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FOCUSED REVIEW

The CRISPR/Cas revolution reaches the RNA world: Cas13, a new Swiss Army knife for plant biologists

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SUMMARY

Application of the bacterial CRISPR/Cas systems to eukaryotes is revolutionizing biology. Cas9 and Cas12 (previously called Cpf1) are widely used as DNA nucleases for inducing site-specific DNA breaks for different kinds of genome engineering applications, and in their mutated forms as DNA-binding proteins to modify gene expression. Moreover, histone modifications, as well as cytosine methylation or base editing, were achieved with these systems in plants. Recently, with the discovery of the nuclease Cas13a (previously called C2c2), molecular biologists have obtained a system that enables sequence-specific cleavage of single-stranded RNA molecules. The latest experiments with this and also the alternative Cas13b system demonstrate that these proteins can be used in a similar manner in eukaryotes for RNA manipulation as Cas9 and Cas12 for DNA manipulations. The first application of Cas13a for post-transcriptional regulation of gene expression in plants has been reported. Recent results show that the system is also applicable for combating viral infection in plants. As single-stranded RNA viruses are by far the most abundant class of viruses in plants, the application of this system is of special promise for crops. More interesting applications are imminent for plant biologists, with nuclease dead versions of Cas13 enabling the ability to visualize RNA molecules *in vivo*, as well as to edit different kinds of RNA molecules at specific bases by deamination or to modify them by conjugation. Moreover, by combining DNA- and RNA-directed systems, the most complex of changes in plant metabolism might be achievable.

Keywords: genome engineering, gene editing, C2c2, RNA processing, RNA editing.

INTRODUCTION

The recent emergence of the CRISPR/Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated) system as a biotechnological tool has revolutionized molecular biology (Jinek *et al.*, 2012). It enables precise and efficient induction of DNA double-strand breaks (DSB) at any site in the genome. Even though targeted DSB induction was possible with different technologies before (Kim *et al.*, 1996; Boch *et al.*, 2009; Voytas, 2013), the CRISPR/Cas system was a breakthrough: its advantage resides in RNA-based sequence specificity, enabling a very simple, fast and reliable design process. After DSB induction, the cell's inherent DSB repair mechanisms can be harnessed for genome-editing purposes, which was demonstrated over 20 years ago (Puchta *et al.*, 1996; Salomon and Puchta, 1998).

The natural CRISPR/Cas systems are adaptive immune systems of prokaryotes and offer enormous diversity. Current classification differentiates two classes, six types and many more subtypes of CRISPR (Koonin *et al.*, 2017; Shmakov *et al.*, 2017). The defining feature differentiating the two classes is the nature of the effector cleaving the target sequence. Class 1 systems (including type I, III and IV) employ several Cas proteins and crRNA to form multi-subunit effector complexes, whereas Class 2 systems (including type II, V and VI) employ a single, large, multi-domain protein. Class 1 systems are the most abundant CRISPR/Cas systems found in both bacteria and Archaea. Class 2 systems are much less common and largely restricted to bacteria (Burstein *et al.*, 2017). The Class 2 type II system of *Streptococcus pyogenes* was the first to be transformed into a genome engineering tool. In the natural system, a complex of the large multi-domain protein Cas9 and two short RNAs, tracrRNA and crRNA, is able to cleave foreign plasmid or viral DNA entering the cell.

Cleavage is dependent on the presence of a short sequence motif next to the target sequence termed protospacer adjacent motif (PAM). Cas9 contains two nuclease domains, RuvC and HNH, each cleaving one strand 3 bp upstream of the PAM (Jinek *et al.*, 2012).

In its simplest form, the CRISPR/Cas system can be used for sequence-specific mutagenesis, which is now a routine procedure. This is mainly used to disrupt genes in order to analyse their function, but can also be used to improve traits in crops (see Pacher and Puchta, 2017 and Scheben *et al.*, 2017 for an overview of modified traits). However, the CRISPR/Cas system is a much more versatile tool (Puchta, 2017). Cas9 can be transformed into a DNA-binding protein by mutating its two nuclease domains. This way, its DNA cleavage activity is deactivated but its DNA-binding potential remains intact. By fusing this nuclease 'dead' Cas9 (dCas9) directly or indirectly to an effector domain, it can then be used to guide diverse enzymatic functions to any specific site in the genome (Gilbert *et al.*, 2013; Konermann *et al.*, 2015). This enables the use of CRISPR/Cas for many diverse site-specific manipulations, such as regulation of gene expression (Piatek *et al.*, 2015; Tang *et al.*, 2017), epigenetic modifications (Hilton *et al.*, 2015; Thakore *et al.*, 2015), imaging of genomic loci in live cells (Dreissig *et al.*, 2017), or base editing without DSB induction enabled by fusion of deaminases (Komor *et al.*, 2016; Zong *et al.*, 2017).

Due to their considerable diversity, natural CRISPR/Cas systems are a very rich, and up to now still largely untapped, resource for further biotechnological tools. Using data-mining of microbial sequence databases along with bioinformatic prediction approaches, more and more CRISPR/Cas systems beyond CRISPR/Cas9 of *S. pyogenes* are being identified (Makarova *et al.*, 2015; Shmakov *et al.*, 2017). However, in order to become a useful genome engineering tool, a newly identified CRISPR system must exhibit activity in eukaryotic cells, a property that is confined to only a small subset of the abundant diversity. Still, extensive screening efforts were fruitful in yielding not only further Class 2 type II systems, whose defining hallmark protein is Cas9, like those from *Streptococcus thermophilus* (Esvelt *et al.*, 2013) or *Staphylococcus aureus* (Ran *et al.*, 2015), but also systems from completely different Class 2 types. The first Class 2 effector bioinformatically identified other than Cas9 was Cas12 (Schunder *et al.*, 2013) (then known as Cpf1), which was assigned to Class 2 type V (Makarova *et al.*, 2015). Zetsche *et al.* (2015) then screened various orthologues of Cas12a, and identified two showing high activity in eukaryotic cells. Due to its unique properties, like requiring completely different PAMs and generating staggered breaks as opposed to the blunt breaks induced by Cas9, this was an extremely useful expansion of the genome engineering toolbox.

Bullet Point Summary

- Cas13 is a newly identified CRISPR effector that specifically cleaves single-stranded RNA in eukaryotic cells, opening up a wide range of new possibilities.
- The RNA cleavage ability can be used for downregulation of specific transcripts with improved specificity over RNAi.
- A first report demonstrated the successful use of Cas13 to combat RNA viruses in plants.
- Analogous to Cas9, Cas13 can be converted into a RNA-binding platform by inactivation of its nuclease domain.
- This enables diverse RNA manipulations, from base editing to base modification to visualization.

A NEW PLAYER THAT TAKES US TO THE RNA LEVEL: CAS13

More recently, Shmakov *et al.* (2015) bioinformatically predicted three novel Class 2 effectors, termed Class 2 candidate1 (C2c1), C2c2 and C2c3. Whereas C2c1 and C2c3 show similarity to Cas12, C2c2 is utterly unrelated. Due to its unique features, this system, now known as CRISPR/Cas13a, was assigned to a completely new type, Class 2 type VI. The presence of HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domains, which are exclusively associated with RNase activity, suggested that Cas13, as opposed to all other functionally characterized CRISPR systems, acts exclusively on RNA. This auspicious hypothesis could later be confirmed (Abudayyeh *et al.*, 2016): Cas13 cleaves single-stranded, but not double-stranded, RNA (Figure 1). Intriguingly, after activation by the target RNA, other RNAs in the solution were cleaved as well in an unspecific manner. This suggests that in the natural system Cas13 elicits programmed cell death or dormancy. The same group also identified a Cas13 orthologue with high activity in eukaryotic cells (Abudayyeh *et al.*, 2017; Cox *et al.*, 2017). Most importantly, the unspecific RNA degradation observed *in vitro* and in prokaryotic cells was absent in eukaryotic cells, enabling highly specific RNA targeting in higher organisms. These findings opened up a wide range of new possibilities by targeted RNA manipulation.

Quickly after the first report on the RNA targeting capability of Cas13, its structure was analysed in detail (Liu *et al.*, 2017a). Similar to other Class 2 effectors, Cas13 adopts a bilobed architecture consisting of a recognition and a nuclease lobe, but the domains involved are utterly distinct and unrelated to other Class 2 effectors. Just like Cas12, Cas13 proteins exhibit the ability to process pre-crRNA on their own without the involvement of tracrRNA. The activity responsible for crRNA maturation is distinct

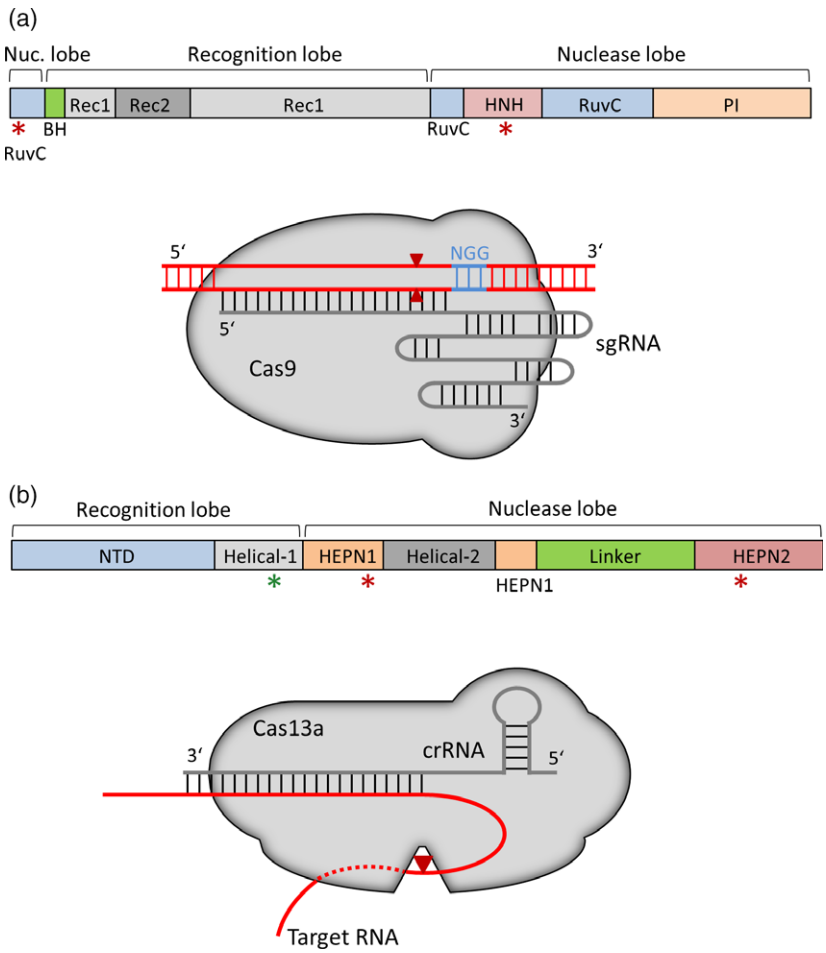

Figure 1. Schematic comparison of the enzyme complexes (a) Cas9 and (b) Cas13a.
The bars show the domain organization. The drawn sizes of the domains correspond to their true size. Red asterisks indicate catalytic sites for target DNA/RNA cleavage. The green asterisk indicates the catalytic site for pre-crRNA processing. Dark red triangles indicate cleavage of the target DNA/RNA. BH, bridge helix; PI, PAM interacting domain; NTD, N-terminal domain.

from the target RNA cleavage activity and is mediated by another domain, the Helical1 domain. Similar to type V systems (containing Cas12), the crRNA in Cas13-containing systems contains a single stem loop in the direct repeat (DR). Cas13 undergoes substantial conformational change not only upon binding of crRNA but also upon target RNA binding. The latter leads to activation of the HEPN catalytic site, which is formed by two very structurally distinct HEPN domains, HEPN1 and HEPN2. In contrast to the catalytic sites in Cas9 and Cas12, which are located deep inside the protein, the HEPN catalytic site of activated Cas13a is located on the outer surface, leading to cleavage of the target RNA outside of the binding region. Intriguingly, it also causes unspecific cleavage of other RNAs in the surrounding solution (East-Seletsky *et al.*, 2016; Liu *et al.*, 2017a,b).

USING CAS13 FOR TARGETED CLEAVAGE OF CELLULAR RNA

In its simplest form, Cas13 can be used for targeted RNA cleavage, for example for downregulation of a specific transcript (Figure 2a). Abudayyeh *et al.* (2017) identified Cas13a from *Leptotrichia wadei* as the most active Cas13a orthologue for targeted RNA knockdown in human cells. Although some Cas13a orthologues require a protospacer flanking site (PFS) analogous to the PAM, there was no such restriction for LwaCas13a. However, its high activity was dependent on a stabilization domain (msfGFP). Concerning cellular localization, fusion of a nuclear localization signal yielded higher efficiencies than nuclear export signals. The level of RNA knockdown was comparable to RNAi but, in terms of specificity, the Cas13a system was clearly superior. Single and double mismatch analysis revealed the presence of a mismatch-sensitive 'seed region' in the centre of the spacer, which can be explained by the structural finding that the central part of the spacer is exposed to the solvent and recognizes the target RNA first (Liu *et al.*, 2017a). Strikingly, transcriptome-wide mRNA sequencing detected hundreds of significant off-targets for RNAi approaches, but no differentially expressed genes other than the target gene for LwaCas13a-mediated RNA knockdown. This suggested a lack of collateral RNA degradation activity as described for *Leptotrichia shahii* Cas13a (Abudayyeh *et al.*, 2016), at least in eukaryotic cells.

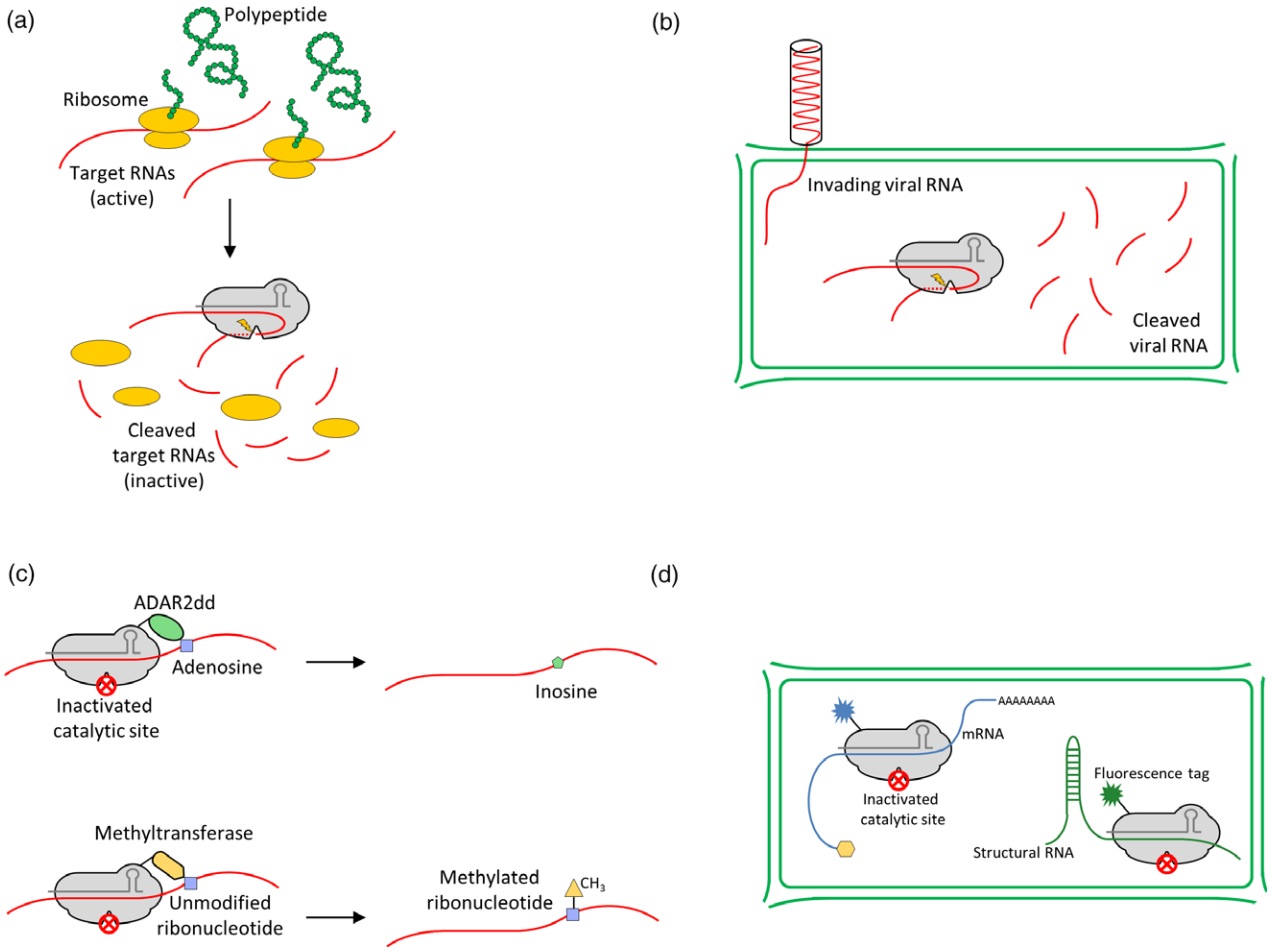

Figure 2. Applications of Cas13 for plant biology.
Active Cas 13 can be used for specific cleavage of target RNAs for downregulation of expression (a) and for defence against plant RNA viruses (b). When the RNA cleavage site is inactivated, dCas13 can be used to direct various effectors to specific RNAs (c) or to visualize specific RNAs in live cells (d).

By the same group, the functionality of LwaCas13a-mediated RNA knockdown was also confirmed for plants: three different genes were targeted in rice protoplasts, with most guides exceeding 50% knockdown at only 48 h after transformation. This demonstrates that Cas13 can rapidly diminish the cytoplasmic RNA pool in plants. In addition, it indicates a broad range of editable organisms beyond human cells for LwaCas13a. Furthermore, Abudayyeh *et al.* (2017) could successfully harness the crRNA maturation activity of LwaCas13a for multiplexing approaches, as was demonstrated for Cas12 before (Zetsche *et al.*, 2017).

Using a computational sequence data-mining approach with altered characteristics, another Class 2 effector containing HEPN domains and acting on RNA was identified. Due to its similarity to Cas13a, it was termed Cas13b (previously C2c6), and assigned to Class 2 subtype VIB (Smargon *et al.*, 2017). Interestingly, these CRISPR systems lack the otherwise ubiquitous Cas1 and Cas2 proteins. Instead they contain two previously uncharacterized associated proteins, Csx27 and Csx28, with Csx27 repressing and Csx28 enhancing Cas13b-mediated RNA cleavage. A large screen for RNA-targeting activity in eukaryotic cells yielded a Cas13b orthologue, from Prevotella sp. P5-125, showing consistently higher efficiencies than LwaCas13a (Cox *et al.*, 2017). In addition, its high activity was not dependent on the msfGFP stabilization domain and, in contrast to LwaCas13a, was most efficient when combined with a nuclear export signal. Just like LwaCas13a, PspCas13b lacked collateral RNA degradation in eukaryotic cells, did not show any PFS requirements and showed similar specificity, including the mismatch-sensitive central seed region. Furthermore, PspCas13b is amenable to multiplexing in the same way as Cas13a due to an inherent crRNA processing activity. This makes PspCas13b the first choice at the moment for targeted RNA cleavage. It would be interesting to test if this applies for plant cells as well.

The RNA-cleaving ability of Cas13 further opens up new opportunities for advances in basic research relating to the

functions of non-coding RNAs (ncRNAs). In theory, all ncRNAs exceeding a certain size should be amenable to Cas13-mediated cleavage. In plants, this mainly applies to long non-coding RNAs (lncRNAs), which now include a category referred to as intermediate sized non-coding RNAs (im-ncRNAs), arbitrarily defined as 50–300 nt long (Wang and Chekanova, 2017). Computational approaches identified over 6000 lncRNAs from Arabidopsis transcriptomic data sets, with mostly tissue-specific or stress-induced expression profiles (Liu *et al.*, 2015). lncRNAs are involved in a wide range of processes in plants: they can regulate gene expression at all stages, function as scaffolds for the assembly of protein complexes, guide protein translocation, influence alternative splicing and modulate chromatin loop dynamics (for review, see Liu *et al.*, 2015; Wang and Chekanova, 2017). However, many more functions of plant lncRNAs and the related mechanisms remain to be discovered, but functional studies on lncRNAs are hampered by a lack of mutants (Liu *et al.*, 2015). In addition to creating these mutants on the DNA-level using Cas9 or Cas12, Cas13 now enables to directly cleave these RNAs *in vivo*, providing greater flexibility in experimental manipulations. For example, using Cas13 it should now be possible to knockdown a specific RNA in the tissue of interest using a tissue-specific promoter, in cases where complete knockout mutants are lethal. Furthermore, different knockdown intensities can be analysed in addition to complete knockout. Opposed to RNAi, Cas13 offers the advantage that it is not limited to cytoplasmic transcripts, but non-coding nuclear transcripts can also be targeted by fusing a NLS to Cas13.

Another possible application of Cas13 for basic research is the functional analysis of the tRNA cleavage mechanism, which involves the release of ribonucleases cleaving in the anticodon loop of mature tRNAs upon stress signals. For example, Arabidopsis seedlings subjected to oxidative stress via H_2O_2 treatment showed a strong increase in cleaved tRNA fragments (Thompson *et al.*, 2008). The cleavage fragments then inhibit translation beyond the reduced availability of functional tRNAs, but the exact mechanism of inhibition is not yet elucidated. Cas13 could now enable artificial cleavage of tRNAs, although there are probably two challenges. First, it is unclear whether the modified nucleotides present in mature tRNA interfere with Cas13 cleavage. The second challenge will be to direct Cas13-mediated cleavage to the correct nucleotides. The tRNA cleavage of the endogenous RNases occurs at varying positions in the anticodon loop (Thompson *et al.*, 2008). Cas13-mediated cleavage was found to occur mostly at Uracil residues at the base of loop structures (Abudayyeh *et al.*, 2016), so the base of the tRNA anticodon loop should be targetable by Cas13. Thus, due to Cas13 the analysis of the tRNA cleavage mechanism might be no longer restricted to observation, but become more accessible for experimental manipulation to dissect its functions.

USING CAS13 FOR COMBATING RNA VIRUSES

It was already shown that the DNA-targeting ability of CRISPR/Cas9 can be used for defence against plant DNA viruses (Ali *et al.*, 2015). Yet, RNA viruses are the most common form of plant viruses, and even many plant DNA viruses contain an RNA intermediate at some stage of their life cycle (Roossinck, 2003; Nicaise, 2014). The yield losses that can be ascribed to plant viruses were estimated to be more than $30 billion annually (Sastry and Zitter, 2014). Worse, viral epidemics are often associated with famine events, for example in the case of Cassava, which constitutes the staple crop for more than 500 million people (Nicaise, 2014). In a pioneering study, Aman *et al.* (2018) demonstrated that the RNA-targeting ability of Cas13 can be used to combat RNA viruses (Figure 2b). They employed LshCas13a for interference against Turnip Mosaic Virus (TuMV), a Potyvirus, in *Nicotiana benthamiana*. They infiltrated leaves with mixed agrobacterium cultures carrying GFP expressing TuMV as well as LshCas13a containing a C-terminal NLS and different crRNAs targeting different parts of the viral genome. Seven days post-infiltration, about 50% reduction in GFP signal was observed for two of the tested crRNAs targets, but only a minor reduction in the case of the other two targets. These results could be confirmed in transgenic *N. benthamiana* lines stably expressing LshCas13a infiltrated with TuMV and crRNAs.

The off-target issue was not systematically addressed in this study, but no adverse effect on plant vitality was reported, suggesting that at least no collateral RNA degradation occurred in plant cells, which was reported for LshCas13a in prokaryotic cells and under *in vitro* conditions (Abudayyeh *et al.*, 2016; Gootenberg *et al.*, 2017). Although the efficiency of RNA virus interference was only modest in this study, it should be noted that it was achieved using the first characterized Cas13 variant. As mentioned above, more efficient Cas13 variants are available by now, which should translate into stronger virus interference. Besides, not all targets on the virus genome were effective, suggesting that target RNA accessibility impacts Cas13a activity, a limitation noted before (Abudayyeh *et al.*, 2017). Accordingly, it is advisable to always test multiple target sequences for each RNA targeted.

In addition to direct interference, there is another way in which Cas13 could be harnessed as a weapon against viral diseases. It relies on the 'collateral' RNA cleavage upon activation by a target RNA observed *in vitro* and in prokaryotic cells. This obviously prevents specific RNA cleavage under these conditions. However, the conformational change leading to activation of Cas13 itself remains

specific. In an elaborate way, this property can be exploited for highly sensitive detection of nucleic acids *in vitro*, as demonstrated by Gootenberg *et al.* (2017). In their technique termed SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing), the target DNA is amplified under isothermal conditions via recombinase polymerase amplification, reverse transcribed into RNA and subjected to Cas13a cleavage in the presence of a quenched RNA reporter that releases its fluorescence signal when collaterally cleaved by Cas13a (Figure 3). A specific RNA can be detected in the same manner, simply by reverse transcribing it into DNA before isothermal amplification. Their system makes use of the Cas13a orthologue from *L. wadei* (LwaCas13a). Remarkably, they achieved single-molecule sensitivity and single-mismatch specificity. Thus, SHERLOCK was able to clearly discriminate between Zika virus (ZIKV) and the related flavivirus Dengue (DENV), and could detect viral particles down to a concentration of 2aM.

Importantly, all SHERLOCK components including Cas13a–crRNA complexes can be lyophilized and later rehydrated, enabling the use of SHERLOCK in the field with paper-spotting. Moreover, all components can be combined in a single reaction, making the procedure simple and fast. This combined with its high sensitivity should enable the use of SHERLOCK for rapid detection of plant pathogenic viruses. Early and reliable detection is a key point for viral disease management in the fields (Boonham *et al.*, 2014), enabling more efficient and affordable countermeasures, for example in order to minimize the spread of the virus, infected plants need to be removed as fast as possible (Nicaise, 2014). Due to its high mismatch-sensitivity, differentiation between highly similar viral strains should also be possible and enable precisely adjusted intervention.

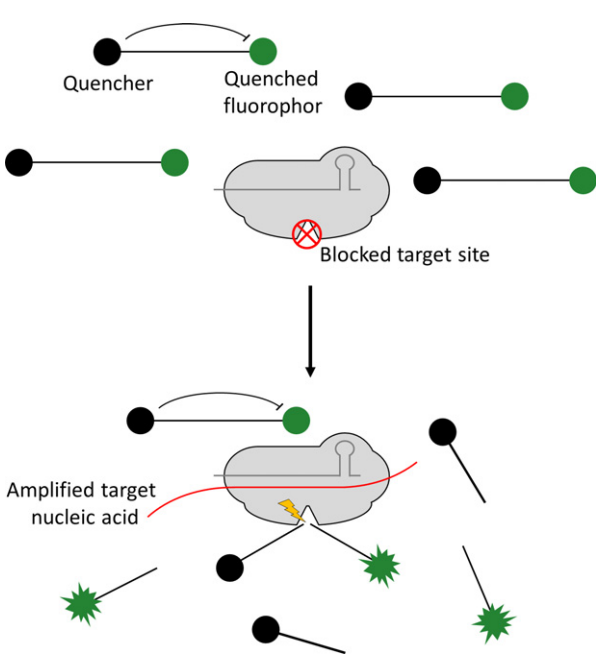

Figure 3. Detection of specific nucleic acids using the SHERLOCK method. After isothermal amplification and reverse transcription, the target nucleic acid leads to activation of Cas13, which then cleaves the reporter. The fluorescence of the released reporter can be detected.

USING CAS13 FOR PRECISE RNA MODIFICATIONS AND VISUALIZATION

Analogous to Cas9 and Cas12, the catalytic residues in the HEPN domains of Cas13 responsible for cleaving the target RNA can be deactivated by point mutations, creating a catalytically 'dead' dCas13. This way, a programmable RNA-binding protein is obtained. Abudayyeh *et al.* (2017) used dLwaCas13a fused to fluorescent proteins for specific imaging of transcripts in live cells. However, due to high levels of background noise from unbound protein, this required a negative-feedback system based upon zinc finger self-targeting and KRAB domain repression. Using this system, they were able to follow mRNA translocation into stress granules in live cells, and the fluorescent signals obtained correlated well with signals from fluorescent *in situ* hybridization. Fluorescent imaging of specific DNA sequences is already established in plants (Dreissig *et al.*, 2017). With the new Cas13 system at hand, visualizing the dynamics of RNA in live plant cells is also within reach now (Figure 2d).

Another new possibility enabled by Cas13 is precise RNA base editing. It was shown before that fusion of a cytidine or adenosine deaminase to deactivated or single-strand break inducing Cas9 can efficiently generate precise point mutations (Komor *et al.*, 2016; Gaudelli *et al.*, 2017) Now, Cox *et al.* (2017) have shown that using Cas13b, RNA can be edited in an analogous manner (Figure 2c). They chose the adenosine deaminase acting on RNA 2 deaminase domain (ADAR2DD) containing a hyperactivating mutation (E488Q) as an effector. This enzyme catalyses hydrolytic deamination of adenosine to inosine, which is functionally equivalent to guanosine in translation and splicing. In the end, this results in targeted A to G replacement on RNA. Editing efficiency was enhanced by using longer spacers (50 nt) that contained a mismatch at the site of the edited adenosine, with an A–C mismatch showing by far the highest efficiency. Using this system, they achieved editing rates robustly at or above 50% in reporter systems. It was also possible to correct two genes related to human diseases, AVPR2 related to X-linked Nephrogenic diabetes insipidus and FANCC related to Fanconi anemia, at 35% and 23% efficiencies, respectively. Concerning specificity, it must be noted that other adenosines on the target RNA were also edited and RNA seq analysis revealed many other off-targets across the entire transcriptome. This issue was addressed by introducing point

mutations into the ADAR2dd domain destabilizing unspecific RNA binding, which increased specificity dramatically, with only 20 instead of 18.385 off-targets, at only slightly decreased target editing rate. Concerning the off-targets on the target RNA, the exact target adenosine can be strongly favoured, as mentioned above, by placing a cytidine across of the target adenosine, creating an A-C mismatch that strongly promotes ADAR editing activity. It should be noted that compared with DNA base editing, RNA base editing is not stable but more easily reversible, allowing a delicate temporal control over the editing process (Cox *et al.*, 2017). In addition, DNA editing affects all transcripts, whereas in the case of RNA editing both edited and non-edited transcripts can be present at the same time, which should also enable a certain dosage control.

Specific RNA manipulations are by no means limited to deaminase domains, but many other effector fusions can be envisaged (Figure 2c). Translational enhancers or repressors could be fused to dCas13 in order to regulate translation of specific mRNAs. dCas13 could also be combined with a trafficking agent. This way, targeted RNAs are translocated to programmed cellular locations. Another valuable application for research purposes could be to use dCas13 to identify proteins bound to specific RNAs. By fusing dCas13 to an affinity tag for protein purification, proteins bound to the targeted RNA can be co-precipitated and further analysed (Mahas *et al.*, 2017).

An interesting target for Cas13-mediated RNA editing in plants for the purpose of basic research is tRNA. In fact, tRNAs contain the highest density and diversity of post-transcriptional modifications among all RNAs: there are 112 known different modifications found across all types of RNAs, 93 of which have been found in tRNAs (Lorenz *et al.*, 2017). However, the exact mechanisms as to how tRNA modifications can regulate translation remain unknown for most cases. The ability of Cas13 to bring modifying effectors to specific tRNAs should now enable targeted manipulation of tRNA modifications. This could help to gain a better understanding of the complex interplay between tRNA modifications and translational regulation.

CONCLUSIONS

Taken together, the results from the studies mentioned above demonstrate that CRISPR/Cas13 is a robust, precise and versatile RNA-targeting system, opening up new research horizons. Compared with previous technologies for RNA manipulation, CRISPR/Cas13 offers several advantages. Its modular composition consisting of a single protein effector module and an RNA guide module enables not only a simple and fast design, but also large scalability by the generation of whole libraries of different guide RNAs. The ability to generate Cas13 mutant versions that function as programmable RNA-binding proteins allows for the first time to efficiently target many different effectors to specific RNAs to induce subtle manipulations. Due to the inherent crRNA biogenesis capability of Cas13, multiple RNAs can be targeted simultaneously. Compared with RNAi, CRISPR/Cas13-mediated manipulations are not restricted to targeting cytoplasmic transcripts, but non-coding nuclear transcripts and pre-mRNA can also be targeted by simply adding a nuclear localization signal. Compared with transcriptional regulation on the DNA level, which affects all splicing isoforms in the same way, CRISPR/Cas13 facilitates targeting of specific isoforms. This could be helpful by eliminating aberrant or pathogenic splicing isoforms without affecting wild-type transcripts (Mahas *et al.*, 2017). In addition, Cas13 enables a temporally much faster downregulation of gene expression by directly cutting away the cytoplasmic mRNA pool, whereas regulation on the DNA level only prevents further mRNA production, relying on the natural degradation rate of the mRNAs present.

As suggested (Puchta, 2016) and recently demonstrated for Cas9 orthologues (Dreissig *et al.*, 2017; Wolter *et al.*, 2018), the simultaneous application of multiple editing systems in the same cell offers promising new options. Analogously, the availability of several functional Cas13 variants enables the employment of different RNA manipulations at the same time by fusing different effectors to each variant. DNA and RNA editing systems could also be combined. This way, some genes can be knocked out while simultaneously others are knocked down by targeting their mRNAs. Likewise, transcriptional and post-transcriptional repression of a specific gene can be combined in a single cell, making it now possible to fine-tune expression at the DNA and RNA levels simultaneously.

The availability of ample structural information regarding Cas13 variants should enable structure guided engineering of more sophisticated Cas13 systems in the future that show enhanced properties in terms of efficiency and specificity, or that are more flexible for RNA manipulation applications. An issue that still needs to be addressed is that the Cas13 variants available to date are sensitive to RNA secondary structure. Thus, it would be very promising to elucidate the underlying determinants of this phenomenon and use this knowledge to engineer more robust versions less sensitive to secondary structure. Regardless of the limitations of the current Cas13 systems still present, it appears that mining deeper in the vast diversity of microbial CRISPR/Cas systems has yet again yielded an invaluable tool for molecular biology applications.

Open Questions

- Can the RNA cleavage efficiency and specificity be further improved by finding new orthologues or structure guided engineering of new variants of existing orthologues?
- What determines the unspecific RNA cleavage observed under some conditions? Is it always absent in eukaryotic cells?
- How does secondary structure of the target RNA affect cleavage, and how does this relate to the choice of the target sequence?
- Does PspCas13b show the same high efficiency in plants as observed in mammalian cells, and would PspCas13b further enhance RNA virus interference in crops?
- How flexible is the dCas13 platform for the wide range of possible RNA modifications?

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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