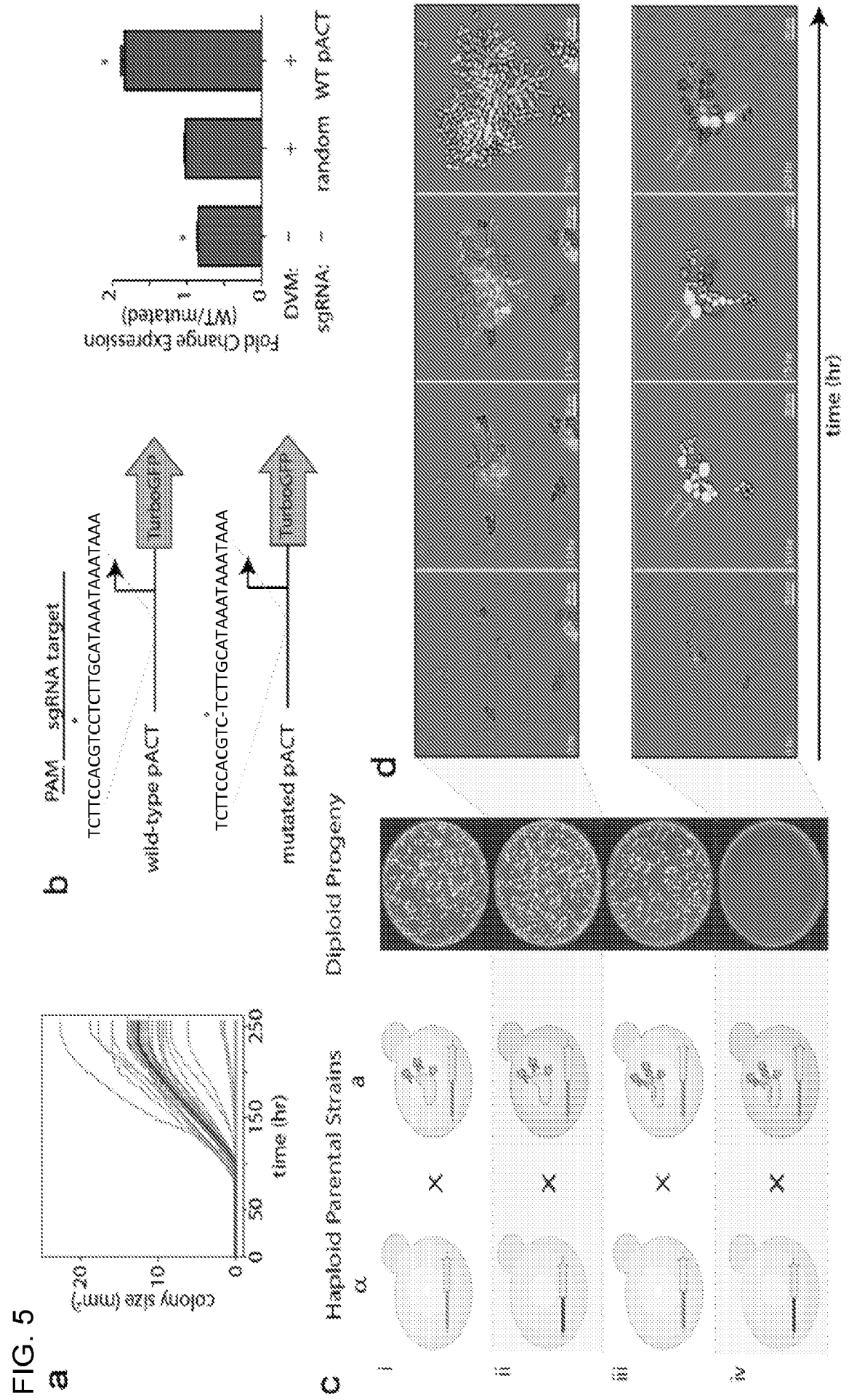

a
colony size (mm²)
time (hr)
b
PAM
sgRNA target
TCTTCCACGTCCTCTTGCATAAATAAATAAA
wild-type pACT
mutated pACT
Fold Change Expression (WT/mutated)
DVM:
sgRNA:
random
WT pACT
c
Haploid Parental Strains
α
a
Diploid Progeny
d
time (hr)

FIG. 6

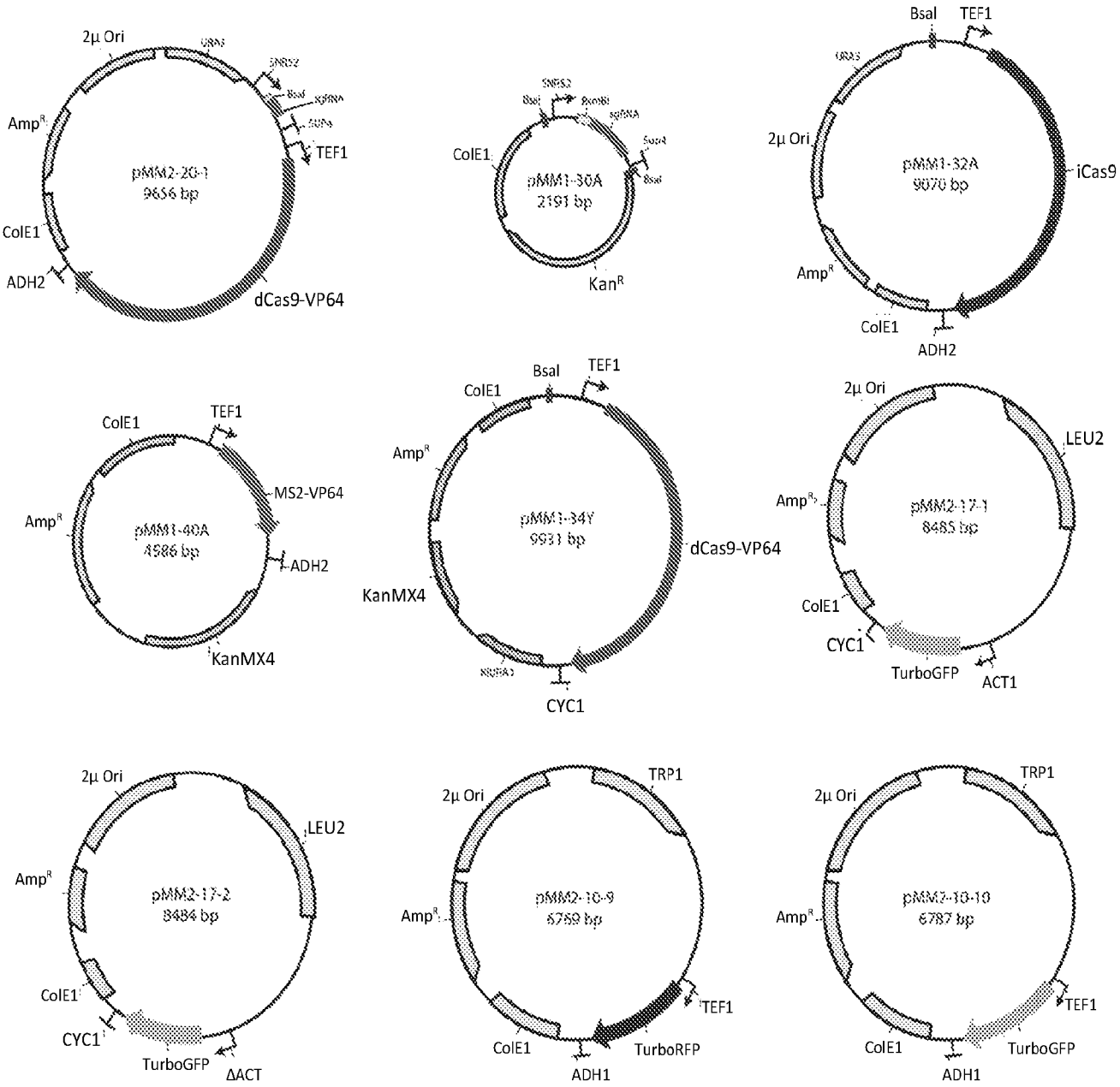

2μ Ori
Amp^R
pMM2-20-1
9656 bp
TEF1
ColE1
ADH2
dCas9-VP64
ColE1
pMM1-30A
2191 bp
Kan^R
Bsal
TEF1
2μ Ori
pMM1-32A
9070 bp
iCas9
Amp^R
ColE1
ADH2
ColE1
TEF1
Amp^R
pMM1-40A
4586 bp
MS2-VP64
ADH2
KanMX4
ColE1
Bsal
TEF1
Amp^R
pMM1-34Y
9931 bp
dCas9-VP64
KanMX4
CYC1
2μ Ori
LEU2
Amp^R
pMM2-17-1
8485 bp
ColE1
CYC1
TurboGFP
ACT1
2μ Ori
LEU2
Amp^R
pMM2-17-2
8484 bp
ColE1
CYC1
TurboGFP
ΔACT
TRP1
2μ Ori
Amp^R
pMM2-10-9
6789 bp
TEF1
ColE1
TurboRFP
ADH1
TRP1
2μ Ori
Amp^R
pMM2-10-10
6787 bp
TEF1
ColE1
TurboGFP
ADH1

FIG. 7

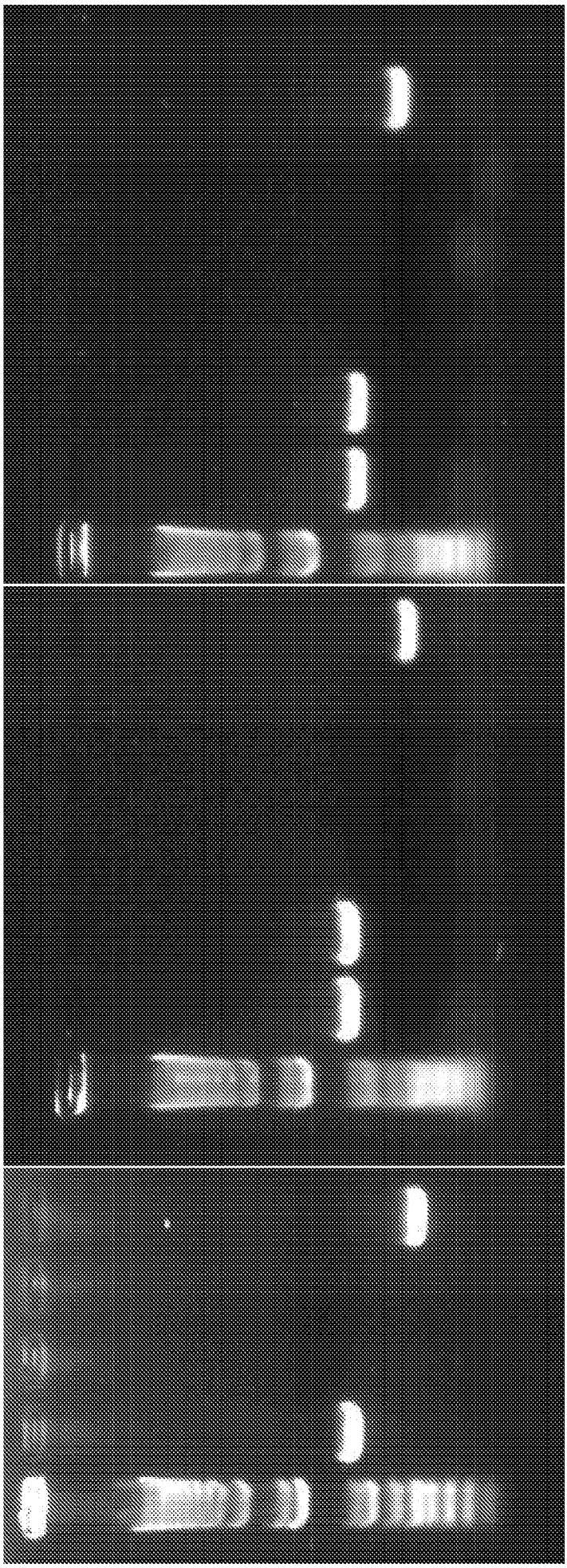
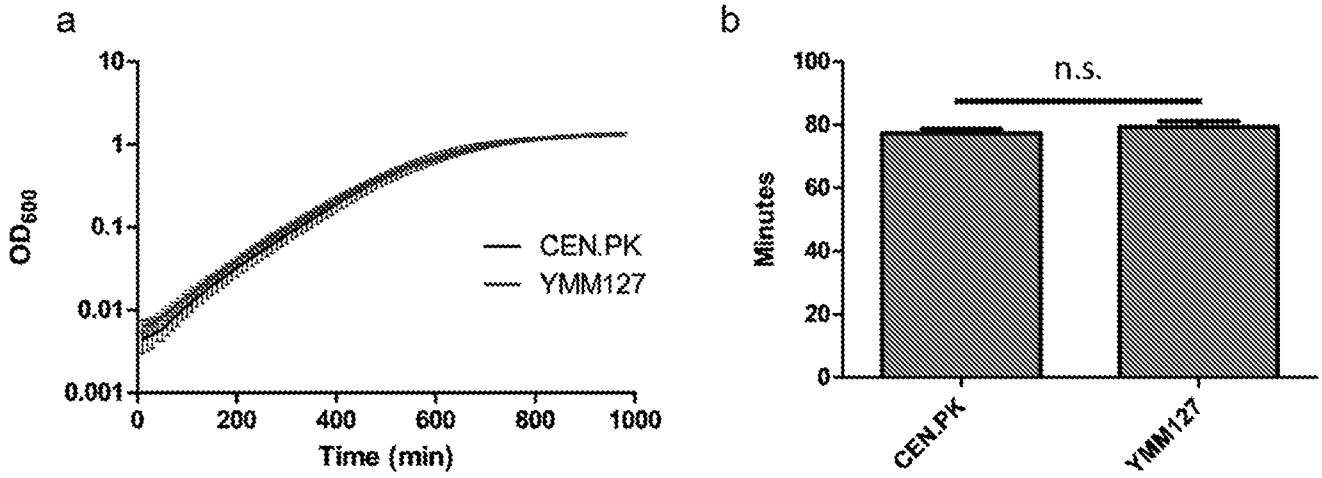

A
MS2-VP64
YMM124 CEN.PK
Wild-Type
YMM124 CEN.PK
B
MS2-VP64
YMM134 YMM155 CEN.PK
Wild-Type
YMM134 YMM155 CEN.PK
C
dCas9-VP64
YMM134 YMM155 CEN.PK
Wild-Type
YMM134 YMM155 CEN.PK

FIG. 8


a
10
1
0.1
0.01
0.001
OD_{600}
0
200
400
600
800
1000
Time (min)
CEN.PK
YMM127
b
100
80
60
40
20
0
Minutes
n.s.
CEN.PK
YMM127

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/61297

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/00; C12N 1/00; C12N 15/00; C07H 21/04 (2016.01)
CPC - C12N 5/06; C12N 1/16; C12N 15/00; C12N 2800/10; C12N 15/52; C12N 15/11
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12N 5/00; C12N 1/00; C12N 15/00; C07H 21/04 (2016.01); USPC - 435/325; 435/254.2; 435/320.1; 536/23.2; 536/23.1
CPC - C12N 5/06; C12N 1/16; C12N 15/00; C12N 2800/10; C12N 15/52; C12N 15/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8) - C12N 5/00; C12N 1/00; C12N 15/00; C07H 21/04 (2016.01); USPC - 435/325; 435/254.2; 435/320.1; 536/23.2; 536/23.1
CPC - C12N 5/06; C12N 1/16; C12N 15/00; C12N 2800/10; C12N 15/52; C12N 15/11 - see keyword below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(USPT,PGPB,EPAB,JPAB); PatBase; Medline, Google: cell, biocontainment, system, coding region, overexpression, alter, growth, apoptosis, death, transcription regulatory region, promoter, operably, upstream, silent mutation, polynucleotide, encode, programmable, transcription activator, CRISPR, Cas9, dCas9, gRNA, yeast, E.coli, cerevisiae, sw

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KIANI et al., Cas9 gRNA engineering for genome editing, activation and repression. Nat Methods. 2015, Vol. 12(11), p. 1051-4. Epub 2015 Sep 7. Abstract; pg 1501, col 2, para 2; pg 1502, col 1, para 1, para 2, and last para, and Fig 1; pg 1503, col 2, lower para, and Fig 2; pg 1505/Online Method, col 1, para 1, and col 2, para 3; and pg 1506/the Last page, col 1, middle para	1-2, 4
Y	CHAPPELL et al., A renaissance in RNA synthetic biology: new mechanisms, applications and tools for the future. Curr Opin Chem Biol. 2015 Oct, Vol. 28, p. 47-56. Epub 2015 Jun 18. Abstract; pg 48, col 1, and col 2, top para; pg 51, col 1, middle para and last para, and col 2, top para; and pg 54, col 2, Ref. 12	1-2, 4
Y	US 2006/0147978 A1 (LORENS et al.) 06 July 2006 (06.07.2006), para [0002], [0073], and [0168]	1-2, 4
Y	US 2009/0155854 A1 (YUEH et al.) 18 June 2009 (18.06.2009), Abstract, para [0008], [0044], and [0057]	1-2, 4
A	BIKARD et al., Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic Acids Res. 2013, Vol. 41(15), p.7429-37. PDF File: pg 1-9. Entire documentation, especially Abstract; pg 2, col 1, top para, and middle para; pg 3, Fig 1; pg 5, Fig 2; and pg 6, col 1, middle para	1-2, 4
A	US 2014/0356958 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 04 December 2014 (04.12.2014), para [0087]	1-2, 4
A	FERNANDO et al., Molecular circuits for associative learning in single-celled organisms. J R Soc Interface. 2009, Vol. 6(34), 463-9. Abstract	1-2, 4

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 January 2017	02 MAR 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/61297

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GALLAGHER et al., Multilayered genetic safeguards limit growth of microorganisms to defined environments. Nucleic Acids Res. 2015 Feb, Vol. 43(3), p.1945-54. Epub 2015 Jan 7. Entire documentation, especially Abstract; pg 1945, col 2; and pg 1946, Fig 1	1-2, 4
A	DICARLO et al., Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res. 2013, Vol. 41(7), p. 4336-43. PDF File: pg 1-8. Entire documentation, especially Abstract, pg 2, Fig 1; and pg 5, Fig 2	1-2, 4
A	US 2015/0064138 A1 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 05 March 2015 (05.03.2015), para [0005], [0016], [0017], [0030], [0056], [0058], [0059], and [0094]	1-2, 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/61297

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-4, directed to a cell comprising a biocontainment system. The cell will be searched to the extent that the cell encompasses the first named species, wherein the cell is a single-celled organism. It is believed that claims 1-2, 4/(1-2) encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass wherein the cell is a single-celled organism. Additional cell will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected cell. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be wherein the cell is a germ cell of a multicellular organism [claims: (1), 3, (4/(1)), 4/(3)].

**********Continued in the extra sheet**********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2, 4/(1-2), limited to a single-celled organism

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/61297

Continuation of:
Box No III (unity of invention is lacking)

se, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature
Among Groups I+, a cell that is a single-celled organism is structurally and functionally different from a cell that is a germ cell of a multicellular organism.

Common Technical Features
The inventions of Groups I+ share the technical feature of a cell comprising a biocontainment system that comprises:
a coding region whose overexpression alters growth of the cell;
a transcription regulatory region operably linked upstream of the coding region, and comprising a silent mutation; and
a polynucleotide that encodes a programmable transcription activator engineered to bind to the transcription regulatory region in the absence of the silent mutation, thereby overexpressing the coding region in the absence of the silent mutation, but does not initiate overexpression of the coding region when the transcription regulatory region comprises the silent mutation (claim 1).

However, these shared technical features do not represent a contribution over prior art as being obvious over an article entitled 'Cas9 gRNA engineering for genome editing, activation and repression' by KIANI et al. (hereinafter 'Kiani'; Nat Methods. 2015, Vol. 12(11), p. 1051-4. Epub 2015 Sep 7), in view of an article entitled 'A renaissance in RNA synthetic biology: new mechanisms, applications and tools for the future' by CHAPPELL et al. (hereinafter 'Chappell'; Curr Opin Chem Biol. 2015 Oct, Vol. 28, p. 47-56), and further in view of US 2006/0147978 A1 to LORENS et al. (hereinafter 'Lorens'), and US 2009/0155854 A1 to YUEH et al. (hereinafter 'Yueh') as follows:

Kiani discloses a cell comprising a system (pg 1502, col 1, para 1 - 'using aptamer-based gRNA tethering systems, we could endow Cas9 in cell lines and organisms already expressing the protein with the ability to ...activate a set of targets') that comprises:
-- a coding region whose expression alters the cell function (pg 1501, col 2, para 2 - 'genes encoding structural proteins (ACTC1 and TTN)...and a protein critical to tissue oxygen delivery (HBG1)', wherein each of 'genes encoding structural proteins (ACTC1 and TTN) ...and a protein critical to tissue oxygen delivery (HBG1)' is 'a coding region whose expression alters the cell function');
-- a transcription regulatory region operably linked upstream of the coding region (pg 1501, col 2, para 2 - 'the promoter regions of genes encoding structural proteins (ACTC1 and TTN) ...and a protein critical to tissue oxygen delivery (HBG1)... expression level for all targets tested', wherein each of 'the promoter regions' is 'a transcription regulatory region' which must be 'operably linked upstream of the coding region' to achieve 'expression... for all targets'; pg 1051, Fig 1, Legend - 'Activation ...endogenous genes in HEK293T cells'; Fig 1(a-c), which shows targeted gene expression; pg 1052, col 2, last para - 'a CRISPR-activatable promoter (CAP) was placed upstream of tdTomato fluorescent protein', indicating a promoter/transcription regulatory region operably linked upstream of the coding region'; Specification: pg 8, ln 5-6 - 'promoter ... Transcription from the mutated promoter was characterized by expressing TurboGFP'); and
-- a polynucleotide that encodes a programmable transcription activator engineered to bind to the transcription regulatory region in the absence of a silent mutation (pg 1056/the last pg, col 1, middle para - 'Vector design and construction.... dCas9-VPR', wherein 'Vector design and construction.... dCas9-VPR' is 'a polynucleotide that encodes a programmable transcription activator engineered to bind to the transcription regulatory region in an absence of the silent mutation', please see the quotations and explanations that follow; pg 1051, col 1, para 1 - 'we fused a potent transcriptional activator (VPR) ...a fusion between nuclease-null Cas9 and VPR (dCas9-VPR)'; pg 1501, col 2, para 2 - 'we targeted ...dCas9-VPR to the promoter regions of genes encoding structural proteins (ACTC1 and TTN), ...and a protein critical to tissue oxygen delivery (HBG1)...Cas9-VPR in conjunction with a 14-nt gRNA was able to generate expression equivalent to at least 40% of the expression level for all targets tested when compared with dCas9-VPR with a 20-nt gRNA', wherein each of 'the promoter regions of genes encoding ... proteins' is 'a transcription regulatory region', and wherein 'targeted ...dCas9-VPR to the promoter regions of genes' is based on 'a polynucleotide that encodes a programmable transcription activator engineered to bind to the transcription regulatory region in the absence of a silent mutation', which is achieved through gRNA recognizing the targeted gene sequence, and wherein different lengths of gRNA control the expression level of the targeted gene expression by 'dCas9-VPR'; pg 1052, Fig 1(a-c), wherein the level of each gene expression induced by 'dCas9-VPR' changes with the length of gRNA; Please Note: without specifying silent mutation indicating 'the transcription regulatory region in the absence of a silent mutation', this explanation will not be repeated further in the following discussion; pg 1055/Online Method, col 1, para 1 - 'HEK293T cells were cotransfected with gRNAs of various lengths'; pg 1055, col 2, para 3 - 'Cells were seeded ... and transfected with 200 ng of Cas9 construct'; Please further see an article entitled 'Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system' by BIKARD et al.; Nucleic Acids Res. 2013, Vol. 41(15), p.7429-37; PDF File, pg 1-9: pg 3, Fig 1A, for the molecular base of gRNA guided dCas9 binding to its targeted gene),
-- thereby expressing the coding region in the absence of the silent mutation (pg 1501, col 2, para 2 - 'we targeted ...dCas9-VPR to the promoter regions of genes encoding structural proteins (ACTC1 and TTN) ...and a protein critical to tissue oxygen delivery (HBG1)...Cas9-VPR in conjunction with a 14-nt gRNA was able to generate expression equivalent to at least 40% of the expression level for all targets tested when compared with dCas9-VPR with a 20-nt gRNA', wherein each of 'the promoter regions' is 'a transcription regulatory region', and wherein 'generate expression... the expression level for all targets tested' is an indication of 'thereby expressing the coding region in the absence of the silent mutation'; Please Note: 'Cas9' has a nuclease cleavage activity, 'Cas9-VPR in conjunction with a 14-nt gRNA was able to generate expression' is because the cleavage activity of 'Cas9' requires '20-nt gRNA', while 'dCas9-VPR' only has activation activity; pg 1051, Fig 1, Legend - 'Activation and cutting of endogenous genes in HEK293T cells'; Fig 1(a-c), which shows 'dCas9-VPR' induces targeted gene expression at the highest level with 'a 20-nt gRNA', with 'a 14-nt gRNA' its induced targeted gene expression is similar with the level induced by 'Cas9-VPR', because with 'a 14-nt gRNA', the 'Cas9' in 'Cas9-VPR' lost its nuclease cleavage activity; See pg 1052, col 1, para 1 for full explanations).

**********Continued in the next extra sheet**********

Continuation of:
The previous extra sheet - Box No III (unity of invention is lacking)

Kiani further discloses wherein the system utilizes CRISPR (clustered, regularly interspaced, short palindromic repeats)-multifunctional Cas9 protein system with Cas9 associated guide RNA (gRNA) (pg 1052, col 1, para 2 - 'we generated a number of synthetic transcriptional devices and layered circuits in human cells using the multifunctional CRISPR (clustered, regularly interspaced, short palindromic repeats)-multifunctional Cas9 protein system to test the utility of such a system for synthetic biology purposes'; Abstract - 'altering the length of Cas9-associated guide RNA (gRNA) we were able to control Cas9 nuclease activity and simultaneously perform ...transcriptional regulation with a single Cas9 protein'; pg 1053, col 2, lower para, see below for the quotation), wherein the system can be extended to safely switch the transcriptional regulation (pg 1053, Fig 2, Legend - 'Design and experimental analysis of human cells with multifunctional CRISPR devices and circuits...(b) Top, schematic of parallel Cas9-VPR and14-nt gRNA-based transcriptional repression and activation devices in a single cell'; Fig 2(b); pg 1053, col 2, lower para - 'nuclease-positive Cas9 can be easily endowed with other previously described dCas9 activities... such as in vivo chromosomal tracking... they could facilitate the development of multifunctional synthetic genetic safety circuits with potential biomedical applications').

Kiani neither specifically teaches wherein the system is a biocontainment system, nor teaches wherein the expression is overexpression and wherein the coding region whose overexpression alters growth of the cell; or the transcription regulatory region comprising a silent mutation, and wherein the programmable transcription activator does not initiate overexpression of the coding region when the transcription regulatory region comprises the silent mutation. Chappell discloses RNA regulators have become new powerful tools for developing programmable genetic regulatory systems including transcriptional regulation system (Abstract - 'a central focus of synthetic biology has been to develop programmable genetic regulatory systems. ... turning to RNA regulators for this task because of their versatility... new powerful RNA design principles'; pg 48, col 1, para 1 - 'New RNA regulatory mechanisms solve key challenges and create new capabilities.. resurgence of RNA synthetic biology has brought about a host of new RNA regulators that control transcription....RNA regulatory switches'), wherein the regulation system includes a biocontainment system for safeguards limiting growth of cells in defined environments (pg 48, col 1, lower para to col 2, top para - 'synthetic regulatory RNAs...have been used in applications ranging from biocontainment [12]'; pg 54, col 2. Ref. 12 - 'Gallagher ...Multilayered genetic safeguards limit growth of microorganisms to defined environments'), and further CRISPR-Cas9-gRNA system is the best regulatory system (pg 51, col 1, middle para - 'Repurposed CRISPR systems - the best of RNAs and proteins... Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) interference (CRISPRi) mechanism combine the best of both worlds in a hybrid system'; pg 51, col 1, last para - 'CRISPR system and consists of two components: a chimeric RNA named the small guide RNA (sgRNA) and a catalytically dead mutant of the Cas9 endonuclease protein (dCas9)').

Chappell also neither specifically teaches wherein the expression is overexpression and wherein the coding region whose overexpression alters growth of the cell; nor teaches wherein the transcription regulatory region comprising a silent mutation, and wherein the programmable transcription activator does not initiate overexpression of the coding region when the transcription regulatory region comprises the silent mutation. Lorens discloses a method for screening cells with altered phenotypes, wherein the cells comprising an expression system including a report protein (para [0002] - 'screening for altered cellular phenotypes using an inducible expression system to enrich for and detect the altered phenotypes' ; para [0073] - 'sort a population or subpopulation of cells having a parent phenotype from a subpopulation of cells having an altered phenotype....an altered phenotype can be detected and collected...the fusion nucleic acids further comprise a sequence encoding a reporter protein'), whose overexpression alters growth of the cell (para [0168] - 'reporter gene encodes a death gene that causes the cells to die when expressed... death results from high expression of the gene ... overexpression of a number of programmed cell death (PCD) proteins known to cause cell death, including, ... caspases, bax, TRADD, FADD, SCK, MEK').

Lorens also does not specifically teach wherein the transcription regulatory region comprising a silent mutation, and wherein the programmable transcription activator does not initiate overexpression of the coding region when the transcription regulatory region comprises the silent mutation. Yueh discloses a method for modulating a targeted gene expression, the method comprising introducing a silent mutation into the promoter region (which is a transcription regulatory region, as discussed above) (Abstract - 'functional flavivirus cDNA in a prokaryotic cell, such as E. coli. ...modified flavivirus cDNA that has one or more silent mutations in one or more prokaryotic promoter regions within a flavivirus cDNA. The silent mutation decreases or abolishes the promoter activity from the prokaryotic promoter region'; para [0008] - 'The silent mutation decreases or abolishes the promoter activity from the prokaryotic promoter region, thus reducing the cryptic expression of one or more toxic polypeptides from the flavivirus cDNA within the prokaryotic cell'), and a method for designing silent mutations for various of promoters based on prediction software (para [0057] - 'Various promoter prediction software can be used to assist the design of silent mutations that can be introduced into a promoter region to decrease or abolish the promoter activity').

Although Yueh also does not specifically teach wherein the programmable transcription activator does not initiate overexpression of the coding region when the transcription regulatory region comprises the silent mutation, one of the ordinary skill in the art at the time the invention was made would have known to introduce a silent mutation to the transcription regulatory region, so that 'wherein the programmable transcription activator does not initiate overexpression of the coding region when the transcription regulatory region comprises the silent mutation', based on the combination of Yueh, Lorens, Chappell, and Kiani, especially Lorens discloses over-expressing a targeted gene induces alteration of growth of cells for cell screening (para [0002]; para [0073]; para [0168]; All quotations as above), while Kiani discloses wherein the programmable transcription activator activating its targeted gene expression is based on the lengths of the matched seqeunces between gRNA of 14-nt (nucleotides) to 20-nt with the targeted gene sequence (pg 1501, col 2, para 2; pg 1052, Fig 1(a-c); All quotations and explanations as above). Therefore, one of the ordinary skill in the art at the time the invention was made would have known to introduce a silent mutation to the transcription regulatory region, so that the gRNA will have less than 14-nt match to the targeted gene transcription regulatory region to obtain 'wherein the programmable transcription activator does not initiate overexpression of the coding region when the transcription regulatory region comprises the silent mutation', based on the combination of Kiani, Yueh, and Lorens, especially it was well known in the art long before the invention was made that gRNA having 14-nt properly match to a targeted gene sequence is required for a Cas9 binding to the targeted gene for carrying out associated regulation, as evident by US 2014/0356958 A1 to PRESIDENT AND FELLOWS OF HARVARD COLLEGE (para [0087] - 'maximally efficient targeting by a gRNA is achieved by. ... mispairing of the six 5'-most nt of a 20 bp gRNA against its genomic target does not abrogate Cas9-mediated cleavage so long as the last 14 nt pairs properly'), and BIKARD et al. (pg 5, Fig 2).

**********Continued in the next extra sheet**********

Continuation of:
The previous extra sheet - Box No III (unity of invention is lacking)

It would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the teachings of Kiani, Chappell, Lorens, and Yueh, to obtain a cell comprising a system that comprises: a coding region whose expression alters the cell function; a transcription regulatory region operably linked upstream of the coding region; and a polynucleotide that encodes a programmable transcription activator engineered to bind to the transcription regulatory region in the absence of a silent mutation, thereby expressing the coding region in the absence of the silent mutation, based on the teaching of Kiani; and wherein the system is a biocontainment system, based on the combination of Chappell and Kaini; further wherein the expression is overexpression, and wherein the coding region whose overexpression alters growth of the cell, based on the combination of Lorens, Kiani, and Chappell; and further wherein the transcription regulatory region comprising a silent mutation, and wherein the programmable transcription activator does not initiate overexpression of the coding region when the transcription regulatory region comprises the silent mutation, based on the combination of Yueh, Lorens, Kiani, and Chappell, in order to combine the methods, technologies, RNA regulation systems, and knowledge available in the art to obtain a cell comprising a biocontainment system for facilitating eliminating unwanted cell contamination based on the change of cell growth with an expected success and without undue experimentation.

Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Continuation of item 4: Claims 5-16 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.