# The double life of CRISPR–Cas13

Jorik F Bot[1,2], John van der Oost[3] and Niels Geijsen[1,2]

Since the discovery of RNA-programmable nucleases from the prokaryotic adaptive immune system CRISPR–Cas, these proteins have seen rapid and widespread adoption for biotechnological and clinical research. A recently discovered system, CRISPR–Cas13, uses CRISPR RNA guides to target RNA. Interestingly, RNA targeting by Cas13 results in cleavage of both target RNA and bystander RNA. This feature has been used to develop innovative diagnostic tools for the detection of specific RNAs. Unlike *in vitro* detection of RNA using collateral RNA cleavage, however, initial studies of mammalian cells only revealed highly specific target RNA-knockdown activity. Although these findings have been confirmed subsequently, several recent publications do report Cas13-mediated toxicity and collateral RNA cleavage when using Cas13 in eukaryotes. Here, we review these conflicting observations and discuss its potential molecular basis.

**Addresses**
[1] Dept. of Anatomy & Embryology, Leiden University Medical Center, Einthovenweg 20, 2300 RC Leiden, the Netherlands
[2] The Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW), Leiden node, the Netherlands
[3] Laboratory of Microbiology, Wageningen University & Research, Stippeneng 4, 6708 WE Wageningen, the Netherlands

Corresponding author: Niels Geijsen (n.geijsen@lumc.nl)

## Discovery of CRISPR–Cas13 — a promising new system for specific RNA knockdown?

Clustered regularly interspaced short palindromic repeats (CRISPR) and their associated proteins (CRISPR–Cas) is a highly diverse prokaryotic adaptive immune system. Key players of this defense system are the Cas nucleases that use CRISPR-derived CRISPR RNA (crRNA) guides to target complementary nucleic acids of invading parasites [1–3]. The most recently discovered CRISPR–Cas system is CRISPR–Cas13 (class 2, type VI) [4,5]. Cas13 effector proteins typically contain two higher eukaryote and prokaryote nucleotide-binding (HEPN) RNase domains [4,6]. These two HEPN domains form a single catalytic site that cleaves RNA upon activation by base pairing of its guide and a matching target RNA [7–12] (see Graphical abstract). Several distinct subtypes have been described, namely Cas13a [5,7], Cas13b [8,12], Cas13c [12], Cas13d [9••,10], Cas13X [11•], and Cas13Y [11•]. *In vitro*, Cas13 has the peculiar property that once it is activated by association with a specific target RNA, it uses its HEPN domains not only to specifically cleave its target RNA, but also to indiscriminately cleave any bystander RNA [7–11•,13,14]. This so-called collateral cleavage activity was immediately seized upon to develop rapid, highly sensitive nucleic acid detection methods [14–18], which has been further spurred on by the coronavirus disease 2019 (COVID19) pandemic [19–21].

Cas13 collateral RNA-cleavage activity was also observed in bacteria [7,8,10], where it is thought to serve an altruistic role by protecting the bacterial population by inducing dormancy after phage infection [22,23]. Surprisingly, initial reports did not find evidence of collateral cleavage in eukaryotic cells [9••,12,24]. Instead, Cas13 was found to specifically knock down its target transcripts. As such, Cas13 has been developed as a more specific alternative to RNAi-mediated knockdown, which is known to display off-target effects [25–27]. Since the first reports, many independent labs have shown that Cas13-based RNA knockdown indeed causes a substantial reduction in off-target transcriptome changes compared with silencing by Argonaute proteins with short-hairpin RNA guides [9••,12,24,28–31]. Furthermore, Cas13 also outperforms silencing by Cas9-based CRISPR interference (CRISPRi) [9••,31]. Because of these promising results, Cas13-based RNA knockdown has seen a flurry of development. Rules that determine spacer efficiency have been elucidated [32•–34]. Specific RNA knockdown with Cas13 has been adapted for use in mice [28,35–37], fish [35,38], yeast [39], Drosophila [31], and plants [40,41]. Recently, two groups used Cas13d for screening the functionality of circular RNAs [29,30]. Finally, Cas13 has been utilized to neutralize viral infection by specifically knocking down viral RNA in animals [42–44] and plants [40,41].

Yet, based on earlier findings of Cas13 *in vitro* and in bacteria, and considering the molecular architecture of Cas13, the lack of collateral cleavage in eukaryotic cells is unexpected. The two HEPN domains of Cas13 co-operate to form a single catalytic site that is required for both specific and collateral cleavage [7,13]. This site is located on the outside of the protein, facing away from the crRNA-target RNA complex [45–49]. Consequently, the nucleotide positions where Cas13 cleaves target RNA are completely independent of the protospacer position [7–9••], instead of cleaving within the proto-spacer such as Cas9 and Cas12 [50–52]. It seems there-fore unlikely that Cas13 can distinguish between target and collateral RNA.

This has led to a somewhat contradictory situation in the field. On the one hand, Cas13 has been adopted as a highly specific RNA-knockdown tool. On the other hand, *in vitro*, after specific detection of a target-of-interest, such as the detection of COVID19 virions, the collateral cleavage properties of Cas13 are employed in highly sensitive RNA-detection methods [19–21]. Re-cently, however, several groups did report toxicity and suggested collateral cleavage after utilizing Cas13 in eukaryotic cells, which seems inconsistent with the previously reported target specificity of Cas13. In this review, we will evaluate these reports, the common elements, and essential gaps in our understanding of Cas13 functionality in eukaryotic cells.

## Reports suggesting Cas13-mediated collateral RNA cleavage in eukaryotic cells

Wang and colleagues first reported that Cas13 is capable of collateral RNA cleavage in eukaryotic cells [53•]. When targeting exogenously overexpressed transcripts using LwaCas13a in U87 cells, they observed an increase in quantitative reverse transcription PCR (RT-qPCR) Ct values of nontarget genes, cleavage of ribosomal RNA, a reduced mapping ratio after RNA-seq, and increased cell death. However, they did not account for any potential off-target activation of Cas13, for example, by including a control that did not express the target. Importantly, they used a different cell line (U87) from earlier pub-lications, which used HEK293T cells. When Wang et al. tried to also confirm collateral cleavage in HEK293T cells, they were unable to detect any. In a subsequent publication, the same team demonstrated rRNA off-target cleavage after targeting a protein-coding en-dogenous gene in two different cell lines (B16F10, GL261) [54]. The same group also utilized the toxic collateral cleavage to trigger cell death in HepG2 and AT2 cells that expressed a fragment of COVID19 [55]. Another group reported Cas13 collateral cleavage effects and reduced viability in U87, HepG2, and mouse em-bryonic stem cells, but again, not in HEK293T cells when targeting with LwaCas13a, PspCas13b, or

RfxCas13d [56••]. However, reduced viability was also observed in the latter study with a nontargeting guide, an indication that overexpression of Cas13 protein itself may be toxic. Indeed, Cas13 protein-mediated toxicity has also been reported in zebrafish, Drosophila, and mice [31,35,57,58], and one report even describes cytotoxicity of catalytically inactive Cas13 (dCas13) [57]. Finally, Xu et al. note that EGFP fluorescent intensity decreased when targeting mCherry with Cas13X.1, LwaCas13a, and RfxCas13d for some spacers [11•], hinting at a col-lateral cleavage effect for all these proteins. However, they did not observe the same when targeting en-dogenous genes. Together, these initial reports suggest that collateral cleavage may occur in certain cell lines for specific targets, however, the exact conditions required remain unclear and some of the observed toxicity may be due to (potentially HEPN-independent [57]) protein-mediated toxicity.

In 2020, two papers reported the use of RfxCas13d in *Drosophila*. One describes target-specific knockdown [31], while the other encountered unexpected toxicity [57]. In the latter study, Buchman et al. found that RfxCas13d was lethal in *D. melanogaster* when targeting either GFP or endogenous genes. The crRNA, a com-plementary target RNA and functional HEPN domains were all required for this observed lethality. Interest-ingly, Huynh et al. claimed successful application of RfxCas13d for specific RNA knockdown in *Drosophila* [31]. One key difference between these studies is that Buchman et al. simultaneously used four guides per target instead of one guide per target. This potentially quadruples the amount of activated Cas13, but also could enhance specific off-target knockdown effects, both of which could explain why they encountered cellular toxicity. It is possible that Huynh and colleagues missed Cas13-mediated cellular toxicity due to the nature of their experimental setup. The transcripts they targeted are all known to be essential for survival, so the observed lethality was expected. Differentiating be-tween lethality due to specific target knockdown versus collateral cleavage is difficult.

Finally, there have been some recent preprints on bioRxiv in which toxicity and likely collateral cleavage is reported [58–60•], one of which was published during the preparation of this paper [61•]. Together, they shed more light on when and where collateral cleavage may occur. The first common element in these reports is that signs of collateral cleavage are only found when targeting highly expressed transcripts. This appears to mirror a recent report which shows that target expression must be above a certain threshold to induce collateral cleavage and dormancy in bacteria [62•]. The second common-ality is that exogenous transcripts, including the Cas13 mRNA itself, seem more susceptible to collateral clea-vage than endogenous RNA. Third, HEK293T cells

(again) show much less, or no signs of, collateral cleavage when compared with other cell lines. Finally, it is important to mention that Li and colleagues found that mice died when targeting known nonessential genes in neurons [60•]. Additionally, Tong et al. found that most transgenic mice constitutively expressing Cas13 alone or with a *Tyr*-targeting guide RNA died within eight and four weeks after birth, respectively [58]. This highlights the potential dangers of unexpected collateral cleavage in clinical applications. Since others have successfully applied Cas13 for specific knockdown *in vivo* in mice using other targets and cell types [28,36,37], this again suggests toxicity depends both on cell type and on target RNA. Interestingly, Li et al. had tested their guides in N2a or HEK293T cells before *in vivo* experiments and found seemingly specific knockdown with both RT-qPCR and RNA-seq, illustrating that these methods may not be suitable for detecting collateral cleavage.

## Seemingly conflicting observations

While aforementioned reports suggest that Cas13 is capable of collateral cleavage activity in eukaryotic cells, some questions remain. If Cas13 is indeed capable of collateral cleavage in eukaryotes, then why has it been missed initially? And why have so many groups been able to successfully apply Cas13 for specific RNA knockdown, even consistently outperforming RNAi-based methods? We propose there are three potential explanations, namely (1) the methods used to detect collateral cleavage have been inadequate, (2) variable expression levels of target RNA, and (3) cell-line-specific differences.

First, the methods used to determine whether there was collateral cleavage were not up to the task. By far, the most commonly used assays to test for collateral cleavage are RT-qPCR and RNA-seq [9••,12,24,29,31,36,42,44]. However, RT-qPCR quantifies the expression of one transcript relative to another. If Cas13 functions as a nonspecific RNase, it would therefore likely cleave both transcripts at the same rate, resulting in identical relative expression. RNA-seq suffers from the same problem, because expression is quantified relative to the number of reads. Some authors argue that collateral RNA cleavage should cause a stress response, and therefore result in differential expression of stress-related genes [24]. While a large amount of RNA cleavage is likely to trigger a stress response, for example, via activation of Toll-like receptors or rRNA cleavage [60•], it should be taken into account that cells also possess endogenous endoribonucleases, hence, a low amount of cleavage is probably tolerated. A low level of cleaved RNAs is quickly cleaned up by the exosome or by other ribonucleases, before inducing a significant stress response. Only when these cleanup systems are overwhelmed, an immune response would be activated. As many studies utilize Cas13 fused to a nuclear localization sequence [9••,24,28,32•,37,57,63–66], another potential limitation is that a nuclear-localized RNase could prevent cells from mounting a coherent transcriptional response by degrading most novel transcripts [59].

There are a couple of metrics from RNA-seq that could be helpful, which unfortunately have not been widely employed. For example, the mapping rates were found to be decreased after targeting an exogenous transcript with LwaCas13a in U87 cells [53•]. Because sequencing library preparation often contains a poly-A pull-down step, we expect the gene-body coverage to show a stronger 3' bias if collateral endonuclease cleavage occurs. Also, checking the fraction of reads that map to introns versus exons could be informative. Shi et al. used spike-in control RNA enabling them to detect an almost uniform 46% decrease in mRNAs, strongly suggesting unbiased collateral cleavage [59]. The use of specific sequencing protocols to detect RNA breaks (such as 5'-end sequencing [22] or long-read sequencing) may be the best approach to definitively prove if and where collateral RNA cleavage does occur.

Second, another common theme in the reports describing collateral cleavage in eukaryotic cells is that target expression needs to be very high for this phenomenon to occur [11•,53•,59–61•]. This leads to difficulty in interpreting experimental results when a toxic effect is found, because highly expressed genes generally are abundant for a reason. It begs the question if the observed phenotype is the result of collateral RNA cleavage, or rather of the specific knockdown of an essential transcript?

Something that we think has been underappreciated in the discussion thus far is the relative size of eukaryotic cells compared with bacteria and archaea, the native hosts of CRISPR systems. Whereas bacteria can have as little as a few hundred to a few thousand mRNA molecules in total [67,68], mammalian cells typically contain about two hundred thousand transcripts [69]. To illustrate the consequence, we performed a simple back-of-the-envelope calculation assuming 2000 mRNA molecules with an average length of 1000 nucleotides (nt) per bacterium, and two hundred thousand mRNA molecules of 2200-nt long per mammalian cell. The number of active Cas13 proteins needed to achieve one cut per kilobase (kb) mRNA was calculated by $\#Active\_Cas13\_needed = \frac{\#mRNA * mRNA\_length}{\#Cuts\_per\_active\_Cas13}$. This calculation shows that in mammalian cells, Cas13 needs to be about two orders of magnitude more active to achieve the same number of cuts per kb mRNA compared with bacteria (Figure 1, horizontal arrow). Alternatively, at a constant level of activity,

**Figure 1**
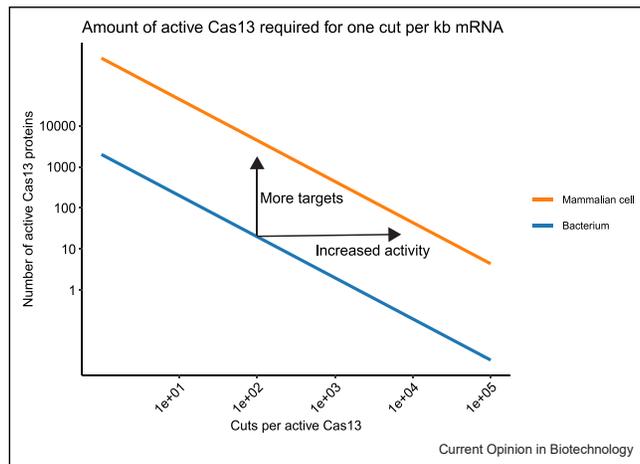


Schematic exemplification of the effect of total number of transcripts and number of active Cas13 molecules on collateral cleavage. The calculation assumes 2000 mRNA molecules of 1000-nt per bacterium, and two hundred thousand molecules of 2200-nt long in mammalian cells. With this simple calculation, a target abundance of 10 molecules per cell requires only 200 cuts per active Cas13 molecule in a bacterium, but forty-four thousand cuts per molecule in mammalian cells to achieve one cut per kb mRNA.

the number of target transcripts needs to be significantly higher (Figure 1, vertical arrow). Yet, the median mRNA copy number in mouse fibroblast is only seventeen [70], and thus very few transcripts meet the threshold for inducing collateral activity. Furthermore, our simple calculation does not take into account compartmentalization of eukaryotic cells, transcription rates, and the potentially suboptimal conditions for bacterial Cas13 protein activity in eukaryotic cells. The Cas13 ortholog with the highest reported activity to date, LbuCas13a, is capable of at least $10^4$ cuts per active molecule during a two-hour *in vitro* incubation period [13,14]. Whether it can reach the same activity in eukaryotic cells is unknown. In summary, the significant size differences between bacteria and eukaryotes could explain why collateral activity only becomes apparent when targeting very abundant transcripts, using highly active Cas13 variants such as LwaCas13a and RfxCas13d.

Finally, most of the reports that are discussed above suggest that HEK293T cells are unusually resilient to collateral cleavage. Yet, pioneering papers showing specific target knockdown are almost entirely based on this cell line [9••,12,24]. HEK293T cells may have a higher tolerance for Cas13, due to either apoptosis inhibition by the SV40 large T antigen [71], or due to lower levels of Cas13 activity in these cells. Since activated Cas13 is bound to its target RNA, the probability of cleaving the

target RNA itself will likely remain relatively high with reduced activity, whereas potential collateral cleavage will decrease linearly. Taken together, the common use of a cell line that is remarkably resilient to collateral cleavage, the requirement for high target-RNA expression levels, and the use of inadequate techniques to detect collateral RNA cleavage could explain why highly specific target knockdown using Cas13 has frequently been reported.

## Where do we go from here?

Recent reports raise the possibility that CRISPR–Cas13 does not inherently behave differently in eukaryotic cells. This calls into question previous results and may explain some unexpected findings. For example, the cell death found by Gao et al. after targeting TERT, EZH2, and RELA with LwaCas13a may not have been the result of specific knockdown as the authors claim [65], but instead due to toxic collateral RNA cleavage. Indeed, the genes they target have been shown to be non-essential in the used cell line (DepMap [72]), making it unlikely that specific RNA knockdown alone is lethal. In their crRNA screen on a viral genome, Freije and colleagues found some spacers to be depleted instead of enriched [42]. This could be explained by assuming that these guides triggered cytotoxic levels of collateral cleavage. Furthermore, Abbott et al. note that mNeonGreen intensity was reduced even when targeting an RNA segment of H1N1 Influenza A virus not containing mNeonGreen [66]. Xu et al. purposefully included four mRNA targets in their LwaCas13a-knockdown screen, which were neither depleted nor enriched in a CRISPR–Cas9 gene knockout screen [64], intending to use them as a negative control. However, crRNAs targeting two of these supposed control mRNAs showed depletion during specific survival challenges. While, as the authors suggest, it is certainly possible that the essential role of these genes was only revealed during a drug challenge, an alternative explanation is that the drug challenge increased their expression to a level where the LwaCas13a collateral cleavage became toxic to the cells. This again illustrates the complications caused by the need for high-target expression, which often encodes proteins that are essential for cellular survival, confounding whether the effects on cell viability are truly the result of target knockdown or collateral cleavage.

If Cas13-based collateral cleavage indeed exists in eukaryotic cells, this probably limits the utility of Cas13 for transcriptome-wide screens, as expression levels are likely to be the main determinant of depletion [62•]. Furthermore, great care needs to be taken when utilizing Cas13 *in vivo*, since this system can even be lethal under certain circumstances [57,58,60•]. Importantly, promising results from cell culture do not guarantee *in*

*vivo* success. Cas13 may still be useful as an antiviral but will likely kill infected cells. This may become dangerous at high viral loads. Although Cas13 could be useful for specifically knocking down transcripts with low expression levels, careful experiments must be performed to confirm the absence of collateral cleavage. On the other hand, collateral RNA cleavage in eukaryotes by Cas13 may be useful in cancer therapy, specifically activating lethal amounts of collateral RNase activity only in cells that (over)express a unique oncogene [7]. Similarly, it could potentially be used as a counterselection tool, eliminating unwanted cells from a heterogeneous population.

There are still many unanswered questions. First, sensitive tools are needed to detect collateral RNA cleavage. Since it appears that Cas13 preferentially cleaves exogenous RNA [11•,59–61•], including its own mRNA, these may be used to provide an indication of collateral RNA cleavage in past and future experiments. Perhaps, endogenous RNAs interact with more RNA-binding proteins, or contain (more) RNA nucleotide modifications, both of which could hinder cleavage by Cas13. Ultimately, specific sequencing methods are needed to determine the extend at which Cas13 cleaves nontarget RNAs, which RNAs get cleaved, and where they get cleaved. Second, exploring why HEK293T cells exhibit no, or much less collateral cleavage, could help improve target specificity in other settings. Third, there may be differences in the rate at which different Cas13 orthologs cleave target RNA versus nontarget RNA. Fourth, it has been noticed that some spacers trigger collateral cleavage and/or toxicity, while other spacers targeting the same RNA do not [53•,58,62•]. It is worth exploring whether certain guide-design rules can bias Cas13 toward target cleavage or collateral activity somehow. Finally, the mechanism by which widespread RNA cleavage triggers cytotoxicity is currently unknown.

Finally, there are some promising new alternatives for Cas13 that avoid any potential collateral cleavage concerns altogether. First, recently, a novel RNA-targeting, type-III CRISPR–Cas system called Cas7–11, was described [56••,73•]. Unlike Cas13, and unlike previously characterized type-III systems [23], this system seems to only cleave the target RNA itself, even *in vitro* and in bacteria. Second, the collateral cleavage of Cas13 can also be prevented by using a catalytically inactive Cas13 to guide an endogenous RNA-degradation-promoting protein to a specific transcript. Rauch and colleagues have shown that this approach works well utilizing m6A reader proteins [74]. They took this approach a step further by engineering RNA-guided RNA tools completely from human proteins, sidestepping concerns about immunogenicity, and greatly reducing the size

compared with Cas13 [75••]. Although these approaches are still in infancy, these tools demonstrate the tremendous potential and interest in RNA-targeting systems and may hold more promise for specific RNA knockdown in the long term.

## Conflict of interest statement

The authors declare not to have conflict of interest. N.G. and J.vdO. are cofounders and scientific advisors of NTrans Technologies.

## Data availability

No data were used for the research described in the article.

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