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Design of Multipartite Transcription Factors for Multiplexed Logic Genome Integration Control in Mammalian Cells

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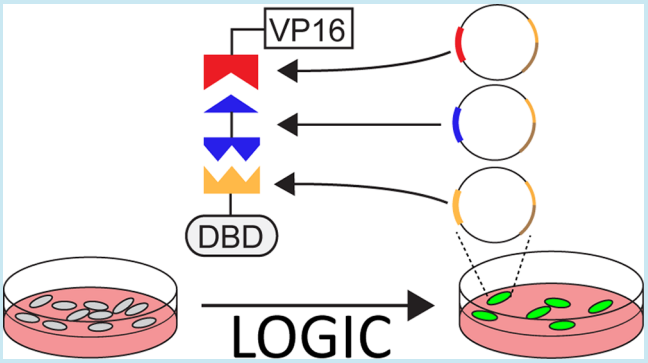
ABSTRACT: Synthetic biology relies on rapid and efficient methods to stably integrate DNA payloads encoding for synthetic biological systems into the genome of living cells. The size of designed biological systems increases with their complexity, and novel methods are needed that enable efficient and simultaneous integration of multiple payloads into single cells. By assembling natural and synthetic protein−protein dimerization domains, we have engineered a set of multipartite transcription factors for driving heterologous target gene expression. With the distribution of single parts of multipartite transcription factors on piggyback transposon-based donor plasmids, we have created a logic genome integration control (LOGIC) system that allows for efficient one-step selection of stable mammalian cell lines with up to three plasmids. LOGIC significantly enhances the efficiency of multiplexed payload integration in mammalian cells compared to traditional cotransfection and may advance cell line engineering in synthetic biology and biotechnology.



Synthetic biology provides new technologies for gene expression control as well as genome editing and has advanced the engineering of mammalian designer cells for biomedicine and biotechnology.[1] For example, human cells are equipped with engineered receptors to detect and respond to disease states according to a predefined logic to increase the precision of cell-based therapy.[2,3] In addition, gene circuits with increasing computational capacity are designed[4,5] that have the ability of cell−cell communication and simultaneous processing of multiple input signals.[6] For the transition from proof-of-concept experiments to real-world applications there is a need for genome engineering technologies that ensure long-term, reliable stability, expression, and functionality of complex, multicomponent synthetic systems in mammalian cells.[7]

Current methods for the generation of stable cell lines are based on random integration vectors, viral vectors,[8,9] transposable elements,[10] or targeted integration using genome-editing technologies such as zinc finger nucleases, TALEN, or CRISPR/Cas9.[11] Recently, it has been shown that targeted integration results in reduced heterogeneity of transgene expression levels compared to random integration in CHO cells.[12]

While the generation of stable cell lines, which express a single transgene is a standard procedure, the integration of multicomponent systems and their long-term functionality remains challenging. Many of the existing technologies require sequential assembly of gene expression cassettes to large, multigene DNA plasmids, which are difficult to propagate in bacteria and often contain repetitive sequences making them unstable in bacterial hosts. Moreover, in order to reach high integration efficiencies, these large plasmids are integrated with site-specific recombinases, which first require the generation of an engineered host cell line harboring landing pads within the genome before the DNA payload can be implemented.[13−15] Another approach is based on baculovirus-mediated transduction of large DNA payloads into mammalian cells, which enables multigene delivery with high efficiencies but lacks long-term expression and functionality due to its transient nature.[9] Multiple transgenes can also be integrated sequentially using multiple antibiotic resistance markers, which is, however, a time-consuming procedure because it includes multiple cycles of cell selection, cells expansion, and genome analysis.[16]

Here, we present a novel technology that allows multiplexed integration of DNA plasmids based on designed multipartite transcription factors (TFs) that control the expression of a single selection marker. The different parts of the TF are distributed among different plasmids that are transfected into mammalian cells. Only cells that have stably integrated all

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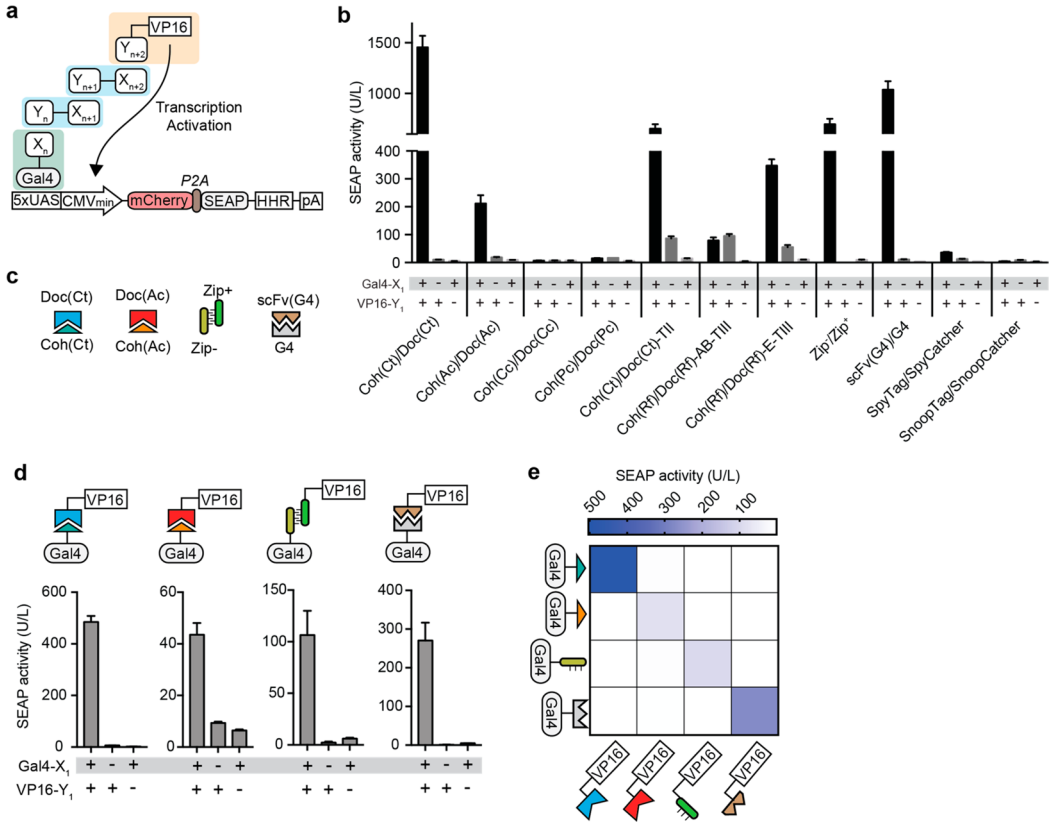

Figure 1. Design of multipartite transcription factors. (a) Assembly of multipartite transcription factors based on protein−protein interaction systems (indicated with X/Y) and the DNA-binding domain Gal4 as well as the viral transactivation domain VP16. Gal4 binds to its cognate response element (UAS) and drives the expression of an mCherry-P2A-SEAP reporter gene with an ribozyme (HHR) in the 3′UTR to reduce leakiness. (b) Assembly of bipartite transcription factors based on protein−protein interaction (PPI) systems (interaction partners are indicated with X/Y) and the DNA-binding domain Gal4 as well as the viral transactivation domain VP16. Performance of the bipartite transcription factors in HEK293T cells based on different protein−protein interaction measured by SEAP reporter activity in cell culture supernatant 48 h after transfection. (c) Cartoons representing protein−protein interaction systems. (d) Performance of the bipartite transcription factors based on each of the four protein−protein interaction measured by SEAP reporter activity in cell culture medium 48 h after transfection into HEK293T cells. (e) Specificity of the protein−protein interactions measured by SEAP reporter gene induction. Error bars represent the means ± s.d. of $n = 3$ replicates.

payloads are able to reconstitute a functional TF and thus produce the selection marker.

RESULTS

Design of Multipartite Transcription Factors. Inspired by the modular principles of the original yeast two-hybrid system,[17] we sought to assemble multiple protein−protein interaction (PPI) modules into multipartite transcription factors (TFs) (Figure 1a). The cohesin-dockerin interaction from *Clostridium thermocellum* (Ct) was utilized for the design of engineered, bipartite TFs consisting of the DNA-binding domain Gal4 fused to the cohesin domain (Gal4-Coh(Ct)) and the viral transactivation domain VP16 fused to the dockerin domain (VP16-Doc(Ct)).[6,18−20] When both parts are mutually expressed the cohesin-dockerin proteins dimerize and reconstitute a functional TF that drives the expression of a P_{5xUAS}-driven engineered secreted-alkaline phosphatase (SEAP) gene expression cassette harboring an sTRSV ribozyme in the 3′UTR to reduce leakiness (Figure 1a). We searched for other protein−protein interactions to expand the set of functional bipartite TFs (Figure 1b). Three other PPIs showed no leakiness in the absence of one of the interaction partners, and at the same time showed a significant induction of SEAP when both parts were expressed in the same cell to reconstitute a functional TF (Figure 1c,d). These included (i) cohesin-dockerin proteins from *Acetivibrio cellulolyticus* (Coh(Ac)/Doc(Ac)),[21] (ii) antiparallel leucine zipper (Zip^-/Zip^+)[22] domains from an engineered, bipartite TF used for gene control in *Caenorhabditis elegans* and (iii) the SunTag system (scFv(G4)/G4)[23] based on a single chain variable fragment of an antibody that specifically binds a yeast-derived 19 amino acid peptide. We observed no crosstalk between the different parts of the protein−protein interactions, which is required for their further assembly to multipartite TFs (Figure 1e).

We used the three best-performing PPIs to engineer tripartite TFs by integrating adaptor modules that bridge the DNA-binding domain Gal4 and the viral transactivation domain VP16 (Figure 2a). Five out of six TF designs resulted in functional transcription factors that were able to induce SEAP expression with different strengths. The best-performing design was based on the Gal4-Coh(Ct) bridged to the VP16-scFv(G4) by the adaptor module Doc(Ct)-$G4_{10x}$. The integration of a second adaptor molecule resulted in tetrapartite TFs, which we assembled from the three dimerizing systems Coh(Ct)/Doc(Ct), Zip^-/Zip^+ and scFv(G4)/G4 in different architectures (Figure 2b). Three out of six TF designs resulted in functional transcription factors that were able to induce SEAP expression. The strongest induction was measured for the design variant, which consists of Gal4-

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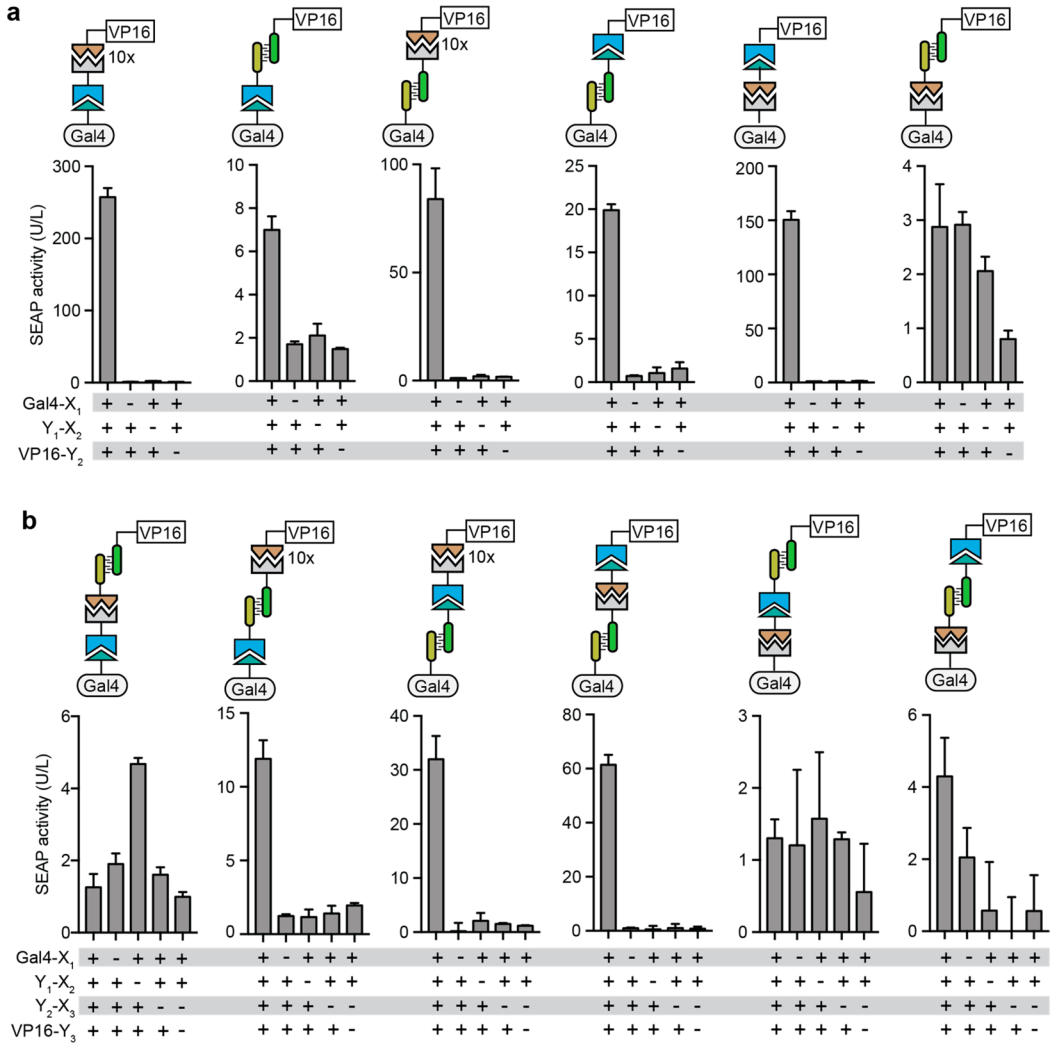

Figure 2. Performance of multipartite transcription factors with orthogonal protein−protein interaction domains. (a) Performance of different variants of tripartite transcription factors based on two protein−protein interactions measured by SEAP reporter gene induction. (b) Performance of different variants of tetrapartite transcription factors based on two protein−protein interactions measured by SEAP reporter gene induction. SEAP reporter assay was performed 48 h after transfection. Error bars represent the means ± s.d. of n = 3 replicates.

Zip^- bridged by the adaptors Zip^+-G4 and scFv(G4)-Coh(Ct) to VP16-Doc(Ct). Since the individual parts of the TFs are encoded on individual plasmids, the multipartite TFs execute a Boolean *AND* logic function, which requires the mutual expression of all parts for efficient SEAP reporter induction (Figure 2a,b).

Multiplexed Logic Genome Engineering Using Transposable Elements. With a set of multipartite TFs engineered and validated in mammalian cells, we implemented the individual parts into piggyBac transposon[10] plasmids (P_{CAG}-driven expression cassettes) for the development of a multiplexed logic genome integration control (LOGIC) platform. To compare LOGIC with a conventional stable cell line selection method, we cloned four different transposon plasmids encoding a P_{CAG}-driven Puromycin resistance gene (Puromycin N-acetyl transferase (PAC)) as selection marker followed by a T2A cleavage site and four different fluorescent proteins, mTagBFP2, mVenus, mCherry, and iRFP720 (Figure 3a). Cotransfection of each plasmid with a transposase in hMSC-TERT cells and selection for 5 days with Puromycin resulted in >98% fluorescence-positive polyclonal cell populations for each fluorescent protein measured by flow cytometry (Figure 3b). To engineer the LOGIC, we replaced the DNA-binding domain Gal4 with VanR, because we were not able to generate transposon-based hMSC-TERT stable cell lines that constitutively express Gal4-VP16 indicating potential interference of Gal4 with endogenous cellular processes in the hMSC-TERT cell line (Figure 3c,d).

Cotransfection of two fluorescent protein-encoding plasmids resulted in a polyclonal cell population of which only 44% expressed both fluorescent proteins after a 7-day selection period (Figure 4a,b). In contrast, the engineered two-plasmid LOGIC system (2-LOGIC), which consists of a P_{CAG}-driven VanR-VP16_T2A_mTagBFP2 and a P_{VanO2}-driven Puromycin_T2A_Citrine gene expression cassette resulted in a higher percentage (>90%) of the double-positive fluorescent polyclonal cell population (Figure 4b). This validated the LOGIC principle, which generates mutual dependencies of the production of the selection marker PAC by distributing parts of the transcription factor and the PAC gene expression cassette onto different plasmids. We further increased the complexity to build a 3-plasmid integration system (3-LOGIC). Conventional selection of three plasmids each encoding for a fluorescent protein and the PAC resistance

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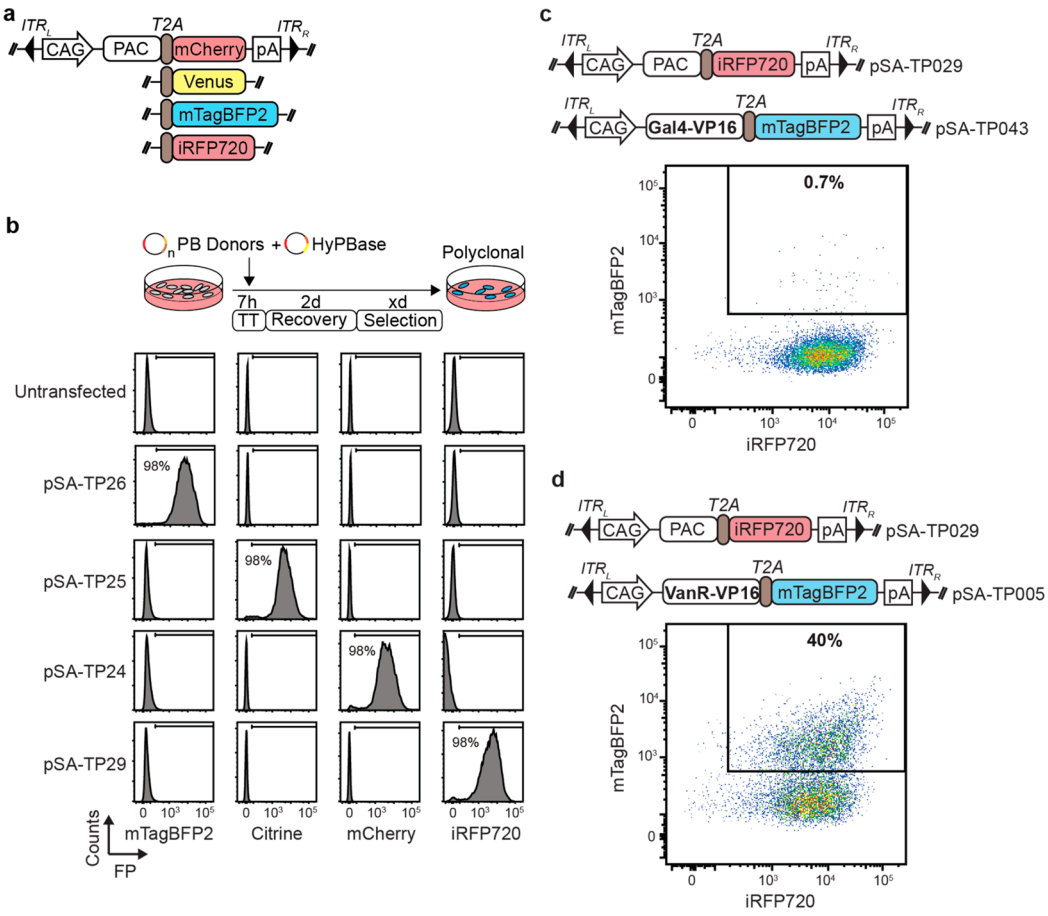

Figure 3. Transposon-based conventional selection for multiplexed plasmid integration in mammalian cells. (a) Transposon donor plasmids encoding for four different fluorescent proteins and a Puromycin resistance marker. (b) Experimental outline of the generation of hMSC-TERT stable cell lines. Transposon-based generation of stable cell lines harboring P_{CAG}-driven gene expression cassettes that produce a PAC-T2A-fluorescent protein transcript (the fluorescent proteins mTagBFP2, mCitrine, mCherry, and iRFP720 are encoded on pSA-TP26, pSA-TP25, pSA-TP24, and pSA-TP29, respectively). Cells were selected with 6 μg/mL Puromycin for 7 days and analyzed using flow cytometry. (c) The 7-day selection of a polyclonal hMSC-TERT stable cell population generated with two transposon-based plasmids where only one plasmid encodes for a PAC selection marker; 0.7% of the polyclonal population contains both fluorescent proteins showing that Gal4-VP16-mTagBFP2 could not be stably propagated in the cells. (d) The 7-day selection of a hMSC-TERT polyclonal stable cell population generated with two transposon-based plasmids where only one plasmid encodes for a PAC selection marker; 40% of the polyclonal population contains both fluorescent proteins showing that VanR-VP16-T2A-mTagBFP2 has been stably integrated into the cells.

gene resulted in only 22% triple-fluorescent positive cells (Figure 4c,d). However, when using a 3-LOGIC system based on the bipartite VanR-Coh(Ct)/VP16-Doc(Ct) TF resulted in stringent selection. More than 80% of the cells in the polyclonal population were triple-fluorescent quantified by the expression of the three fluorescent proteins Citrine, mTagBFP2, and mCherry using flow cytometry (Figure 4d). Thus, LOGIC selection systems enable efficient and rapid selection of desired multicomponent polyclonal mammalian cell populations with a single antibiotic selection marker.

DISCUSSION

Protein−protein interactions are valuable tools in synthetic biology and have been used for the design of bipartite transcription factors enabling cancer cell detection[19,20] and design of small molecule-programmable logic gates.[6,18] We demonstrate that multiple protein−protein interactions can be assembled into higher-order transcription factors that control gene expression in mammalian cells. Removal of one of the parts results in impaired TFs and thus no target gene expression. In the future, other protein−protein interactions could be implemented to design new sets of multipartite TFs. We found that some TF architectures were not functional and assume that either the protein structure or expression levels of some adaptor modules were disturbed. Thus, further modifications in linker sequences or addition of stabilizing domains could lead to improved versions of multipartite transcription factors. We have recently shown that Gal4 and VanR as well as TetR and TtgR DNA-binding domains can be assembled to functional, bipartite transcription factors with similar performance.[6] Swapping DNA-binding domains could therefore be standard practice to tune the performance of multipartite transcription factors and/or adapt it for a particular cell type.

Since these multipartite TFs strictly require all parts for functionality, the system resembles *AND* Boolean logics. By distributing the TF's parts on different plasmids and controlling the expression of a resistance marker, we developed a technology for multiplexed genomic manipulations in mammalian cells. While we used an antibiotic resistance gene for the selection of cell populations, it may also be possible to place a fluorescent protein or a cell surface marker under the

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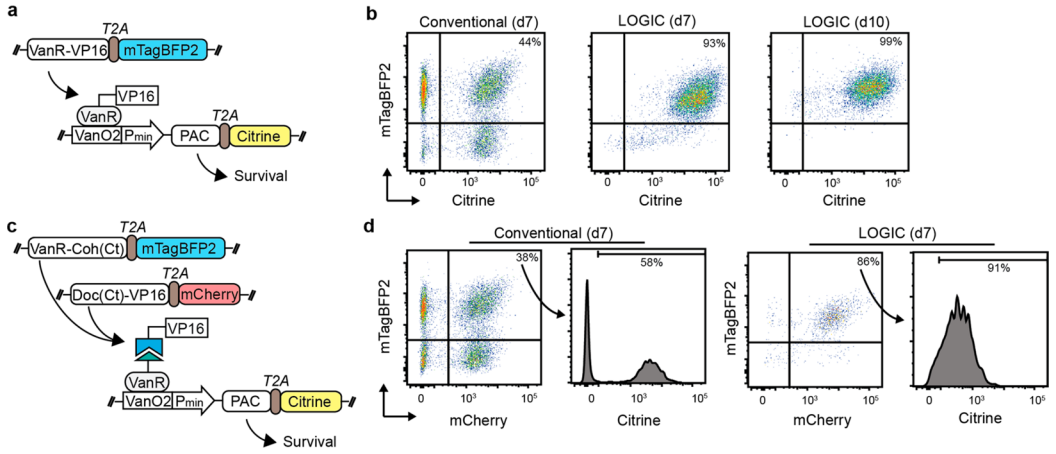

Figure 4. Transposon-based LOGIC for multiplexed plasmid integration in mammalian cells. (a) 2-LOGIC based on the distribution of the transcription factor VanR-VP16 and the corresponding inducible PAC resistance gene expression cassette on two different transposon donor plasmids each encoding also for a specific fluorescent protein. (b) Comparison of 7-day selection of polyclonal stable cell populations generated with either the conventional method (both plasmids encode for PAC resistance marker) or 2-LOGIC with Citrine and mTagBFP2 as DNA payload. (c) 3-LOGIC based on the distribution of the transcription factor VanR-Coh(Ct), transactivation domain VP16-Doc(Ct), and the corresponding inducible PAC resistance gene expression cassette on three different transposon donor plasmids each encoding also for a specific fluorescent protein. (d) Comparison of 7-day selection of polyclonal stable cell populations generated with either the conventional method (three plasmids encode for PAC resistance marker) or 3-LOGIC with Citrine, mCherry, and mTagBFP2 as DNA payload.

control of multipartite TFs enabling FACS or magnetic cell enrichment of desired cell populations. In contrast to previous efforts to develop one-step methods for manipulations of multiple genomic regions, our work is based on the principle of genetic circuit design and biocomputation. Recently, miRNA patterns have been exploited for the detection of specific cell populations, such as cancer cells[24] or differentiated cell types,[25] and may complement the LOGIC technology for additional logic control options. In addition, other logic circuit design strategies based on RNA elements[26,27] or recombinases[28,29] could also be adapted for LOGIC.

To fully unleash the potential of mammalian synthetic biology in biomedicine and biotechnology, multiplexed and precise genome editing will become key to deliver designer cells that consist of synthetic systems and tailored genomes. Recently, CRISPR/Cas9-facilitated genetrapping has been used to insert a chimeric antigen receptor (CAR) into the TCRα genomic locus, which resulted in simultaneous knockout of the endogenous TCRα and targeted insertion of the CAR driven by the endogenous promoter in primary T cells.[30] In theory, LOGIC could be used to introduce additional genomic manipulations, for example, knockout of immunoinhibitory receptors, such as PD-L1 or CTL4A, to produce the next generation of engineered autologous T cells, which require extensive genome engineering in a one-step procedure. Especially in primary cells in which the expansion of cell numbers is limited, LOGIC may prove to be a valuable technology for the generation of engineered cells enabling simultaneous DNA insertions and manipulation of endogenous genes.

■ ONLINE METHODS

Vector Design. Comprehensive design and construction details for all expression vectors are provided in Supplementary Tables 1 and 2.

Mammalian Cell Culture and Transfection. Human embryonic kidney cells (HEK293T/17, ATCC: 11268) or hMSC-TERT cells[31] were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, cat. no. 31966-021) supplemented with 10% (v/v) fetal calf serum (FCS, Sigma-Aldrich, lot no. 022M3395) in antibiotic-free conditions at 37 °C in a humidified atmosphere containing 7.5% CO_2. Cell viability and number were quantified using an electric field multichannel cell counting device (Casy Cell Counter and Analyzer Model TT, Roche Diagnostics GmbH). For experiments, 2.4×10^6 cells were seeded into a multiwell plate 1 day before transfection in DMEM. After 16 h, plasmid DNA was mixed with polyethylene imine (PEI, MW 40 000, stock solution: 1 mg/mL in ddH_2O, Polysciences, cat. no. 24765-2) in a 1:6 ratio (w/w) and incubated in FCS-free DMEM for 30 min at 22 °C before it was added dropwise to the cells. After 7 h, the transfection medium was replaced with FCS-supplemented DMEM. Transfection mixture recipes can be found in Supplementary Table 3.

Generation of Stable Cell Lines. At 48 h after transient transfection, the cells were trypsinized and centrifuged. One quarter of the cells were reseeded in a multiwell plate of the same layout in DMEM containing indicated Puromycin concentrations. Part of the cells were used for flow cytometry analysis. Puromycin-containing medium was replaced every day for indicated days with fresh selection medium. After selection, cells were trypsinized and used for flow cytometry analysis. If indicated, polyclonal cell populations were further cultivated in DMEM without Puromycin. For the generation of monoclonal cell lines by limiting dilution, cell concentration was adjusted to 0.7 cells/100 μL and seeded into 96-well plates. Resulting colonies of cells were trypsinized, analyzed by flow cytometry and further cultivated in DMEM.

Flow Cytometry. Cell populations were analyzed with a LSRII Fortessa flow cytometer (Becton Dickinson) equipped for mTagBFP2 (405 nm laser, 445/15 emission filter), mCitrine (488 nm laser, 505 nm long-pass filter and 530/11 emission filter), mCherry (561 nm laser, 600 nm long-pass filter and 610/20 emission filter), and iRFP720 (640 nm laser, 760/50 emission filter) detection and set to exclude cell debris and cell doublets. A total of 10 000 single cells were recorded

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per sample and were analyzed with FlowJo software (version no. 10; FlowJo LLC, Oregon, USA).

SEAP Reporter Gene Assays. The production of human placental secreted alkaline phosphatase was quantified in cell culture supernatants (Schlatter et al., 2002) as described before. In brief, absorbance of 4-nitrophenol at 405 nm was monitored for 30 min using an Infinite M200 PRO multiwell plate reader (Tecan Group Ltd., Männedorf, Switzerland).

Statistics. No statistical methods were used to predetermine sample size.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00413.

Plasmids and oligonucleotide sequences used in this work; transfection details of engineered cells (PDF)

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Author Contributions

S.A. and D.A. designed the project. S.A., D.A., P.L., and M.F. analyzed the results and wrote the manuscript. S.A., P.L., and M.K. performed the experimental work.

Notes

The authors declare no competing financial interest.

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