CellPress

Molecular Cell
Technology Preview

Putting Non-coding RNA on Display with CRISPR

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http://dx.doi.org/10.1016/j.molcel.2015.07.002

In a recent issue of *Nature Methods*, Shechner et al. (2015) reported the development of CRISPR Display (CRISP-Disp), which is a sophisticated, flexible, modular, and multiplexable platform for targeting different types of non-coding RNAs (ncRNAs) to genomic loci. CRISP-Disp will facilitate synthetic-biology applications and enable the elucidation of ncRNA functions.

Non-coding RNAs (ncRNAs) are a diverse set of transcripts that play myriad roles in regulating critical cellular functions. The rapid improvement of deep-sequencing technologies has helped uncover novel ncRNAs belonging to different classes such as long non-coding RNAs (lncRNAs), microRNAs, circular RNAs, piwi-interacting RNAs, and many others (Cech and Steitz, 2014). The abundance, conservation, and structural variety of ncRNAs, as well as the multiplicity of their functions in the regulation of gene expression and genome remodeling, have enriched our knowledge of cell biology and challenged longstanding assumptions about the organization of eukaryotic genomes. However, for many ncRNAs, the precise functions and the mechanisms by which they are executed remain largely unknown. New technologies that enable targeting of ncRNA to specific loci and manipulation of protein partners are needed to elucidate ncRNA functions and adapt them for new applications. To address this need, Shechner et al. (2015) recently developed a platform that they named CRISPR-Display (CRISP-Disp) to interrogate or repurpose ncRNA function. This platform utilizes CRISPR-Cas9-based genome-targeting technologies to deliver ncRNA cargoes to predetermined loci.

The CRISP-Disp system uses a catalytically dead Cas9 (dCas9) that acts as a programmable DNA binding protein whose specificity is determined by a short RNA sequence known as a guide RNA (gRNA) and whose function is determined by fusion with heterologous effector domains (Figure 1A). Traditionally, a gRNA consists of about 20 base pairs (bp) that specify the target sequence followed by a loop approximately 80 bp long that provides the tridimensional structure needed for dCas9 to appropriately recognize the gRNA-genomic DNA complex (Farzadfard et al., 2013; Maeder et al., 2013; Perez-Pinera et al., 2013; Qi et al., 2013). Alternatively, a minimal hairpin aptamer that binds the MS2 protein can be appended to the gRNA tetraloop and stem loop 2. Fusion of MS2 with a transcriptional activation domain has been shown to efficiently activate gene expression (Figure 1B) (Konermann et al., 2015).

Shechner et al. (2015) demonstrate that the DNA-targeting sequence of the gRNA can be part of a much larger cargo without affecting dCas9 binding and activity. The authors accomplished this by performing a set of experiments in which they fused aptameric "accessory RNA domains" with gRNAs at different positions in the gRNA architecture, including internally as well as at the 5′ or 3′ ends. The integrity of the targeting mechanism was tested by measuring reporter gene activation with a dCas9-VP64 fusion protein ("direct activation") or with dCas9 and, separately, an aptamer-binding protein coupled with VP64 ("bridged activation"). Direct activation demonstrated the integrity of the targeting mechanism, while bridged activation showed that the fused RNA accessory domain was sterically and functionally accessible to protein binding partners. These experiments identified several gRNA-ncRNA fusion topologies that permit targeting of ncRNAs to specific genomic DNA loci without disrupting the functionality of dCas9. This modified CRISPR-Cas system points to a wide range of possibilities for synthetic biology applications or mechanistic characterization of ncRNAs (Figure 1C).

CRISP-Disp is an attractive system for the synthetic biologist due to its flexibility, modularity, and multiplexability. Shechner et al. (2015) demonstrated the potential of the CRISP-Disp system to accommodate gRNAs with large accessory RNA domains up to ~4.8 kb in size, approaching the length of natural lncRNAs. The authors expressed these modified lncRNAs from RNA polymerase II promoters that permit synthesis of transcripts longer than those possible with typical RNA polymerase III promoters that have been previously used to express gRNAs (Nissim et al., 2014). However, since lncRNAs represent only a fraction of all ncRNAs, the authors also tested this platform with other natural and artificial ncRNAs including RNA domains with protein-binding motifs and artificial aptamers that bind proteins or small molecules (Figure 1C) and found that the functionality of CRISP-Disp is preserved independently of the "accessory ncRNA domain."

Furthermore, the CRISP-Disp platform is multiplexable and modular because the gRNA can be coupled with multiple ncRNA domains that can function simultaneously and independently of each other. These properties can be unlocked by using orthogonal RNA-binding proteins that perform distinct tasks at multiple genomic loci while sharing the same pool of dCas9. For instance, Shechner

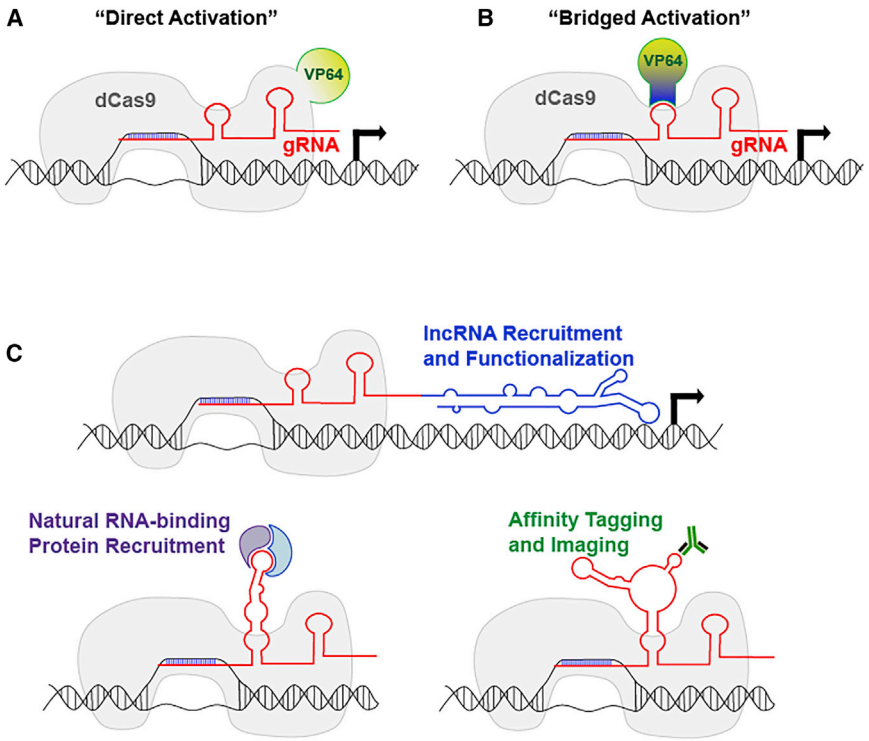


Figure 1. Structure and Applications of CRISP-Disp

(A and B) Schematic representation of two CRISPR systems for gene activation. Catalytically inactive Cas9 (dCas9) is targeted to a particular locus by a short RNA sequence known as a guide RNA (gRNA). The VP64 transcriptional activation domain can be recruited to the complex by fusion to dCas9 (direct activation, A) or by fusion to RNA-binding proteins that bind to the gRNA (bridged activation, B).

Shechner et al. (2015) demonstrated that dCas9 can be targeted to genomic DNA loci by modified gRNAs that are part of larger RNA cargoes. This system, named CRISP-Disp, enables ncRNA targeting for studies that require ncRNA recruitment and functionalization, natural RNA-binding protein recruitment, or affinity tagging (C).

et al. (2015) co-expressed two gRNAs targeting a reporter gene and a telomeric sequence, respectively, each fused to orthogonal aptameric accessory domains. With this strategy the authors were capable of activating reporter gene expression at one locus while imaging a different locus. These two functions could be deployed simultaneously and controlled independently with the CRISP-Disp framework. As the CRISP-Disp system affords control over the recruitment of RNA-protein complexes to specific loci, one can envision its potential uses in engineering sophisticated gene regulatory circuits, such as genetic logic gates based on recruiting different transcriptional activators and repressors to synthetic promoters in response to defined inputs.

In addition to the applications of CRISP-Disp in synthetic systems, it has the potential to become a powerful tool to study the function of biological ncRNAs, including lncRNAs, which are an abundant and intensively researched class of ncRNAs. Yet despite deep efforts to understand the functions and mechanisms of action of lncRNAs, platforms to systematically construct, target, and characterize lncRNAs have been relatively lacking. One particularly outstanding question is: what are the RNA-intrinsic functions of lncRNAs? If a lncRNA locus, for instance, activates gene expression, is it due to the effect of transcription from the lncRNA locus, or is there a modular, sequence-dependent effect mediated by the RNA itself? CRISP-Disp can be used to address this question by probing the contribution of lncRNAs to a spectrum of lncRNA-associated phenomena including epigenetic modification, chromatin remodeling, or transcriptional regulation. For example, the lncRNA Xist has been shown to regulate X chromosome inactivation in *cis* through recruitment of the Polycomb repressive complex 2 (PRC2) (Zhao et al., 2008). This RNA-intrinsic repressive mechanism was tested by Shechner et al. (2015) by fusing the RepA domain of Xist to a gRNA and targeting it to a reporter gene. Consistent with canonical Xist function, they observed significant, albeit modest, repression of the target reporter by the Xist fusion gRNA. This finding shows that a significant portion of the repressive function of Xist lies in the RepA domain and, importantly, that this function is retained when abstracted from the context of the X chromosome. It is also known that other protein complexes in addition to PRC2 are necessary for Xist-mediated chromatin silencing (McHugh et al., 2015). Immunoprecipitation of dCas9 in the CRISP-Disp system followed by mass spectrometry could offer a novel method to identify proteins involved with functional lncRNA domains. The flexibility of CRISP-Disp also raises the possibility that Xist or other lncRNAs could be used as experimental tools to direct chromatin modifications at loci of choice.

Other types of lncRNAs are associated with the activation of gene expression. For example, RNAs transcribed from enhancer regions (eRNAs) are, in many cases, necessary for the expression of adjacent genes (Cech and Steitz, 2014). However, it remains unclear whether eRNA function is due to the act of transcription in general, to RNA-guided topological changes bringing the enhancer in close proximity to a promoter, or to other sequence-specific properties of eRNAs. If the gene-activating function of eRNAs were intrinsic to their sequence, then they could be appended to a CRISP-Disp system and employed to activate an arbitrary locus. Shechner et al. (2015) tested this proposition by fusing the *FALEC* and *TRERNA1* eRNAs to gRNAs targeting a reporter gene, but they observed only extremely modest activation with *TRERNA1* and no significant activation with *FALEC*. Though it will be necessary to test eRNAs more thoroughly with CRISP-Disp, it seems that some eRNAs cannot carry out strong activation of gene expression in a synthetic system. Therefore, these preliminary

results are consistent with the hypothesis that eRNAs are components of three-dimensional, locus-specific enhancer complexes.

Further work will be needed to test the limitations of this technology with a wider range of artificial and natural ncRNAs, to improve the efficiency of encoded functions, and to demonstrate its ability to uncover novel biological mechanisms. In summary, CRISP-Disp provides a broadly useful toolkit for targeting ncRNAs to specific genomic loci with the potential for deploying multiple, independently controllable functions.

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