RESEARCH INNOVATION VALUE

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An inducible CRISPRa system for gene manipulation in cell culture cells

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Abstract

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The invention of the CRISPR (clustered regularly interspaced short palindromic repeat) technology for guided gene knock-out is one of the major scientific findings within the last decades. It is derived from anti-viral bacterial defense mechanisms and was adapted for biotechnology applications in 2013 (Jinek et al. 2012; Qi et al. 2013). The main application of the technique is based on the finding, that the bacterial nuclease Cas9 (CRISPR associated protein 9) can be directed site specifically to the genome by the use of small complementary guide RNAs (gRNAs) and causes DNA double strand breaks due to its nuclease activity. These lesions are then either repaired by non-homologous end joining or by homologous recombination leading to a disruption of the genomic context. If the strand break is within the coding region of a gene a small insertion or deletion appearing after non-homologous end joining leads to a change in the reading frame of the protein and most likely to a non-functional protein equivalent to a knock-out of the gene. This system was rapidly adapted for a broad range of application in a variety of organisms from plants to mammalian cell culture cells to in vivo model organisms such as mouse and fish (Belhaj et al. 2013; Mao et al. 2013; Ran et al. 2013; Yang et al. 2013; Wang et al. 2013; Irion et al. 2014). The design of the optimal complementary small gRNAs has been investigated thoroughly (Ran et al. 2013; Doench et al. 2014; Doench et al. 2016) and only follows very loose rules regarding the length (20 nt) and a Cas9 specific protospacer adjacent motif (PAM) sequence (NGG for Cas9 of *Streptococcus pyogenes*) being present at the cutting site downstream of the gRNA binding site

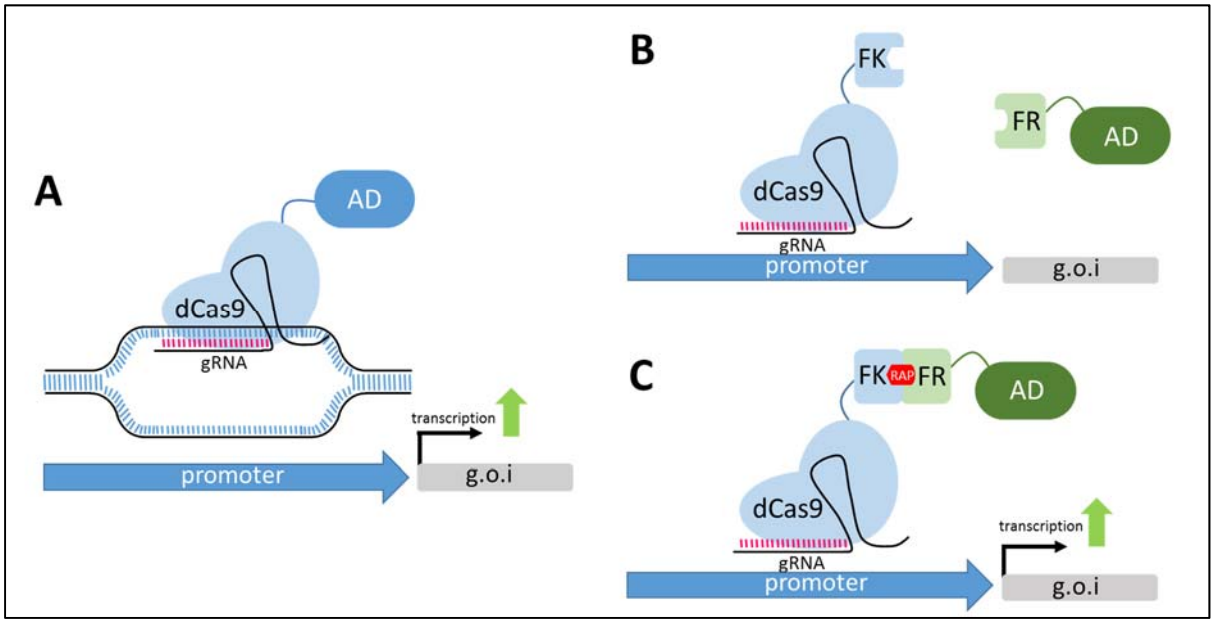

Figure 1. CRISPRa system for gene activation. A catalytically dead Cas9 (dCas9) protein is directed to the promoter region of a gene of interest (g.o.i.) via sequence specific small guide RNA (gRNA). In the conventional system (A) dCas9 is fused to an activation domain (AD) to induce transcription. For the small molecule induced CRISPRa system (B,C), the dCas9 is fused to an FK and the AD domain Is fused to FR. If no dimerizer (RAP) is present dCas9 is located to the promoter region (B) but only after RAP addition the AD is recruited to the complex to initiate transcription of the g.o.i. (C).

A further application of the CRISPR system is the manipulation of gene expression with the use of catalytically dead Cas9 (dCas9) mutants that still have the ability to bind specifically to their genomic target site (directed by the sgRNA) but do not exhibit nuclease activity (Gilbert et al. 2013) . Of special interest for these proteins is the targeting of promoter regions within the genome. Promoter regions normally contain binding sites for specific transcription factors and gene expression is regulated by signalling pathways modulating the activity of the transcriptions factors. General transcription factors have a modular design consisting of a DNA binding domain that mediates the binding to the specific sequences in the promoter region, and an activation domain that recruits the polymerase II complex for transcription (Ansari / Mapp 2002). To artificially induce gene activation without the need for transcription factor activation the dCas9 proteins can be fused to transcriptional activation domains and, when gRNAs target the promoter region of a gene of interest, initiate gene transcription. Due to the simple design rules of gRNAs it is therefore possible to activate transcription of any given gene with this system only by transfecting a dCas9-activator fusion and a gRNA construct. This method of CRISPR guided gene activation (CRISPRa) is already used for targeted gene manipulation of single genes as well as in large screening projects to find out gene function and regulation networks (Cheng et al. 2013; Kearns et al. 2014; Gilbert et al. 2014)

Our approach is to combine this CRISPRa technique with the system of inducible transcription by a small molecule dimerizer system. The small molecule rapalog is a derivative of rapamycin from *Streptomyces hygroscopicus* and specifically dimerizes two protein domains (FK and FR) without having any negative influence on normal cell function (Liberles et al. 1997; Pollock et al. 2002). This hetero-dimerization system has already a broad range of applications *in vitro* and *in vivo* (Rivera et al. 1996). One major application is the hyper-activation of transcription by recruiting multiple strong activation domains via FR fusion to DNA bound FK domains (Natesan et al. 1999). For the inducible CRISPRa system instead of directly fusing the activation domain to the dCas9, dCas9 is fused to 3 FK domains

whereas the activation domain is fused to FR. The three components (gRNA, dCas9-FK fusion protein and FR-activation domain fusion protein) are then transfected and constitutively expressed in cell culture cells without effect on target gene transcription. Only upon rapalog addition FK and FR domains dimerize and the activation domain is brought into close proximity to the transcription start site on the promoter, leading to an immediate gene activation. Instead of only looking at an activated state compared to untreated cells, the inducibility of the system provides convenient control reactions by comparing plus and minus rapalog conditions.

One critical point for induced transcription is the choice of the activation domain. For establishment of this system we tested different activation domains of viral (VP16, VP96) (Triezenberg et al. 1988; Balboa et al. 2015), mammalian (p65) (Ballard et al. 1992) or hybrid (VPR) (Chavez et al. 2015) origin and found the most potent transactivation with the VPR activation domain consisting of the activation domain VP48 and the activation domains of NFκ-B p65 and Rta. For a first validation of the method, a luciferase reporter system was used. The reporter construct contains multiple artificial binding sites (ZFHD) (Pomerantz et al. 1995) for a specific gRNA (sgZF) upstream of a minimal promoter and a firefly luciferase gene (Fig. 1). Upon binding of the gRNAs to its target sequence on the reporter, the dCas9-FK fusion protein is recruited to the reporter plasmid within the cell but only after addition of rapalog the activation domain is brought to the complex via FK-FR dimerization. The presence of the activation domain then leads to the expression of firefly luciferase. This bioluminescent enzyme produces light after substrate addition and can be detected with great sensitivity with a luminometer plate reader after cell lysis. The results of transient experiments showed a concentration dependent, strong inducibility of reporter gene expression upon rapalog addition with its maximum 24 hrs after rapalog addition. The firefly luciferase activity of the rapalog treated cells increased up to 3000-fold while the non-rapalog control still stayed at basal luciferase levels. The time and concentration dependency provides a wide spectrum of regulation of the system and can be used to induce different levels of gene activation. This might give further information on the tight regulation of signalling networks. For a proof of concept of the inducible CRISPRa system for endogenous target genes we selected the heat shock protein 72 (Hsp72). This protein is encoded by the HSPA1A gene and functions as a chaperone. Upon stress its expression is upregulated and the protein helps to ensure correct folding of native peptides as well as refolding of denatured proteins. We designed four different gRNAs targeting the HSPA1A promoter to activate transcription. First, again a luciferase assay was used to help select the most promising gRNA. Therefore the HSPA1A promoter region was cloned upstream of a firefly luciferase gene (Ortner et al. 2015) and co-transfected with the components for induced CRISPRa. Using this artificial reporter assay together with one gRNA, the reporter was activated up to 5-fold. These preliminary reporter experiments gave first informations on the function of the individual components. Nevertheless, most important is a proof of its functionality in the genomic context. As a read out the mRNA level of the target gene was determined with quantitative PCR after RNA isolation and cDNA synthesis. Therefore, the best sgRNA targeting the HSPA1A promoter was used and Hsp72 mRNA levels were determined with quantitative PCR. Also in this context the system showed reliable induction of 2.5-fold on HSPA1A mRNA level. The relatively low activation can be explained by the insufficient transfection efficiency of the 3 different constructs (sgRNA expression construct, dCas9-FK expression construct, FR-AD expression construct). To circumvent this problem and establish a reliable and reproducible system for gene activation we created stable cell lines expressing dCas9-FK together with FR-VPR. For the generation of the stable cell line a two-step approach was applied using lentiviral transduction. First lentiviral particles for the constitutive expression of dCas9-3xFK were created and subsequently a stable dCas9-FK cell line was generated using HEK293 cells. Second, lentiviral particles for the introduction of FR-VPR were created and the Cas9-FK stable cell line was transduced. The resulting cell line was then again screened with the sgZF reporter system and the cell clone with the highest reporter induction was selected for further experiments. To further improve the activation of endogenous target genes a combined vector for multiple sgRNAs targeting the same promoter was established. To facilitate easy and rapid production

of an expression vector containing up to 4 different sgRNAs a golden gate cloning strategy was established (Kabadi et al. 2014). By using type II restriction enzymes this technique allows the combination of 4 different sgRNA expression cassettes within a single cloning step. The generated cell line is an ideal tool to study gene function with controlled and inducible overexpression of target genes. In our hands it will be used to study single components of signalling pathways by observing changes in pathway activities upon up-regulation of individual components. To activate the gene of interest sgRNAs can either be expressed from a multiplex vector with up to 4 different sgRNAs, or brought into the cell directly as RNAs for even higher transfection efficiencies. The rapalog based induction can lead to higher induction levels due to recruitment of multiple activation domains to one dCas9 protein by using fusions with three FK domains. The most important improvement over already existing CRISPRa approaches is the addition of an uninduced control with all components (even the sgRNAs) present in the system. This control provides optimal conditions to find out the specificity of the observed effects after target gene induction. Based on these improvements the presented induced CRIPSRa method provides yet another valuable application in the ever growing family of CRISPR/Cas9 technologies.

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