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POINT OF VIEW

CRISPR-Cas type II-based Synthetic Biology applications in eukaryotic cells

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ABSTRACT

The CRISPR-Cas system has rapidly reached a huge popularity as a new, powerful method for precise DNA editing and genome reengineering. In Synthetic Biology, the CRISPR-Cas type II system has inspired the construction of a novel class of RNA-based transcription factors. In their simplest form, they are made of a CRISPR RNA molecule, which targets a promoter sequence, and a deficient Cas9 (i.e. deprived of any nuclease activity) that has been fused to an activation or a repression domain. Up- and downregulation of single genes in mammalian and yeast cells have been achieved with satisfactory results. Moreover, the construction of CRISPR-based transcription factors is much simpler than the assembly of synthetic proteins such as the Transcription Activator-Like effectors. However, the feasibility of complex synthetic networks fully based on the CRISPR-dCas9 technology has still to be proved and new designs, which take into account different CRISPR types, shall be investigated.

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Introduction

The CRISPR-Cas system represents an RNA-based component of the immune system of prokaryotic organisms.[1] Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are organized, in the chromosome, into a repeat-spacer array that is accompanied by several Cas (CRISPR associated protein) genes. Repeat sequences are roughly from 20 up to 50 nucleotides long and varies from species to species. Spacers can be longer (over 80 nucleotides) and correspond to pieces of foreign DNA that entered the cell in the past.[2,3] A new spacer is acquired[4] when a small portion of an intruder DNA (virus or plasmid) is cut, probably in the proximity of the protospacer adjacent motif (PAM), and then inserted at the leader end of the CRISPR locus.[5,6] Therefore, the CRISPR sequence is an archive of the infections met by the cell.

Among the 6 types of CRISPR-Cas systems so far encountered,[7] the most studied and used for bioengineering purposes is the so called CRISPR-Cas type II. Here–as in every other CRISPR-Cas type–an infection due to a foreign DNA triggers the transcription of a long precursor CRISPR RNA (pre-crRNA) molecule. The CRISPR-Cas type II system shows a peculiar crRNA maturation pathway where 3 different molecules (tracrRNA–trans-activating crRNA, RNAse III, and Cas9) contribute to the formation of CRISPR RNA molecules able to bind the invading DNA and direct its cleavage.[8,9]

tracrRNAs are short RNA molecules (about 75 nucleotides) that contain a 25 -nucleotide long sequence complementary to the repeat. They bind, by base-pairing, the pre-crRNA at each repeat sequence and trigger the formation of active Cas9-crRNA:tracrRNA complexes. Following Deltcheva et. al,[8] Cas9 acts as a "molecular anchor" and facilitates the binding between tracrRNA and pre-crRNA. RNase III is then recruited and cuts the tracrRNA:pre-crRNA complex at 2 different positions, one inside the repeat, the other along the spacer. tracrRNA is also required in DNA recognition by orienting crRNA to interact with its DNA target, as suggested by Jinek et al.[10] A mature crRNA molecule is 42 -nucleotide long. The 5′ end contains 20 nucleotides from the spacer, hence representing the DNA binding domain. The other 22 nucleotides (3′ end) belong to the repeat and are paired to the tracrRNA sequence [10,11] to constitute a handle for Cas9 binding. Interestingly, only 26 nucleotides of the tracrRNA sequence (from position 23 to 48) are required to have an active Cas9-crRNA:tracrRNA complex. According to this finding, Jinek et al.[10] designed a minimal chimeric RNA molecule, able to drive Cas9-mediated DNA cleavage, by bridging the 3′ end of the crRNA to the 23–48 tracrRNA region via 4 nucleotides (GAAA). Overall, the chimeric RNA is made of the DNA recognition sequence followed by a harpin structure that interacts with Cas9. This has inspired the design of (single) guide RNA molecules–(s)gRNA–that have been largely used in Synthetic Biology applications of the type II CRISPR-Cas system, as we will discuss below.

Cas9-crRNA:tracrRNA is able to recognize and bind a pathogenic element only if the target sequence on the DNA is followed, in the 5′–3′ direction, by the protospacer adjacent motif. Moreover, to have DNA cleavage, no mismatches are allowed between the crRNA DNA recognition domain and the foreign DNA protospacer in a region (referred to as seed) that is reported to cover up to 13 nucleotides upstream the PAM.[10-14] Upon DNA binding, Cas9 breaks both strands of the foreign DNA and stimulates its degradation. Due to the similarity to eukaryotic RNA interference, the crRNA-based DNA degradation pathway is also referred to as CRISPR-mediated interference or CRISPRi (see Fig. 1).

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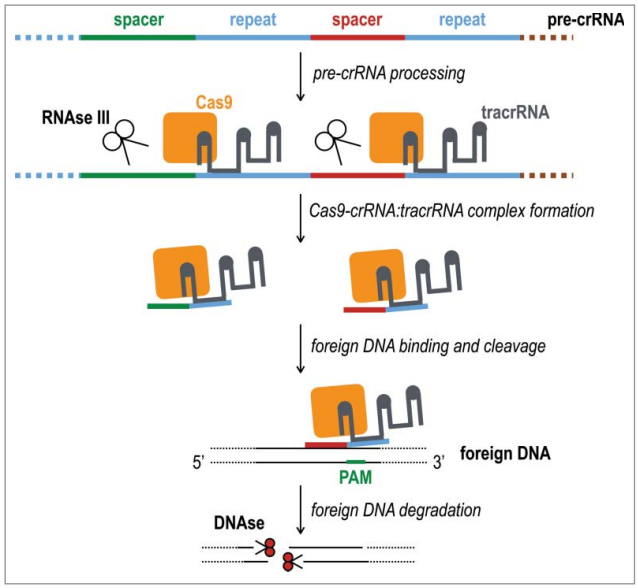

Figure 1. CRISPR-Cas9-based DNA degradation pathway. A long pre-crRNA chain, which contains pieces of previously-encountered foreign DNA, is processed into Cas9-crRNA:tracrRNA molecules. They bind and cut foreign DNA upon recognition of the protospacer adjacent motif. Double-strand cleavage triggers the degradation of foreign DNA.**

So far, the main biotechnology application of the CRISPR-Cas9 system has been DNA editing.[15,4] To this aim, Cas9 from Streptococcus pyogenes (SpCas9) has been characterized deeply and largely exploited. We know that SpCas9 PAM is the NGG triplet. However, SpCas9 has been reported to recognize, though with lower efficiency, the NAG protospacer adjacent motif too.[13] The nuclease activity of SpCas9 relies on 2 domains (termed RuvC and HNH) that induce a double-strand DNA cleavage with blunt-ends 3 nucleotides upstream the PAM.[5,10,11] These 2 nuclease domains can be silenced via just 2 mutations (D10A and H841A[16]). A deficient SpCas9 (dSpCas9), which is unable to carry out DNA cleavage, has however become, together with the corresponding guide RNAs, the key element for the construction of a new kind of transcription factors. They have proved their utility in the implementation of synthetic gene circuits in eukaryotic cells.

CRISPR-dCas9-based transcription factors: Main ideas

Synthetic gene circuits are represented as networks of DNA sequences that interact via the exchange of molecules such as transcription factor proteins and small RNAs.[17,18] Circuit functionalities arise, mainly, by exploiting transcription and translation regulation. A requirement for the correct working of a genetic circuit is the orthogonality between the circuit components and its chassis i.e., the organism where the circuit is placed. Orthogonality means that the circuit, by carrying out its function, does not interfere with, nor is disturbed by, the normal cellular activity of the hosting cell. For instance, bacterial repressors such as TetR, LacI, and LexA are orthogonal to the yeast genome i.e., they bind a chromosome and interact with DNA only if their operators have been previously integrated into a specific locus.[19-21]

A new and powerful way to build orthogonal transcription factors, which are able to bind and regulate a vast amount of promoter sequences, is given by the usage of dSpCas9 together with guide RNAs. Following,[10] guide RNAs are conceived as small RNA molecules made of a DNA-recognition sequence at the 5′ end and a handle to interact with dSpCas9 at the 3′ end. Within a eukaryotic synthetic gene circuit gRNAs are usually produced by RNA polymerase III promoters–such as the murine U6 promoter[22] or the yeast SNR52 promoter[23]–provided that the gRNA sequence does not contain 4 or more adjacent thymines that could be interpreted by RNA polymerase III as a termination signal.[23] It should be noted that gRNAs can be transcribed also under RNA polymerase II promoters, as a part of a longer mRNA chain. However, in this case the gRNA has to be flanked by nuclease sites or hammerhead ribozymes [24-26] to be cut off the mRNA and become functional.

dSpCas9 proteins are fused to one (or more) activation or repression domain such that, once they are bound to a gRNA and brought to the DNA, they can either promote or shut down transcription initiation. Therefore, inside a synthetic gene circuit a promoter can be potentially either down- or upregulated by dSpCas9:gRNA complexes if it contains protospacer adjacent motifs along its sequence. A dSpCas9:gRNA-based transcription factor is orthogonal to the circuit chassis if the gRNA cannot bind anywhere in the original genome of the hosting cell.

gRNA design

As we have seen above, the DNA sequence targeted by an SpCas9:gRNA complex is 20 nucleotide long. Mismatches in the first 7/8 nucleotides have basically no effects both in DNA recognition and cleavage, whereas a single mismatch in the following seed region prevents DNA cleavage. Furthermore, according to,[27] DNA binding requires perfect match only with the 5 nucleotides just upstream the PAM. This implies that, along a given genome, there might be for any SpCas9:gRNA a large number of off-target sites where SpCas9 would not carry out DNA cleavage but could interfere with the cell transcriptional machinery. This further complicates the design of orthogonal guide RNAs to be associated with dSpCas9 for the regulation of the activity of the promoters involved into synthetic gene circuits.

Rational design of unique (hence orthogonal) gRNAs is performed computationally. In our Synthetic Biology laboratory, we use the web-server CRISPRdirect.[28] The program takes as inputs a DNA sequence where to look for gRNA targets, the PAM (triplets only), and the genome where to search for off-target matches. Possible target sequences on both DNA strands are returned together with the number of matches within the whole selected genome. If, for instance, we need a gRNA able to bind a viral promoter integrated into the S. cerevisiae genome, all the sequences found by CRISPRdirect whose seed region (here defined as the 8 nucleotides upstream the PAM) has at least one match along the yeast genome should be discarded.

RNA-guided activators and repressors

Originally, CRISPR-Cas9-based synthetic activators for eukaryotic cells have been engineered by fusing dSpCas9 to a single

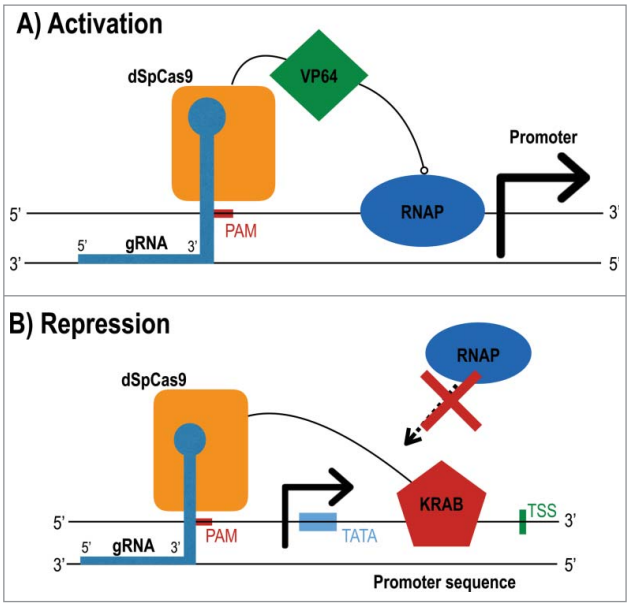

Figure 2. Synthetic transcription factors built on the dSpCas9:gRNA system. The fusion of an activation domain such as VP64 (A) or a repression domain like KRAB (B) to dSpCas9 confers to the dSpCas9:gRNA system the ability to regulate transcription initiation from eukaryotic promoters. A line ending with an empty circle is used here (an in the next Figures) to depict the recruitment of RNA polymerase II molecules (RNAP) to the DNA.[**]

strong activation domain such as VP64,[22,29,30,31] p65[22], VP48 and VP160,[32] and the plant EDLL[33] (see Fig. 2A). As a general result, to achieve a substantial increase in gene expression promoters need to be targeted by gRNAs at multiple sites (from 3 up to 12). Furthermore, they should be placed within 300 nucleotides upstream the TSS (transcription start site). Synergistic interactions among the activator molecules binding at different promoter location is what fosters transcription activation. A comparative study on activators constructed with 3 different dCas9 proteins (from S. pyogenes, S. thermophilus, and N. meningitidis) fused to VP64 did not underline any significant difference in their transcription regulation activity in human cells.[34] Remarkably, these activators proved to be orthogonal to each other since they activated only the promoter they targeted i.e., they did not show any crosstalk. Improvements have been achieved by fusing to dSpCas9 either 2 copies of VP64 (VP64-dSpCas9-VP64)[35] or a hybrid tripartite activation domain made of VP64, p65 and the Rta activation domains (hence named VPR).[36] dSpCas9-VPR, increased endogenous gene expression in human cells from about 20 to over 300 folds compared with dSpCas9-VP64. However, it still required that each gene was targeted by 3 or 4 gRNAs, whereas Chakraborty et al.[35] pointed out that at least one human locus (Myod1) was clearly activated by a single guide RNA in conjunction with VP64-dSpCas9-VP64.

A different strategy was followed by Hilton et al.[37] They did not fused dSpCas9 to a canonical activation domain but to the catalytic core of the human acetyltransferase p300 domain. Hence, transcription activation was not achieved by enhancing RNA polymerase II recruitment to the DNA but via chromatin modification. Remarkably, this configuration proved to be able to activate most of the targeted genes (in human cells) with a single gRNA.

Chromating remodelling is commonly exploited to engineer synthetic repressor proteins for mammalian promoters. To this aim, the KRAB domain is usually fused to a DNA binding domain.[38] The KRAB domain was shown to outperform other chromatin remodelling domains (such as WRPW and Hes1) when fused to dSpCas9.[22] However, to maximize repression, the gRNA target sequence should be chosen within a region (−50… +250 nucleotides) around the TSS [39,40] (see Fig. 2B).

Bare dSpCas9:gRNA was also shown to work as a transcriptional repressor in mammalian cells.[16,41] In this case, promoter steric occupation prevents the binding of RNA Polymerase II (or III). This competition mechanisms is usually used in yeast cells. Interestingly, Farzadfard et al.[30] showed that even dSpCas9-VP64:gRNA works as a repressor if the gRNA target sequence is placed less than 20 nucleotides upstream the TATA box or between the TATA box and the TSS of the minimal CYC1 yeast promoter.[42] Gilbert et al.,[22] however, proved that dSpCas9 fused to the mammalian Mxi1 repression domain, which is known to work in yeast,[43] is a much stronger repressor–on the yeast constitutive pTEF1 promoter[44]–than the bare dSpCas9.

In Synthetic Biology the importance of the dCas9:gRNA systems lies also in the fact that they represent an “easy” alternative to TALes for the construction of synthetic transcription factors. A TAL effector DNA binding domain that targets a 20 -nucleotide-long operator (i.e., the same length of a gRNA) demands to put together 20 modules (repeats), each of them made of 34 amino acids–a part from the last one that contains only 20 amino acids.[45] Despite the fact that kits and cloning techniques have been developed or modified ad hoc,[46,47] the overall assembly of a TALe is, by far, more complex than the simple design of a guide RNA. However, Lebar and Jerala[48] pointed out that, in mammalian cells, transcription activation is higher when based on TAL effectors rather than on dSpCas9-VPR:gRNA. Repression of transcription–achieved by fusing KRAB both to a TALe and a dSpCas9–gives, in contrast, comparable results. Nevertheless, layered logic circuits return a more faithful truth table representation when the bridges between 2 adjacent layers are TALe- rather than CRISPR-dSpCas9-based repressors.

CRISPR-dCas9 as a scaffold

In almost all the works mentioned so far, activation or repression of gene synthesis was achieved by fusing an activator or a repressor domain to dSpCas9, whereas the guide RNA played the only role of DNA binding domain. Mali et al.[49] proposed a new design where the gRNA was used also to recruit 2 copies of VP64, whereas no modification was made on dSpCas9. The 3′ end of the gRNA was modified with the insertion of 2 stem loops working as binding sites for the MS2 bacteriophage coat-protein that had been fused to VP64. Activation of endogenous genes in human cells necessitated multiple gRNA sites along the target promoters. Compared to the standard design, where it is dSpCas9 that carries VP64, this new approach, however, turned out to be less effective.

Konermann et al.[50] showed how to exploit the gRNA as a scaffold to build a powerful activator that leads to substantial gene expression in presence of a single guide RNA. As in,[49] 2

aptamers binding MS2 proteins were added to the gRNA, though in different positions. dSpCas9 was fused to VP64, whereas MS2 was fused to 2 other activation domains: p65 and HSF1, to create hetero-synergistic activation from the interaction of overall 3 different activation domains. The whole system made of dSpCas9-VP64, MS2-p65-HSF1 and the double-aptamer-containing gRNA was termed SAM (synergistic activation mediator). On 10 endogenous human genes, it displayed stronger activation–with a single gRNA target sequence–than a standard dSpCas-VP64 associated with 8 different gRNAs. Moreover, activation was shown to be optimal when the single gRNA target is located within 200 nucleotides upstream the TSS.

Zalatan et al.[51] redesigned the guide RNA to be a scaffold (which they called scRNA) for more than a single protein. Together with MS2, 2 more viral proteins (PP7 and COM) are considered in this study. Here, a gRNA hosts up to 2 different aptamers able to bind as many different proteins. Both activation and repression domains (VP64 and KRAB) are no longer fused to dSpCas9 but to the RNA-binding proteins (see Fig. 3A). In this way, a single dSpCas9:scRNA is a multifunctional complex since it can either activate or repress its target promoter depending on the domain fused to the corresponding RNA-binding proteins. As a remarkable application, the bacterial violacein biosynthesis pathway (5 genes) was re-engineered in budding yeast cells and proved to be functional, upon expression of dSpCas9, once 3 diverse scRNAs and 2 different RNA-binding proteins were constitutively expressed into the cells.

Another kind of scaffolding design was realized by exploiting a particular family of RNA-binding proteins called Pumilio/FBF (Puf). Several types of Puf proteins have been identified in eukaryotic cells. Generally, they bind the 3′ end of an mRNA chain and trigger its degradation or promote its spatial localization (e.g. into mitochondria).[52] Puf main feature is a modular mRNA-binding domain (also referred to as Pumilio Homology Domain–Pum-HD) made of 8 repeats (each one containing 36 amino acids) that are flanked by 2 half repeats.[53] This mRNA-binding domain can be reengineered to bind, in principle, any chosen octamers[54] and has been fused to other protein domains to either repress or activate translation.[55]

Cheng et al.[56] extended the gRNA with a variable number of Puf binding domains (PBS) and modified the bare Pum-HD with the addition of a transcriptional activation (VP64, p65, and HAT) or repression (KRAB) domain. The overall system, termed Casilio, was engineered in different variants by changing the Pum-HD and the number of the corresponding PBS along the gRNA. Optimal performances were achieved with 5 PBSes (see Fig. 3B).

Differently from the all the previous works, in[57] a scaffold was designed on dSpCas9 rather than on the gRNA. dSpCas9 was tagged with a peptidic tail (termed SunTag) containing epitopes of the GCN4 antiboby. GCN4 molecules were fused to VP64 such that a single tagged dSpCas9 could recruit multiple VP64 domains (10 giving optimal results) to the target promoter. Interestingly, all the CRISPR-dCas9-based activators here described (with the exception of the Casilio system) were the subject of a comparative study in both human and D. melanogaster cells. VPR, SAM, and SunTag showed best performance in both organisms.[58]

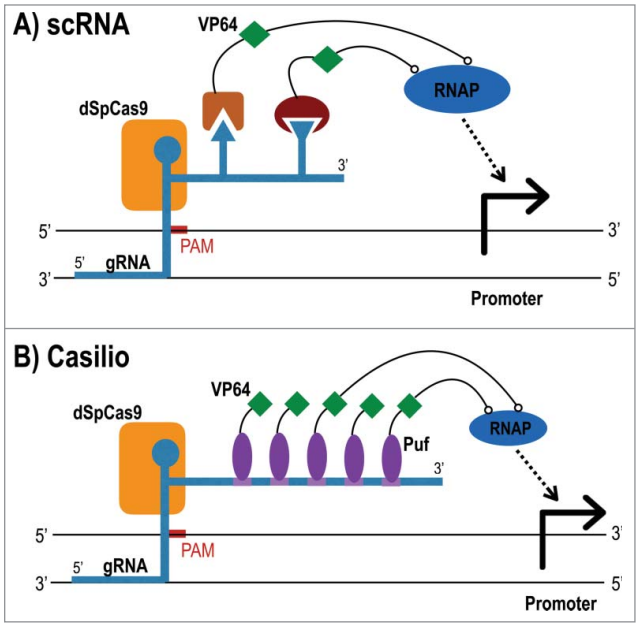

Figure 3. Examples of gRNA used as a scaffold. (A) An scRNA is built by extending a guide RNA with aptamers that are anchor points for RNA-binding proteins. The latter are fused either to repression or activation domains and regulate transcription initiation. (B) The Casilio system gives its name to the usage of dSpCas9:gRNA in conjunction with Pumilio/FBF proteins that are fused to an activation or a repression domain. Best performance was achieved by extending the gRNA with 5 PBSes, as shown here.[**]

Gene expression regulation via active Cas9

Catalitically active Cas9 was reported to be unable to cleave DNA when bound to a gRNA truncated at its 5′ end i.e., with no more than 16 nucleotides complementary to the target DNA sequence.[59] Coherently to this finding, Dahlman et al.[60] showed that Cas9 can be used to induce gene expression when associated with a guide RNA that is shortened, up to 5 nucleotides, at its 5′ end. The truncated gRNA was termed "dead RNA" (dRNA). Activation domains were recruited by dRNAs fused to aptamers (the same as in[50]) able to bind the MS2-P65-HSF1 complex (which in[50] is part of the SAM system). A dRNA truncated to have from 11 up to 15 complementary nucleotides with its target promoter activated gene expression strongly without producing a significative number of indels. Therefore, a single kind of Cas9 can be expressed, in eukaryotic cells, together with both complete guide RNAs and dRNAs to upregulated and knock down the expression of multiple genes. Similar results were presented in.[61] Here, Cas9 (from S. thermophilus and S. aureus) was fused to the VPR activation domain and proved to be able to both activate and repress promoters when interacting with short gRNAs sharing a 14 -nucleotide-complementary sequence with their target promoters.

A Cas9-based classifier

Cell classifiers are complex synthetic biosensing devices that are particularly useful in medical diagnostics. They respond only to the presence of multiple signals such that they can discriminate, within high accuracy, between healthy and sick cells. Cancer

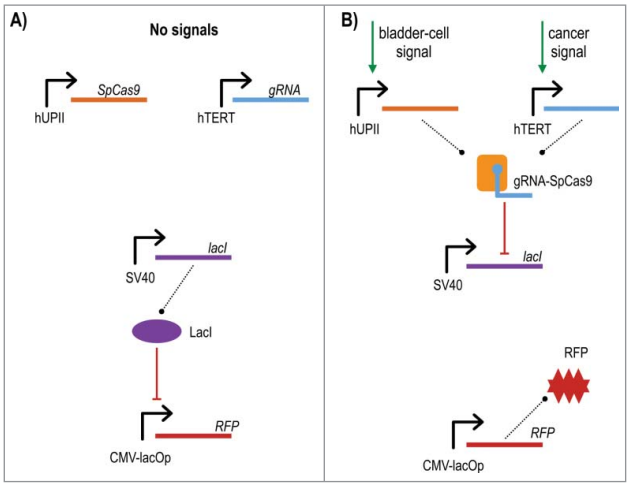

Figure 4. An AND gate as a bladder cancer cell classifier. (A) In absence of any of the 2 input signals, both hUPII and hTERT promoter are inactive and the SpCas9:gRNA complex targeting the *lacI* gene is not synthesized. LacI, constitutively produced by the SV40 promoter, binds the synthetic CMV-lacOp promoter and turns off the expression of red fluorescence proteins (RFP), the circuit output. Hence, if the classifier is inserted into non-bladder cells or healthy bladder cells, no fluorescence can be detected. (B) When both input signals are present, the SpCas9:gRNA system is assembled. Upon *lacI* gene cleavage by SpCas9, RFP is expressed in considerable quantity by the CMV-lacOp promoter. Therefore, bladder cancer cells induce the production of a high red fluorescence signal from this circuit. Green arrows indicate promoter activation, red lines ending with a bar represent repression of transcription, dashed lines ending with a circle stand for gene expression.**

cell classifiers have been previously realized as RNAi-based networks.[62] More recently, Liu et al.[63] proposed a novel strategy, based on the usage of SpCas9 nuclease, to detect bladder cancer cells. Here, 2 different input signals–one associated with cancer, the other with bladder cells–are combined into an AND gate. Upon detection of the corresponding signal, the bladder-specific hUPII promoter drives the synthesis of SpCas9, whereas the cancer-specific hTERT promoter leads gRNA production. Hence, an SpCas9:gRNA system is assembled when the 2 promoters are activated i.e., only in presence of both input signals. gRNA binds (and SpCas9 cleaves) the DNA sequence of the LacI repressor. This bacterial protein binds a synthetic CMV promoter (modified with the insertion of a lac operator) and dowregulates the production of a red fluorescent protein, the AND gate output. Human cells reengineered with the insertion of this classifier circuit are analized in vitro. Bladder cancer cells are revealed by the production of a strong red fluorescence signal (see Fig. 4).

Inducible CRISPR-dCas9 systems

In the cell classifier described above, input signals do not interact with SpCas9 or the guide RNA but with the cell pathways that lead to their synthesis. To make CRISPR-dCas9 directly responsive to an input signal, dCas9 is modified via the fusion of a protein domain sensitive to an environmental stimulus.

A "light-activated CRISPR-Cas9 effector" (LACE) was realized by Polstein and Gersbach[64] by means of the CRY2 and CIB1 proteins from A. thaliana. They form a heterodimer upon exposure to blue light. The highest promoter activation in human cells was obtained by fusing dSpCas9 with CIB1 (both at the N and C end) and either the full CRY2 or just its photolyase homology region (CRY2PHR) to VP64. Maximal gene expression (comparable to the one reached with the reference dSpCas9-VP64:gRNA system) was achieved in presence of 4 different guide RNAs. The same CRY2-CIB1 system was used by Nihongaki et al.[65] Here, however, the best performance (minimal background from and highest fold induction in the activity of a lucipherase reporter) was obtained by fusing a truncated CIB domain to dSpCas9, and p65 to CRY2PHR.

Zetsche et al.[66] proposed a split-dSpCas9 system to construct an activator that responds to rapamycin drug. The 2 dSpCas9 fragments, named dSpCas9(N) and dSpCas9(C), were attached to 2 different domains (FRB and FKBP, respectively) of the mechanistic target of rapamycin (mTOR). To avoid auto-reassembly of dSpCas9, dSpCas9(N)-FRB was fused to a nuclear export sequence, whereas dSpCas9(C)-FRKP was fused to 2 nuclear localization sequences (NLS). Finally, VP64 was added to dSpCas9(C)-FKBP-2xNLS. In presence of rapamycin, FRB and FKBP dimerize and allow the reconstruction and the nuclear import of the dSpCas9-VP64:gRNA activator. A consequent 57 -fold activation in the expression of the targeted human gene (ASCL1) was reported.

In a recent work Gao et al.[67] tested 6 different pairs of heterodimerization domains to construct 3 light and 3 chemical CRISPR-dCas9 inducible systems. Only 2 of them–responding to abscisic acid (ABA) and gibberellin (GA)–led to the construction of strong activators. As in,[64,65] one domain was fused to dSpCas9, the other to VPR (see Fig. 5A) Other activation domains were tested too but turned out to be less efficient. Inducible repressors were built by exchanging VPR with KRAB. The same architecture was followed to build trascription factors on dCas9 from S. aureus. They were not able to enhance the expression of a green fluorescent protein to the same levels reached by dSpCas9-based activators (only about 20–30% of them). However, transcription factors build on the 2 different dCas9 were shown to be orthogonal and, hence, able to carry out in human cells simultaneous regulation of 2

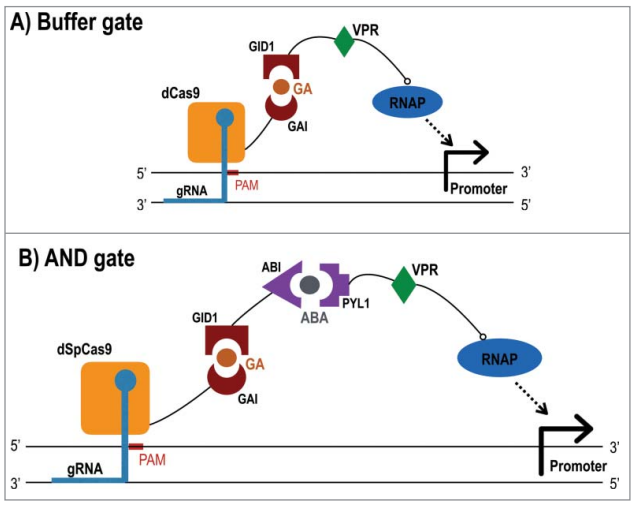

Figure 5. CRISPR-dCas9-based inducible systems. (A) In presence of GA, GAI dimerizes with GID1 and the VPR activation domain can recruit RNA polymerase II to the promoter targeted by dCas9:gRNA. In logic terms, this is a *buffer* gate. (B) Only under simultaneous induction with GA and ABA, both GAI-GID1 and ABI-PYL1 dimerize. This double heterodimerization bridges dCas9:gRNA to the VPR and results in promoter activation. In this configuration, a single chemical is not enough to trigger protein expression. Hence, the whole system behaves as an AND gate.**

different genes without interfering to each other. Furthermore, a 2-input OR gate was built by fusing dSpCas9 to 2 different domains, one bound by ABA (called ABI) and the other by GA (GAI), with their pairing monomers (PYL1 and GID1, respectively) fused to VPRs. A more complicate structure was required for engineering an AND gate. Here, dSpCas9 was fused to GAI, GID1 was linked to ABI and, finally, PYL1 was fused to VPR (see Fig. 5B). Hence, only in presence of the 2 chemicals VPR is wired to dSpCas9 and the target promoter is activated.

Toward the use of different nucleases

dSpCas9:gRNA has proved to be a useful instrument for the relatively easy engineering of new synthetic transcription factors. However, its applicability to the construction of complex genetic networks, such as multi-input digital circuits,[68] has still to be clarified. In particular, dSpCas9:gRNA usage as a repressor might be limited by the fact that it recognizes only DNA sequences followed by the protospacer adjacent motif NGG or NAG. Although both dSpCas9 PAMs are not difficult to be found in eukaryotic promoters, their position relatively to the TATA box or the TSS might be non-optimal for an efficient transcription downregulation. Engineering variants of SpCas9 able to recognize new PAM triplets, as shown in,[69] is a possible solution to this issue. In alternative, SpCas9 orthologs, which recognize PAMs different from NGG or NAG,[70] could be used. Moreover, as discussed above, orthologous dCas9 can be used into the same circuit and regulate the expression of multiple genes without crosstalk.[34,67]

Beside that, different CRISPR-associated nuclease proteins are under study and might be adopted for Synthetic Biology purposes. For instance, the DNA editing properties of the CRISPR type V Cpf1 endonuclease in human cells are already well-know.[71,72] Moreover, Cpf1 crystal structure in complex with a crRNA[73] and also a target DNA sequence[74] have been reported recently. With respect to Cas9, Cpf1 recognizes a different PAM (such as TTN in Francisella novicida–FnCpf1), has a single RuvC-like nuclease domain–which can be silenced via point mutations–and binds a shorter crRNA, roughly half the length of the one associated with Cas9. Therefore, dCpf1:gRNA might be a favorable template for the construction of new transcription factors. To date, the only Synthetic Biology application based on FnCpf1 is a method for the assembly of plasmids whose expression, however, is limited to E. coli.[75] We envisage that the construction of multi-component CRISPR-based synthetic gene circuits, able to achieve high performances in eukaryotic cells, will demand soon to go beyond the usage of the only CRISPR-Cas type II system.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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