Catalytically active Cas9 mediates highly specific transcriptional interference to facilitate bacterial virulence

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Summary

Beyond defense against foreign DNA, the CRISPR-Cas9 system of pathogenic *Francisella novicida* represses expression of an endogenous immunostimulatory lipoprotein and is essential for virulence. We investigated the specificity and molecular mechanism of this regulation, demonstrating that Cas9 has a highly specific regulon of four genes which must be repressed for bacterial virulence. Regulation occurs through a PAM-dependent interaction of Cas9 with its endogenous DNA targets, directed by a non-canonical small RNA (scaRNA) duplexed with tracrRNA. The limited complementarity between scaRNA and the endogenous DNA targets precludes cleavage. This highlights the evolution of the scaRNA to direct transcriptional interference via interaction with endogenous DNA without lethally targeting the chromosome. We show that scaRNA can be reprogrammed to repress other genes, and with engineered, extended complementarity to an exogenous target, the repurposed scaRNA:tracrRNA-Cas9 machinery can also be licensed to direct cleavage of target DNA. Natural Cas9 transcriptional interference likely represents a broad paradigm of regulatory functionality, which is potentially critical to the physiology of numerous Cas9-encoding pathogenic and commensal organisms.

Introduction

CRISPR-Cas systems are prokaryotic adaptive immune systems that restrict infection by potentially harmful foreign genetic elements (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). CRISPR-Cas9 systems use the single effector protein Cas9 for target recognition and cleavage (Gasiunas et al., 2012; Jinek et al., 2012). Cas9 forms a complex with a duplex of small RNAs, one being a crRNA that is transcribed and processed from a genomic CRISPR (clustered regularly interspaced short palindromic repeats) array (Deltcheva et al., 2011). The other is

tracrRNA, a small RNA transcribed from the CRISPR-Cas9 locus that contains an inverted sequence that is complementary to the repeat sequence conserved in each crRNA derived from a CRISPR array (Deltcheva et al., 2011). Another portion of the crRNA, the spacer sequence, is often complementary to an exogenous DNA target (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Upon infection with a nucleic acid threat, the crRNA spacer binds to the complementary sequence on the incoming DNA (the protospacer), leading Cas9 to cleave the DNA target, resulting in a double strand break (Gasiunas et al., 2012; Jinek et al., 2012).

Before the crRNA spacer can interact with the complementary sequence on the DNA target, the Cas9 complex must first recognize a short nucleotide sequence on the opposite strand and adjacent to the protospacer, called a protospacer adjacent motif (PAM) (Anders et al., 2014; Jinek et al., 2012; Jinek et al., 2014; Nishimasu et al., 2014; Sapranaukas et al., 2011). This stage of target recognition is necessary to prevent cleavage of the genomic CRISPR array from which the crRNAs are transcribed (Gasiunas et al., 2012; Jinek et al., 2012). There are no PAM sequences next to the crRNA spacers in the genome (Marraffini and Sontheimer, 2010; Mojica et al., 2009).

Interestingly, CRISPR-Cas systems can have additional roles in bacterial physiology that extend beyond defense against foreign nucleic acids (Louwen et al., 2014; Ratner et al., 2015; Westra et al., 2014). Of particular interest are Type II CRISPR-Cas systems, which include CRISPR-Cas9, because they are uniquely abundant in pathogenic and commensal organisms (Chylinski et al., 2013; Fonfara et al., 2014; Sampson et al., 2013). Mutants lacking *cas9* in *Streptococcus agalactiae*, *Campylobacter jejuni*, *Neisseria meningitidis*, and *Francisella novicida* are impaired in processes such as attachment and invasion of host cells and intracellular survival (Louwen et al., 2013; Ma et al., 2018; Sampson et al., 2013). These defects correspond

to reduced pathogenicity of *cas9* mutants in *S. agalactiae*, *C. jejuni*, and *F. novicida* during infection (Louwen et al., 2013; Ma et al., 2018; Sampson et al., 2013).

In *F. novicida*, which can cause human infections, the attenuation of the *cas9* mutant is due in part to *F. novicida* Cas9 (FnoCas9) regulation of the expression of an endogenous mRNA encoding a bacterial lipoprotein (BLP), FTN_1103 (1103) (Jones et al., 2012). Regulation of 1103 by FnoCas9 is controlled by *tracrRNA* and *scaRNA*, which is a distinct small RNA transcribed from an independent promoter near the CRISPR locus (Chylinski et al., 2013; Postic et al., 2010; Sampson et al., 2013). Interestingly, FnoCas9, *tracrRNA*, and *scaRNA* together enable robust repression of 1103 transcript levels in a crRNA-independent manner (Sampson et al., 2013). Since BLPs are ligands for mammalian innate immune proteins, repression of 1103 helps facilitate the evasion of these sensors by *F. novicida* (Jones et al., 2012; Sampson et al., 2014; Sampson et al., 2013). However, reduction of 1103 levels alone is not sufficient to completely restore the virulence of a *cas9* mutant, suggesting that additional factors are involved (Jones et al., 2012; Sampson et al., 2013).

To comprehensively characterize the endogenous regulatory role of FnoCas9, we performed a genome-wide transcriptional analysis which revealed that FnoCas9 has an exquisitely specific regulon of just two transcripts encoding four genes, including 1103. Regulation is PAM-dependent and uses a catalytically active Cas9 bound to an RNA duplex (*scaRNA:tracrRNA*) that is distinct from the duplex used for targeting foreign DNA (*crRNA:tracrRNA*). *scaRNA* is complementary to the template strand of the 5' UTRs of the two transcripts, thus targeting FnoCas9 to specific sites on the endogenous genomic DNA to repress transcript levels. Repression of all four genes contributes to the virulence of *F. novicida*. These findings show for the first time that a cleavage-competent Cas9 complex can exist in two distinct

states in the bacterium to mediate two different functions: binding to endogenous DNA as a transcriptional repressor and cleaving foreign DNA to prevent infection. We further demonstrate that the scaRNA can be reprogrammed to guide FnoCas9 to repress other genes in *F. novicida*, highlighting the potential usefulness of this system in the control of gene expression. Taken together, these findings likely represent a broad paradigm in the way CRISPR-Cas9 systems mediate non-canonical functions that are distinct from DNA cleavage and contribute to bacterial physiology.

Results

FnoCas9 has a highly specific regulon

To identify regulatory targets of FnoCas9, we performed a genome-wide analysis of RNA levels in a *cas9* deletion mutant compared to wild-type (WT) *F. novicida*. Only 4 out of 1,782 genes (0.22%) were significantly up-regulated in the *cas9* mutant by more than 2-fold, including *FTN_1103* (1103) encoding a bacterial lipoprotein (BLP), which we previously demonstrated to be regulated by FnoCas9 (Figure 1A, S1A, C-E) (Jones et al., 2012; Sampson et al., 2013). Similar experiments in *tracrRNA* and *scaRNA* deletion strains revealed the identical regulon of only four genes (Figure 1A, S1A). These results, validated by Northern blots, indicated that similar to 1103, repression of the *FTN_1104* (1104), *FTN_1102* (1102), and *FTN_1101* (1101) transcripts was dependent on FnoCas9, *tracrRNA*, and *scaRNA*, but independent of *crRNA* (Figure 1C-F, S1A). Interestingly, the four genes localize to the same genomic region (Figure 1B, S1A), and Northern blot (Figure 1C-F) and PCR analysis (Figure S1B) revealed that they are located on two separate transcripts, one containing the operon 1104-

1102 and another with 1101. These data highlight that the FnoCas9 machinery has a regulon that is highly specific to a region of the genome.

FnoCas9 represses transcript levels by targeting the 5' UTR of target genes

We next investigated whether the two repressed transcripts contained regions that could serve as targeting sites by the scaRNA:tracrRNA-FnoCas9 machinery. We identified a 17 bp region in both the 1104 and 1101 5' UTRs with 100% sequence identity as a potential site of repression (Figure 2A). To test whether the 5' UTRs can confer FnoCas9-dependent repression, we constructed chromosomal promoter fusions driving expression of a non-native sequence *gfp** in place of the 1104-1101 locus (Figure 2B, S2A). *gfp** was placed directly downstream of the 1104 promoter and the 1104 5'UTR. Expression of *gfp** was greatly enhanced in the *cas9* mutant as compared to WT, consistent with a role for Cas9 in mediating repression (Figure 2C). No such repression of *gfp** was observed in the WT strain from a similar reporter construct lacking the 1104 5' UTR (Figure 2C). These data indicate that the 1104 5' UTR can direct a non-native transcript to be under scaRNA:tracrRNA-Cas9-dependent repression.

To test whether the 1104 5' UTR could promote FnoCas9-dependent repression downstream of non-native promoters, we generated reporter constructs in which the 1104 promoter was replaced with either p146, a synthetic promoter with constitutive expression in *F. novicida*, or the broad host range T5 promoter from *E. coli* containing a *lac* operator (Bryksin and Matsumura, 2010; McWhinnie and Nano, 2014). Irrespective of the promoter used, the 1104 5' UTR conferred FnoCas9-dependent repression of *gfp** (Figure 2C). Furthermore, the 1101 5' UTR was also capable of conferring FnoCas9-dependent repression of *gfp** when located downstream of the native 1101, synthetic, or *lac* promoters (Figure 2D). Taken together, these

data demonstrate that the 1104 or 1101 5' UTRs can direct a transcript to be under FnoCas9 regulatory control independent of the promoter used.

scaRNA has complementarity to the 1104 and 1101 5' UTRs

To determine how the FnoCas9 machinery might target the 5' UTRs of 1104 and 1101, we bioinformatically searched for predicted interactions between the scaRNA or tracrRNA and these regions. We precisely defined the sequences of the scaRNA and tracrRNA using a small RNAseq analysis (Figure 3A, S2E-F)(Chylinski et al., 2013). We identified a sequence of 11 bases of perfect complementarity between the template strand of the 1104 5' UTR and the tail of the scaRNA (which was included in a larger region of 15 bases of complementarity over 19 bases in the scaRNA tail; Figure 3A). This site overlapped with the 17 bp stretch of homology between the 1104 and 1101 5' UTRs (Figure S2A), with the template strand of the 1101 5' UTR also having 11 bases of perfect complementarity to the scaRNA tail (and 12 bases of complementarity over a region of 15 bases; Figure S2B). This suggests that scaRNA may direct FnoCas9 to the DNA encoding the 1104 and 1101 5' UTRs since the sequence of the template strand is not encoded on the mRNA.

FnoCas9 uses a PAM to interact with target 5' UTR DNA

The Cas9 complex requires recognition of a PAM sequence in the dsDNA target before the crRNA spacer can interact with its complementary sequence in the DNA. We identified an NGG PAM sequence on the coding strand adjacent to the predicted scaRNA binding site on the template strand of both 5' UTRs (Figure 3A, S2B). To test if the PAM was required for Cas9-dependent repression, we mutated the PAM of the 1104 5' UTR from TGG to TGA, TAG, or

TAA in the *gfp** reporter strain with the 1104 promoter. All of the PAM mutations greatly reduced the ability of FnoCas9 to repress *gfp** expression, consistent with the possibility that FnoCas9 binds the 1104 5' UTR DNA (Figure 3B). Similarly, we constructed the TAA PAM mutation in the 1101 promoter and 5' UTR reporter strain and observed a loss of FnoCas9-dependent repression (Figure S2C).

To determine whether FnoCas9 interacts with the 1104 5' UTR DNA in *F. novicida*, we crosslinked intact cells expressing either a FLAG epitope-tagged WT Cas9 or a FLAG-tagged point mutant (Cas9:R59A-FLAG) that is unable to interact with any of the FnoCas9-associated RNAs due to a mutation in the RNA binding domain. We next immunoprecipitated Cas9-FLAG from bacterial lysates, fragmented and isolated crosslinked DNA, and amplified the 1104 5' UTR sequences by qPCR. The 1104 5' UTR was enriched in the WT Cas9-FLAG pulldown relative to the Cas9:R59A-FLAG mutant, demonstrating that FnoCas9 does indeed interact with the 1104 5' UTR DNA (Figure 3C). Furthermore, this interaction was dependent on the scaRNA since Cas9-FLAG in a scaRNA deletion strain behaved similarly to Cas9:R59A-FLAG in the WT (Figure 3C). The 1101 5' UTR followed the same trend of enrichment in the WT Cas9-FLAG pulldown compared to the Cas9:R59A-FLAG and Δ scaRNA Cas9-FLAG strains (Figure S2D). Collectively, these data indicate that scaRNA mediates the interaction of FnoCas9 with 1104 and 1101 5' UTR DNA, representing the first natural example of Cas9-mediated transcriptional interference.

Complementarity to scaRNA and proximity to the TSS modulate the level of transcriptional interference

We next evaluated which factors control the level of transcriptional interference exhibited by FnoCas9. The importance of complementarity between the scaRNA and 1104 5' UTR was tested by measuring *gfp** transcript level from fusion constructs with either 0, 8, 11, or 15 bases of complementarity to scaRNA (Figure S3A). Compared to the strain without any complementarity between scaRNA and the 1104 5' UTR, the highest level of *gfp** repression was observed in the strain with 15 bp of complementarity to scaRNA, with lesser repression in the strain with 11 bp of complementarity (Figure 4A, S4). Repression of *gfp** was alleviated in the strain with only 8 bp of scaRNA complementarity (Figure 4B, S4). These data indicate that 11 to 15 bp of identity between the scaRNA and its target is sufficient for repression, with 15 bp providing the strongest effect (Figure 4A, S4).

The degree of transcriptional interference correlated with the affinity of FnoCas9 binding. Electrophoretic mobility shift assays (EMSAs) revealed that the affinity of the scaRNA:tracrRNA-FnoCas9 complex for the DNA increased with the amount of scaRNA complementarity (8, 11, 15 bp) (Figure 4C-E). The FnoCas9 complex did not bind to DNA with no scaRNA complementarity or in the absence of the scaRNA:tracrRNA duplex (Figure 4B).

We also attempted to investigate the ability of scaRNA to bind and repress a construct to which it had 20 bases of complementarity. However, we observed a significantly reduced number of transformants while attempting to make such a strain, and the transformants had mutations in the scaRNA target (Figure S3B). We reasoned that this might be due to lethal targeting of recombinants by the scaRNA:tracrRNA-FnoCas9 complex, and set out to test this. First, we validated that the scaRNA:tracrRNA-FnoCas9 complex was indeed able to bind a 20 bp target *in vitro* (Figure 4F). We next used a transformation inhibition model to directly test whether modulating the amount of complementarity between scaRNA and artificial exogenous

205 targets (both a linear allelic exchange fragment and a plasmid) harboring the 5' UTR region with
206 the PAM was sufficient to block transformation. As expected, transformation of constructs
207 containing 8, 11, and 15 bases of complementarity to scaRNA into WT and *cas9* mutant strains
208 resulted in an equal number of transformants. However, when the region of complementarity
209 between the artificial target and the scaRNA tail was artificially extended to 20 bases, we
210 observed a significantly reduced number of plasmid transformants in the WT but not the *cas9*
211 mutant strain (Figures 4G). These results suggest that scaRNA is capable of guiding the Cas9
212 complex to cleave a DNA target when the extent of complementarity is high enough. The
213 viability of *F. novicida* indicates that the native complementarity between scaRNA and the 1104
214 and 1101 5' UTRs is not sufficient to cause cleavage of the genomic DNA.

215 We next investigated the importance of proximity between the scaRNA target in the 5'
216 UTR to the transcriptional start site (TSS). To do this, we measured *gfp** transcript level from
217 fusion constructs with either 0, 5, 10, or 20 bases between the TSS and the 1104 5' UTR region
218 with complementarity to scaRNA (Figure S3A). We observed that constructs with 0, 5, and 10
219 bases between the TSS and the scaRNA complementarity region effectively repressed *gfp**
220 (Figure 4H). However, the construct with 20 bp between the TSS and the scaRNA
221 complementarity region exhibited significantly reduced repression (Figure 4H). To test if this
222 was the result of altered binding, we conducted *in vitro* DNA binding assays with constructs that
223 have 0, 5, 10, or 20 bases between the TSS and an 11 bp scaRNA target region. All of the
224 constructs had comparable binding affinities to FnoCas9 in complex with a scaRNA:tracrRNA
225 duplex, suggesting that the difference in transcriptional inhibition observed when the FnoCas9
226 binding site is moved further from the TSS is not the result of decreased binding affinity (Figure
227 4I-J). These data highlight that the scaRNA complementarity region must be in close proximity

to the TSS for effective FnoCas9-dependent transcriptional interference to occur. Together, these results indicate that binding affinity through complementarity to scaRNA can modulate the level of transcriptional interference nearby a TSS, until the number of bases alters Cas9 function from DNA binding to cleavage.

Cleavage-capable FnoCas9 binds competing RNA duplexes to form two distinct complexes with different functions

We demonstrated that repression of 1104-1101 was crRNA-independent, and that scaRNA with artificially extended complementarity to a target could inhibit transformation with a target-containing plasmid (Figure 1C-F, 4C, S3B). To test whether DNA targeting by the CRISPR array was independent of scaRNA, we transformed WT *F. novicida* and deletion mutants of crRNA, scaRNA, tracrRNA, and *cas9* with a plasmid containing a target of an endogenous crRNA (with and without a PAM), and evaluated the ability of each strain to restrict transformation relative to WT. All strains were transformed with the plasmid lacking a PAM at the same frequency. However, *cas9*, tracrRNA, and crRNA mutants were unable to restrict transformation with a target plasmid containing a PAM, while the scaRNA mutant inhibited transformation similarly to WT (Figure S5A). Together, these results led us to hypothesize that Cas9 forms two distinct cleavage-capable complexes in *F. novicida*, one with a scaRNA:tracrRNA duplex, and another with a crRNA:tracrRNA duplex. To test this, we measured the interaction between purified FnoCas9 and preformed complexes with either scaRNA:tracrRNA or crRNA:tracrRNA. This analysis revealed that FnoCas9 can indeed form complexes with both small RNA duplexes (Figures S5B-C), and raised the question of how these distinct complexes may affect each other.

We performed Northern blot analysis for tracrRNA, crRNA, and scaRNA from a panel of mutant and complemented strains. As expected, the abundance of each small RNA was dependent on the presence of FnoCas9 since each was undetected in a *cas9* mutant but restored in the complement strain. In addition, analysis of the tracrRNA mutant and complemented strains indicated that the presence of crRNA and scaRNA was dependent on tracrRNA. While deletion of scaRNA did not significantly alter the levels of crRNA, deletion of crRNA led to an increase in scaRNA levels (Figures 5A-C). To investigate the impact of the increased abundance of scaRNA in a crRNA mutant, we measured the level of 1104-1101 in a crRNA mutant compared to WT by qRT-PCR. In a crRNA mutant, 1104-1101 were expressed at lower levels than in WT *F. novicida*, consistent with enhanced transcriptional repression (Figure 5E-H, 1C-F). Complementation of the crRNA mutant restored wild-type levels of crRNA expression and repression of 1104-1101 (Figure 5D-H). Taken together, these results suggest that there is competition for FnoCas9 between the scaRNA:tracrRNA and crRNA:tracrRNA complexes.

Repression of each gene in the FnoCas9 regulon contributes to virulence

It was unclear whether repression of each gene in the FnoCas9 regulon contributes to *F. novicida* virulence. To test this, we infected mice subcutaneously with *cas9* double mutants also lacking one of the repressed genes. This revealed that 1104 expression plays a major role in the attenuation of the *cas9* mutant, similar to 1103 (Figure S6B). Deletion of *1104* from a *cas9* mutant led to a greater than 4-log enhancement in the levels of bacteria recovered after infection as compared to the *cas9* mutant (Figure S6B). 1102 and 1101 played more minor roles, but still contributed to attenuation of *F. novicida* when expressed, reducing virulence by 1-2 logs (Figure S6C-E). To test if virulence could be completely restored to the *cas9* mutant by deletion of the

entire 1104-1101 locus, we infected mice with a $\Delta cas9\Delta 1104-1101$ strain and evaluated the bacterial burden in the spleen 48 hours post-infection. We found that unlike the $\Delta cas9\Delta 1103$ mutant, virulence was restored to WT levels by deletion of the four genes in the FnoCas9 regulon from a $\Delta cas9$ strain (Figure 6). These data demonstrate that *F. novicida* uses Cas9 to specifically repress four genes whose expression would otherwise lead to attenuation of bacterial virulence.

scaRNA can be reprogrammed to guide FnoCas9 to repress non-native targets

To determine whether scaRNA can be reprogrammed to repress new targets, we replaced the 1104-1101 targeting portion of the scaRNA tail with a 15 bp sequence complementary to a portion of the 98 bp intergenic region between *FTN_0544* (0544; *naxD*, new NCBI locus tag: *FTN_RS02820*) and *FTN_0545* (0545; *flmF2*; new NCBI locus tag: *FTN_RS02825*). These two genes are required for the modification of outer membrane lipid A that leads to resistance to the antibiotic polymyxin B, and are transcribed in opposite directions (Figure 7A) (Kanistanon et al., 2008; Llewellyn et al., 2012). In the scaRNA reprogrammed strain, we observed a significant reduction in transcript levels of both *0544* and *0545* compared to WT (Figures 7B, C). This suggests that the FnoCas9 CRISPR-Cas9 system can be engineered to repress the expression of new targets, and that repression is independent of the strand targeted. Furthermore, this repression was dependent on FnoCas9, since a *cas9* mutant harboring the reprogrammed scaRNA exhibited WT levels of *0544* and *0545* (Figures 7B, C). In addition, when the natural scaRNA was reprogrammed for *0544-0545*, the strain lost the ability to repress *1104* (Figure 7D). The repression of *0544* and *0545* in the scaRNA reprogrammed strain led to an increased susceptibility to polymyxin B of almost 100-fold, similar to a *0544* deletion strain (Figure 7E).

The susceptibility was reversed by deletion of *cas9* from the reprogrammed scaRNA strain (Figure 7E). These results indicate that FnoCas9 can be reprogrammed to repress expression from new targets in a scaRNA:tracrRNA-dependent manner, highlighting the scaRNA:tracrRNA-FnoCas9 machinery as a potential new tool to control gene expression and modulate bacterial physiology.

Discussion

Using an analysis of the native FnoCas9 transcriptome to elucidate the specificity of endogenous gene regulation, we located the site of interaction between the FnoCas9 complex and the DNA of the 5' UTR of each transcript in its regulon. FnoCas9 uses scaRNA to interact with the template strand of the 5' UTR by recognition of a PAM and a scaRNA-complementary sequence on the DNA target. By targeting the 5' UTR DNA, FnoCas9 functions as a transcription factor, repressing gene expression. Through this interaction, FnoCas9 regulates the expression of four endogenous genes with remarkably high specificity. We determined that repression is dependent on a PAM in the 5' UTR, and that the sensitivity of natural FnoCas9 regulation could be modulated by the length of the RNA-target interaction and proximity of the scaRNA binding site to the TSS. We demonstrated that the extent of complementarity between scaRNA and the DNA target alters the binding affinity of the dual-RNA-FnoCas9 complex to the DNA. We also observed that the distance of the TSS from the scaRNA target region does not affect the binding affinity of the complex to the DNA. Using this knowledge, we reprogrammed scaRNA to target FnoCas9 to repress desired genes, highlighting the potential use of scaRNA:tracrRNA-FnoCas9 in the control of gene expression.

We hypothesize that the ability of Cas9 to repress transcription could be an expansion of the phage defense toolkit, to not only block replication of phage DNA, but also repress the transcription of harmful phage genes. It is hypothesized that scaRNA evolved from a degenerated CRISPR array that contains repeats with impaired complementarity to the inverted repeat of tracrRNA (Chylinski et al., 2013). Yet, we found that scaRNA is still capable of interacting with tracrRNA to form a hairpin that binds FnoCas9, competing with crRNA for this interaction. We propose that the degenerated array, which scaRNA evolved from, was retained because it conferred protection against invading DNA. This protection may have been due to transcriptional repression from the phage DNA by FnoCas9, a function that was later co-opted for 1104-1101 repression and evasion of intracellular innate immune responses.

It is particularly interesting that in spite of the degeneration of its repeat sequence, scaRNA has retained the ability to direct DNA cleavage. When *F. novicida* is transformed with an artificial target containing 20 bases of identity to the scaRNA, FnoCas9 restricts transformation. However, the 11 consecutive bases of perfect complementarity between scaRNA and the native 1104 and 1101 5' UTRs is sufficient for robust transcriptional repression, which we hypothesize is due to the inability of FnoCas9 to enter a cleavage-favorable conformation with a partial scaRNA-DNA target interaction. If so, this would be similar to what has been observed with shortened crRNA spacers, which guide Cas9 to bind but not cleave a DNA target (Bikard et al., 2013; Sternberg et al., 2015). Thus, modification of the length of the targeting sequence of the guiding scaRNA:tracrRNA duplex determines whether FnoCas9 represses transcription or cleaves its DNA target. We utilized this knowledge to reprogram scaRNA to target genes involved in polymyxin resistance. This led to efficient repression of the targeted genes and greatly increased sensitivity to polymyxin.

The use of a catalytically active Cas9 for gene repression makes this system unique compared to engineered CRISPRi technologies that use catalytically inactive mutants of the protein (dCas9) in complex with an RNA guide containing 20 bases of complementarity to its target (Bikard et al., 2013; Larson et al., 2013; Qi et al., 2013). This highlights the potential for scaRNA:tracrRNA-FnoCas9 complexes to be used to control gene expression, and especially for applications that seek to multiplex DNA cleavage and transcriptional control.

The limited scaRNA:target complementarity required for FnoCas9 transcriptional repression, as compared to 20 bp crRNA or guide RNA complementarity to cleavage targets, could increase the risk of off-target effects. Typically, analyses to identify off-target cleavage sites are performed with full length (20 bp) target sequences, however, we propose that for any use of Cas9, such analyses should include an examination of potential off-target transcriptional effects as well. Similarly, potential endogenous regulatory functions of native CRISPR-Cas9 systems could be identified by decreasing the stringency of self-targeting spacer identification.

Previous work from our lab suggested that a tracrRNA interaction with the 1103 mRNA contributes to repression, however the precise molecular mechanism of this regulation has remained elusive (Sampson et al., 2013). The mechanism of gene regulation via transcriptional inhibition that we have described here is unique compared to other examples of RNA targeting by Cas9 orthologs. SpyCas9 can be repurposed to target RNA when supplied with a short PAMmer sequence, while NmeCas9, SauCas9 and CjeCas9 have recently been shown to degrade ssRNA in a PAM-independent manner that requires the HNH catalytic domain and perfect or near perfect interaction with a crRNA spacer (Dugar et al., 2018; O'Connell et al., 2014; Rousseau et al., 2018; Strutt et al., 2018). ssRNA cleavage by Cas9 has been proposed to have roles in endogenous gene regulation (CjeCas9) and foreign nucleic acid defense (SauCas9)

(Dugar et al., 2018; Strutt et al., 2018). However, FnoCas9 is not capable of this mechanism of ssRNA targeting (Strutt et al., 2018), neither the HNH nor RuvC domains of Cas9 are required for repression (Sampson et al., 2013), and our results strongly suggest that 1104-1101 are repressed through an interaction with the Cas9 complex and DNA rather than RNA.

FnoCas9 transcriptional repression is critical for *F. novicida* virulence and we found that repression of each gene in the FnoCas9 regulon has a distinct contribution to virulence in a mouse model (Figure S6B). We find that the attenuation of a *cas9* mutant of *F. novicida* can be reversed by deletion of the 1104-1101 locus (Figure 6). 1104, 1103, and 1101 all exhibit conserved features of Gram-negative bacterial lipoproteins, while 1102 has some but not all of these features (Figure S6A).

Together, these results highlight a novel role for CRISPR-Cas9 systems in endogenous gene regulation and provide a mechanistic explanation of the role Cas9 plays in the virulence of *F. novicida*. Interestingly, *F. novicida* utilizes two distinct RNA duplexes for foreign DNA restriction and transcriptional repression, although both are capable of DNA restriction. The prevalence of these systems and the minimal base pair requirements needed for a shift between DNA cleavage and transcriptional interference suggest that a role of Cas9 as a transcriptional regulator may be a broader phenomenon in bacterial physiology than previously expected.

Materials and Methods

Construction of cDNA libraries for total RNA analysis

Biological triplicates of *Francisella novicida* U112 WT, $\Delta cas9$, $\Delta tracrRNA$ and $\Delta scaRNA$ were grown overnight on LB plates (37°C), cultured into LB medium (37°C shaking), and grown until OD_{620 nm} = 0.1. Twenty-five mL of bacterial culture were mixed with 25 mL of 1:1 acetone/ethanol and total RNAs were extracted using TRIzol (Ambion) and treated using turbo DNase (Ambion). RNA integrity was checked using a bioanalyzer (RIN > 8). cDNA libraries were prepared at the HZI genome analytics platform in Braunschweig, Germany as described with some modifications (Dötsch et al., 2012). Briefly, rRNAs were removed using the MICROBExpress kit (Ambion) and samples were treated with TAP (tobacco acid phosphatase). The RNAs were fragmented using sonication (Covaris) to fragments of 200 nucleotides. T4 Polynucleotide kinase (Fermentas) was used to phosphorylate 5' ends and remove the 3' phosphate. Successively, 3' and 5' adapters were added using T4 RNA ligase. Reverse transcription was performed using SuperScript II (Invitrogen) followed by 15 cycles of PCR with Phusion (New England Biolabs) and agarose gel purification. The sequencing was performed using 50 nucleotide single end reads (HiSeq2500). cDNA libraries for small RNA analysis were generated as in Chylinski et al., 2013 (Chylinski et al., 2013).

Analysis of total RNA sequencing

The raw data files were demultiplexed using the specific barcode sequences. The reads were trimmed from adapter sequences and the read quality was assessed using fastQC. The reads were mapped using STAR to the *Francisella novicida* U112 reference genome (NC_008601.1). We retrieved from 201525 to 518855 of uniquely mapped reads. The number of reads for each gene

were counted using HTseq and the differential expression analysis was done for each deletion mutant compared to the WT using DESeq2.

Northern blot analysis

After RNA extraction (see above), RNAs were resolved on 1% agarose containing 0.74% formaldehyde and transferred by capillarity to a nylon membrane (Hybond™ N+, GE healthcare). Forty pmol of oligonucleotide probes were 5' radiolabeled using [γ -³²P] ATP (Hartmann Analytics) and T4 polynucleotide kinase (Fermentas) and purified over Microspin™ G-25 columns (GE Healthcare) following the manufacturers' instructions. The probes were hybridized at 55°C using Rapid-hyb buffer (GE healthcare). The radioactive signal was visualized using a Thyphoon FLA-9500 phosphorimager (GE healthcare) and the transcript sizes were determined using a 3'-end radiolabeled RiboRuler high range RNA ladder (Thermo Scientific™). 16S rRNA was used as a loading control.

Electrophoretic mobility shift assay

tracrRNA, crRNA and scaRNA were *in vitro* transcribed from annealed oligonucleotides or PCR fragments (Table S2) using the AmpliScribe™ T7-Flash™ Transcription Kit (Epicenter). scaRNA:tracrRNA or crRNA:tracrRNA duplexes were hybridized by heating to 95°C and cooling to room temperature in hybridization buffer (200 mM NaCl, 20 mM HEPES pH 7.5). DNA substrates were generated by annealing complementary DNA oligonucleotides (Table S2) in a similar manner. Annealed oligos were 5' radiolabeled using [γ -³²P] ATP (Hartmann Analytics) and T4 polynucleotide kinase (Fermentas) according to the manufacturers' instructions, and purified using Microspin™ G-50 Columns (GE Healthcare). Cas9 was pre-

incubated at 37°C with two-fold molar excess of prehybridized scaRNA:tracrRNA duplex for 15 min in DNA-binding buffer (20 mM Tris HCl pH 7.4, 100 mM KCl, 5 mM CaCl₂, 1 mM DTT, 5% (w/v) glycerol, 40 ng/μl poly(dI-dC)), then 1 nM labelled DNA substrate was added. For duplex RNA EMSAs, tracrRNA was dephosphorylated using FastAP Alkaline Phosphatase (Thermo Scientific™) and then 5' radiolabeled as described above, before hybridization with scaRNA or crRNA. The reaction was performed as described above using 100 ng/ul tRNA as competitor. Binding reactions were incubated for 1 h. The samples were loaded on a native 5% polyacrylamide gel, which was run in 0.5X TBE supplemented with 5 mM CaCl₂. The gels were exposed on autoradiography films and visualized by phosphorimaging.

***Francisella novicida* strain construction and growth conditions**

All strains are listed in Table S1. *Francisella novicida* U112 derived strains were grown in Tryptic Soy Broth (VWR International) supplemented with 0.2% cysteine (BD Biosciences) at 37 °C with shaking. Strains were plated on Tryptic Soy Agar (VWR International) plates supplemented with 0.1% cysteine. Deletion and fusion construct mutants were constructed by allelic exchange (primers listed in Table S3). Promoter fusions were inserted in place of the 1104-1101 locus in the chromosome in $\Delta 1104-1101$ background strains. Chromosomal promoter and 5' UTR fusions were made using a fragment of *gfp* derived from the pBav-kGFP vector, *gfp** (bases 170-499). CRISPR-Cas9 system mutants $\Delta cas9$, $\Delta scaRNA$, $\Delta crRNA$, $\Delta tracrRNA$, their respective complemented strains, and $\Delta cas9\Delta 1103$, Cas9-FLAG, Cas9:R59A-FLAG, and $\Delta scaRNA$ Cas9-FLAG were described previously (Table S1) (Jones et al., 2012; Sampson et al., 2013; Weiss et al., 2007). Phusion high-fidelity DNA polymerase (New England Biolabs) was used to amplify homologous sequences (500-1000 bp) flanking the region of interest from

genomic DNA isolated with Qiaquick Tissue Extraction Kit (Qiagen). Overlapping PCR was used to construct the allelic exchange substrate by inserting a kanamycin selectable marker containing Flp recombinase target sites (FRT) between the flanking sequences (Llewellyn et al., 2011). Allelic exchange substrates were transformed into chemically competent *F. novicida* (Llewellyn et al., 2011). Mutants were selected on media supplemented with kanamycin sulfate (30 µg/ml, Fisher Scientific). Mutants were confirmed by PCR amplification from outside of the recombined region followed by sequencing (Genewiz) using “seq” primers (Table S3). The selection cassette was removed from the mutants using a temperature sensitive suicide vector, pFFlp, carrying the Flp recombinase in *trans*, as described previously (Gallagher et al., 2008).

Quantitative real-time PCR

RNA was isolated from bacterial cultures at OD_{600 nm} of 0.8-1.0 using TRI-reagent and a Direct-zol RNA MiniPrep Kit (Zymo Research). DNA was removed with Turbo DNaseI (Ambion Biosciences). qRT-PCR was performed with biological triplicates using the primers indicated in Table S3 and Power Sybr Green RNA-to-CT one-step kit (Applied Biosystems). *CT* values for each gene were normalized to the *Francisella novicida* housekeeping gene DNA helicase II (*uvrD*, *FTN_1594*) to determine $2^{-\Delta\Delta ct}$ for each condition (Sampson et al., 2013). Results are plotted as relative transcript levels of percent transcript in WT (intact Cas9) compared to the transcript level in a *cas9* mutant.

Cas9-FLAG crosslinking and immunoprecipitation

DNA was crosslinked and immunoprecipitated as described previously, with the indicated modifications to optimize for *F. novicida* and Cas9-FLAG (Jaggavarapu and O'Brian, 2014).

Cultures grown to OD₆₀₀ nm of 0.6-0.8 were crosslinked by adding formaldehyde to a final concentration of 1% in 10 mM PO₄ buffer, and shaking for 10 min at RT. Reactions were quenched using 1:10 volume of 100 mg/ml glycine and shaken for 30 min at 4°C. Cells were pelleted, washed 2X in PBS, and concentrated 20X in lysis buffer (100 mM Tris pH 8.0, 300 mM NaCl, 10 mM EDTA) with protease inhibitor cocktail (Sigma). Samples were further lysed and DNA was fractionated to 500-3000 bp fragments by sonication (15x 10 sec pulses with 15 sec pauses). Lysates were cleared by centrifugation and Cas9-FLAG was pulled-down from the supernatant using anti-Flag M2 agarose beads (Sigma). Following elution from the anti-FLAG beads, DNA was uncrosslinked from Cas9 by adding NaCl (final concentration 0.2 M) and incubating overnight at 65°C. DNA was purified (Qiagen PCR purification kit), and used as a template for qPCR. Results were normalized to the input DNA levels and a housekeeping gene (see qRT-PCR methods).

PCR amplification from cDNA

RNA and extracted from WT and $\Delta cas9$ (see above) and converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Segments were PCR amplified with Phusion high-fidelity DNA polymerase from the cDNA of each strain and compared to amplification from WT gDNA (New England Biolabs) (Table S3).

Plasmid construction

Plasmids were constructed using the primers indicated in Table S3. The broad host range vector pBAV1K-T5-GFP (pBAV) was used as the control plasmid and backbone for the plasmids in all assays (Bryksin and Matsumura, 2010). Plasmids that were used to measure repression and

transformation inhibition were made by replacing the promoter and RBS driving expression of *gfp* in the pBav vector with the synthetic constitutive promoter (p146) followed by different amounts of complementarity to the scaRNA tail and a PAM sequence directly upstream of *gfp* (McWhinnie and Nano, 2014). Plasmids were constructed using a Gibson Assembly Cloning Kit (NEB) and transformed and isolated from competent *E. coli* (NEB 5-alpha) using Zypzy Mini, Midi and Maxi prep kits (Zymo Research). Cas9 target plasmids containing spacer 13 with and without a PAM were previously described (Price AA, 2015).

Transformation Assays

Competent cells of *F. novicida* were made by concentrating cultures at an OD_{600 nm} of 0.8-1.0 10X in 4°C chemical transformation buffer (CTB) (Llewellyn et al., 2011). For the transformations, DNA was added to 100 µl competent cells, and transformations were incubated by shaking at 37°C for 20 minutes. 1 ml of recovery media (TSB+0.2% cysteine) was then added and transformations were incubated for another 2 hours (shaking, 37°C). Transformations were plated on TSA plates with kanamycin selection and incubated at 37°C overnight. For transformations with plasmid vectors, 500 ng of plasmid was used. For transformation inhibition experiments, transformants per 100 ng plasmid were enumerated and compared between strains to determine transformation efficiency. To measure Cas9 repression of plasmid *gfp* expression, plasmids used to transform WT and *cas9* mutants. Transformants from each strain were isolated and grown in TSB+0.2% cysteine+kanamycin 30 µg/ml selection to an OD_{600 nm} of 0.8-1.0, RNA was isolated and *gfp* transcript level was measured (see qRT-PCR methods) and normalized to the kanamycin resistance cassette on pBAV to account for variations in plasmid copy number. For transformation assays with allelic exchange fragments, DNA of purified allelic exchange

fragments were normalized by concentration and transformed. Transformation efficiency after 24 hours was measured by comparing the number of transformants into WT and a *cas9* mutant.

Polymyxin susceptibility assay

Overnight cultures of WT, *scaRNA_0544/0545*, $\Delta 0544$ and $\Delta cas9+scaRNA_0544/0545$ strains of *F. novicida* were prepared as described previously (Llewellyn et al., 2012). Strains were incubated with 100 μ g/ml polymyxin B (Tokyo Chemical Industries Japan) shaking at 37°C for 6 hours and then plated to enumerate CFU surviving bacteria from each condition. Results are presented as % survival of each strain treated with polymyxin relative to untreated.

Mouse infections

Specific-pathogen free mice were kept in filter-top cages at Yerkes National Primate Center, and provided food and water *ad libitum* (Sampson et al., 2013). Emory University Institutional Animal Care and Use Committee (protocol #YER-2000573-061314BN) approved all procedures (Sampson et al., 2013). Female C57BL/6J mice (Jackson) between 8 and 12 weeks were infected subcutaneously with $\sim 2 \times 10^5$ cfu bacteria (Weiss et al., 2007). Spleens were harvested at 48 hours post infection, homogenized in PBS, and the bacterial burden per organ was determined.

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Author contributions

H.K.R. A.E.M, A.L.R., E.C. and D.S.W conceived and designed this study. H.K.R, A.E.M, and A.L.R conducted the majority of the experiments. S.J. designed and assisted with the Cas9 pulldown assays and made the $\Delta 1104-1101$ mutant. J.E.W. conducted the polymyxin killing assay. E.K.C. constructed the $\Delta cas9 \Delta 1104$ mutant. H.K.R., A.E.M., A.L.R., E.C., and D.S.W. wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

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685

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Figure Legends

Figure 1. FnoCas9, scaRNA, and tracrRNA regulate transcript levels in a specific genomic

region of *F. novicida*. A) Differential transcript expression in the indicated *F. novicida* deletion mutants ($\Delta cas9$, $\Delta tracrRNA$, and $\Delta scaRNA$) relative to expression in wild-type (WT). Table represents all genes with a log fold change ($\log_2FC$) > 1 and an adjusted p-value (p_{adj}) < 0.05 in each strain compared to WT. The names used for these genes throughout the paper are 1104, 1103, 1102, and 1101. The genome of *F. novicida* has recently been re-annotated in NCBI and the new locus tags are indicated. B) Schematic of the chromosomal locus encoding the four FnoCas9-regulated genes (1104, 1103, 1102, and 1101) and the two mRNA products of the locus. C-F) Northern blots for (C) 1104, (D) 1103, (E) 1102, (F) 1101, in wild-type (WT) and deletion mutants for each component of the *F. novicida* CRISPR-Cas9 system ($\Delta cas9$, $\Delta tracrRNA$, $\Delta scaRNA$, $\Delta crRNA$) and their respective complementation strains.

Figure 2. FnoCas9 targets sequences coding for 5' untranslated regions (UTRs) leading to

transcriptional interference. A) Alignment of 1104 (teal) and 1101 (red) 5' UTRs with the location of the transcriptional start site (TSS) and start codon (ATG) highlighted. Brackets indicate the number of nucleotides in each UTR flanking the aligned sequence. B) Schematic indicating the design of the fusion constructs used to interrogate the roles of the promoters and 5' UTRs in transcriptional repression by FnoCas9. C-D) Relative *gfp** transcript level measured by quantitative real time PCR (qRT-PCR) from constructs with either the native promoter ("1104" in C, and "1101" in D), p146 synthetic promoter, or *lac* promoter, and with or without the native 5' UTR (1104 in C, and 1101 in D), in a wild-type (Cas9+) or *cas9* mutant (Cas9-) background. ($n=3$, error bars represent s.e.). ** $p \leq 0.005$; *** $p \leq 0.001$.

Figure 3. A PAM motif is required for FnoCas9 transcriptional interference. A) Schematic of predicted interactions between scaRNA (orange), tracrRNA (blue) and the 1104 5' UTR. The underlined region represents the identical sequence conserved between the 1104 and 1101 UTRs. The transcriptional start site (TSS) and PAM are shown on the coding strand of the UTRs. B) Relative *gfp** transcript levels were measured by qRT-PCR in strains with the native 1104 promoter driving expression of the 1104 5' UTR containing the indicated PAM variations, followed by *gfp**. Data is shown in both wild-type (Cas9+) and *cas9* mutant (Cas9-) strains. * $p \leq 0.05$; ** $p \leq 0.005$. C) Cas9-FLAG, Cas9:R59A-FLAG, and Δ scaRNA Cas9-FLAG strains were crosslinked and Cas9 was immunoprecipitated from bacterial lysates. Quantitative PCR was performed on immunoprecipitated DNA and the enrichment of 1104 DNA was determined relative to a housekeeping gene ($n=3$, error bars represent s.e.).

Figure 4. FnoCas9 transcriptional interference is controlled by degree of scaRNA complementarity and target proximity to the TSS. A) Relative *gfp** transcript levels were measured by qRT-PCR from constructs containing sequences with different lengths of complementarity to the scaRNA tail (0, 8, 11, 15 bp) between a synthetic promoter and *gfp** sequence ($n=3$, error bars represent s.e., *** $p \leq 0.001$). B-F) Electromobility shift assays (EMSA) with a Cas9:scaRNA/tracrRNA complex (1:2 molar ratio Cas9:RNA duplex) and DNA target (1 nM) containing different extents of complementarity to scaRNA: (B) 0 bp (9025/9026; DNA sequences in Table S2), (C) 8 bp (9027/9028), (D) 11 bp (9029/9030), (E) 15 bp (9031/9032), (F) 20 bp (9033/9034). G) Plasmid inhibition assay of WT *F. novicida* and Δ *cas9* with plasmids containing PAM-adjacent target sequences with 0, 11, 15, and 20 bases of complementarity to

scaRNA between a synthetic promoter and *gfp**. Results are presented as percent transformation into WT relative to $\Delta cas9$ ($n=3$, error bars represent s.e., $**p \leq 0.005$). H) Relative *gfp** transcript levels were measured by qRT-PCR in strains with varying numbers of additional bases (0, 5, 10, 20 bp) placed between the TSS of a synthetic promoter and a sequence with 11 bp of complementarity to the scaRNA, followed by *gfp**. A strain with *gfp** placed downstream of the synthetic promoter and 0 bp of complementarity to scaRNA was used as a control ($n=3$, error bars represent s.e., $**p \leq 0.005$; $***p \leq 0.001$). I-K) EMSAs with a Cas9:scaRNA/tracrRNA complex (1:2 molar ratio Cas9:RNA duplex) and DNA targets (1 nM) with 5, 10, or 20 bp between the TSS and the 11 bp region of complementarity to scaRNA: (I) 5 bp from TSS (9035/9036; DNA sequences in Table S2), (J) 10 bp from TSS (9037/9038), and (K) 20 bp from TSS (9039/9040).

Figure 5. Cas9 forms complexes with two competing RNA duplexes. (A-C) Northern blots for (A) tracrRNA, (B) crRNA, and (C) scaRNA in wild-type (WT), mutants for each component of the Cas9 complexes ($\Delta cas9$, $\Delta tracrRNA$, $\Delta crRNA$ and $\Delta scaRNA$), and the indicated complementation strains. D) qRT-PCR for transcript levels of crRNA in WT, $\Delta crRNA$, and a $\Delta crRNA$ + crRNA complemented strain. E-H) qRT-PCR for transcript levels of (E) *1104*, (F) *1103*, (G) *1102*, and (H) *1101* in WT, $\Delta crRNA$, and $\Delta crRNA$ + crRNA complemented strains. $*p \leq 0.05$; $**p \leq 0.005$; $***p \leq 0.001$.

Figure 6. Deletion of *1104-1101* restores virulence of a *cas9* mutant. Mice were subcutaneously infected with either WT *F. novicida*, $\Delta cas9$, $\Delta cas9 \Delta 1103$, or $\Delta cas9 \Delta 1104-1101$. Spleens were homogenized and plated for enumeration of colony forming units (CFU) at 48 hr

post-infection. ** $p \leq 0.005$.

Figure 7. scaRNA can be reprogrammed to repress new targets. A) Schematic of the scaRNA target site when reprogrammed to interact with the intergenic region between two *F. novicida* genes, *0544* and *0545*, that are transcribed in opposite directions. *0544* and *0545* ORFs are 98 bp apart in the genome. B-D) qRT-PCR for transcript levels of (B) *0544*, (C) *0545*, and (D) *1104* in WT, scaRNA_0544/0545 (WT with the scaRNA tail reprogrammed to target *0544/0545*), and $\Delta cas9$ +scaRNA_0544/0545 ($\Delta cas9$ with the scaRNA tail reprogrammed to target *0544/0545*) ($n=3$, error bars represent s.e., * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$). E) Percent survival of WT, scaRNA_0544/0545, $\Delta 0544$ and $\Delta cas9$ +scaRNA_0544/0545 strains 6 hours after polymyxin treatment (100 $\mu\text{g/mL}$) relative to untreated strains ($n=3$, error bars represent s.e., * $p \leq 0.05$).

Supplemental Figure Legends

Figure S1. Validation of the 1104-1103-1102 and 1101 transcripts. A) RNA sequencing coverage of the Cas9-regulated region in WT, $\Delta cas9$, $\Delta tracrRNA$ and $\Delta scaRNA$. B) Quantitative PCR over gene junctions. The location of the amplified regions are indicated in relation to the transcribed RNAs determined by RNAseq. Each primer set was used to amplify from “1” WT U112 gDNA as a control, “2” WT U112 cDNA, and “3” $\Delta cas9$ cDNA. (C-E) qRT-PCR for transcript levels of each gene in the FnoCas9 regulon in WT and $\Delta cas9$ strains; (C) *1104*, (D) *1103*, (E) *1102*, (F) *1101* (error bars represent s.e.; $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$).

Figure S2. PAM-dependent transcriptional repression by FnoCas9. A) Alignment of the

coding strands of the 1104 and 1101 5' UTRs. B) Schematic of predicted interactions between scaRNA (orange), tracrRNA (blue), and the 1101 5' UTR. The underlined region represents the sequence conserved between the 1104 and 1101 5' UTRs. The transcriptional start site (TSS) and PAM are shown on the coding strand of the UTRs. C) Relative *gfp** transcript levels measured by qRT-PCR. Expression of *gfp** is driven by the 1101 promoter and 5' UTRs containing either a WT TGG PAM sequence or a TAA mutation to the PAM, in strains WT (Cas9+) or $\Delta cas9$ (Cas9-) strains. * $p \leq 0.05$. D) Cas9-FLAG, Cas9:R59A-FLAG, and $\Delta scaRNA$ Cas9-FLAG strains were crosslinked and Cas9 was immunoprecipitated from bacterial lysates. Quantitative PCR was performed on immunoprecipitated DNA and presented as the enrichment of 1101 DNA relative to a housekeeping gene ($n=3$, error bars represent s.e.). E-F) tracrRNA and scaRNA detected by small RNA sequencing. (E) RNAseq of tracrRNA (blue) and scaRNA (orange) in the context of the CRISPR locus. (F) The DNA sequence, total coverage, and read coverage of the 5' and 3' ends are indicated.

Figure S3. scaRNA restricts transformation of exogenous targets with 20 bases of complementarity. A) All fusion constructs driving expression of *gfp** were made in the chromosome in place of the 1101-1104 locus, using a synthetic constitutive promoter. Between the promoter and *gfp**, the distance of the scaRNA targeting site from the TSS or amount of scaRNA complementarity to the target were modulated to evaluate their effect on transcriptional repression. B) Linear DNA fragments of the fusion constructs with 8, 11, 15, or 20 bases of complementarity to scaRNA between the promoter and *gfp** were transformed into WT *F. novicida* and a $\Delta cas9$ mutant, and recombinants were selected. Results are presented as the percent transformation efficiency into WT relative to a $\Delta cas9$ mutant for each DNA fragment.

*** $p \leq 0.001$.

Figure S4. Identity to scaRNA determines repression level from a plasmid-based reporter construct. pBAV-derived plasmid constructs with 0, 8, 11, or 15 bases of complementarity to scaRNA, inserted between a synthetic promoter and *gfp**, were transformed into WT *F. novicida* and a $\Delta cas9$ mutant. *gfp** transcript levels were measured by qRT-PCR. * $p \leq 0.05$; ** $p \leq 0.005$.

Figure S5. crRNA restriction of foreign DNA is scaRNA-independent. A) pBav-derived plasmids containing a spacer sequence from the CRISPR-Cas9 locus, with and without an adjacent PAM sequence, were transformed into wild-type (WT) and deletion mutants for each component of the *F. novicida* CRISPR-Cas9 system ($\Delta cas9$, $\Delta tracrRNA$, $\Delta scaRNA$, $\Delta crRNA$). Transformation efficiency of the plasmids into each strain was determined relative to WT. B-C) EMSA showing that Cas9 binds scaRNA:tracrRNA and crRNA:tracrRNA pre-formed duplexes. Increasing concentrations of Cas9 were incubated with a stable concentration of the RNA duplex: (B) scaRNA:tracrRNA or (C) crRNA:tracrRNA.

Figure S6. Repression of each gene in the Cas9 regulon contributes to virulence of *F. novicida*. A) Lipoprotein prediction scores for the Cas9-regulated proteins were generated using the Gram-negative lipopredict software as well as direct analysis of the sequence. The N-terminal amino acid sequences of 1104, 1103, 1102, and 1101 were annotated for canonical lipoprotein features: charged N-terminal region (red), hydrophobic region (blue), and the lipobox (bolded). The cysteine at which signal sequence cleavage occurs is underlined. B) CFU burden in mouse spleens 48 hr post-infection with $\Delta cas9 \Delta 1104$, $\Delta cas9 \Delta 1103$, $\Delta cas9 \Delta 1102$, or

825 $\Delta cas9 \Delta 1101$. * $p \leq 0.05$; ** $p \leq 0.005$. C) CFU burden in mouse spleens 48 hours post-infection
826 with *cas9* mutant strains expressing only one of the Cas9-regulated genes ($\Delta cas9 \Delta 1104-1102$;
827 only expresses 1101) or ($\Delta cas9 \Delta 1104-1101:1102$ complement; only expresses 1102) were
828 measured. * $p \leq 0.05$; ** $p \leq 0.005$. D-E) qRT-PCR of relative (D) *1102* and (E) *1101* expression in
829 WT and $\Delta cas9$ strains compared to $\Delta 1104-1102$ and $\Delta 1104-1101:1102$ complemented strains +/-
830 *cas9*.

Figure 1

A

B

Figure 3

A

B

C

Figure 4

Figure 5

Figure 6

Figure 7

A

Polymyxin resistance genes 0544 and 0545:

B

0544

C

0545

D

1104

E

Supplemental Figure 1

Supplemental Figure 2

A

1104 5' UTR 5' GCTTTATATAAATTAGCTGTAATGGAGGTATTAAATAT
 |||||
1101 5' UTR 5' GCTATTACTTAGTAATTAGCTGTAATGGAGAAATCCAAATGTTAATAATAATAAAAAATAAGGATGATAAT

B

C

D

E

F

Supplemental Figure 3

A

B

Supplemental Figure 4

Supplemental Figure 5

A

B

C

Supplemental Figure 6

A

FTN 1104

Lipopredict Spl score=19.6362

MRCNGLIMKDKLLIGVAISSAALLGCGKTET

FTN 1103

Lipopredict Spl score=18.4244

MKYGNLMMTKKKLLIGMVTISGIVILGSCGKTET

FTN 1102

Lipopredict Spl score=26.2134

MITLFYYAYINSSLRTVTQYSNT

FTN 1101

Lipopredict Spl score=18.1717

MKITKKKVVGAAIISGTVLLASCGKTTT

B

C

D

E

$\Delta cas9 \Delta 1104-1101:1102$ complement expresses 1102 only
 $\Delta cas9 \Delta 1104-1102$ expresses 1101 only

Table S1. Strains and plasmids used in this study

Name	Genotype (Δ mutants::insertion constructs)	Reference
CRISPR-Cas9 system mutants and complement strains		
WT	<i>Francisella novicida</i> U112 (background strain for all mutants)	(Weiss et al., 2007)
Δ cas9	Δ cas9	(Weiss et al., 2007)
Δ tracrRNA	Δ tracrRNA	(Sampson et al., 2013)
Δ scaRNA	Δ scaRNA	(Sampson et al., 2013)
Δ crRNA	Δ crRNA	(Sampson et al., 2013)
Δ tracrRNA + tracrRNA	Δ tracrRNA +tracrRNA complement in <i>cis</i>	(Sampson et al., 2013)
Δ scaRNA + scaRNA	Δ scaRNA +scaRNA complement in <i>cis</i>	(Sampson et al., 2013)
Δ crRNA + crRNA	Δ crRNA +crRNA complement in <i>cis</i>	(Sampson et al., 2013)
cas9-FLAG	cas9-FLAG	(Sampson et al., 2013)
cas9:R59A-FLAG	cas9:R59A-FLAG	(Sampson et al., 2013)
Δ scaRNA ^a cas9-FLAG	cas9-FLAG Δ scaRNA :: kan ^r	(Sampson et al., 2013)
1104 and 1101 promoter and UTR fusion constructs		
1104 promoter + 5' 1104 UTR (WT) ^b	Δ 1104-1101::1104 promoter +1104 5' UTR + <i>gfp</i> *+kan ^r	This study
1104 promoter + 5' 1104 UTR (Δ cas9)	Δ cas9 Δ 1104-1101::1104 promoter +1104 5' UTR + <i>gfp</i> *+kan ^r	This study
1104 promoter only (WT)	Δ 1104-1101::1104 promoter + <i>gfp</i> *+kan ^r	This study
1104 promoter only (Δ cas9)	Δ cas9 Δ 1104-1101::1104 promoter + <i>gfp</i> *+kan ^r	This study
Synthetic promoter + 5' 1104 UTR (WT) ^c	Δ 1104-1101::p146 +1104 5' UTR + <i>gfp</i> *+kan ^r	This study
Synthetic promoter + 5' 1104 UTR (Δ cas9)	Δ cas9 Δ 1104-1101::p146 +1104 5' UTR + <i>gfp</i> *+kan ^r	This study
Synthetic promoter only (WT)	Δ 1104-1101::p146 + <i>gfp</i> *+kan ^r	This study
Synthetic promoter only (Δ cas9)	Δ cas9 Δ 1104-1101::p146 + <i>gfp</i> *+kan ^r	This study
Lac promoter + 5' 1104 UTR (WT) ^d	Δ 1104-1101::lac promoter+1104 5' UTR + <i>gfp</i> *+kan ^r	This study
Lac promoter + 5' 1104 UTR (Δ cas9)	Δ cas9 Δ 1104-1101::lac promoter+1104 5' UTR + <i>gfp</i> *+kan ^r	This study
Lac promoter only (WT)	Δ 1104-1101::lac promoter+ <i>gfp</i> *+kan ^r	This study
Lac promoter only (Δ cas9)	Δ cas9 Δ 1104-1101::lac promoter+ <i>gfp</i> *+kan ^r	This study
1101 promoter + 5' 1101 UTR (WT)	Δ 1104-1101::1101 promoter +1101 5' UTR + <i>gfp</i> *+kan ^r	This study
1101 promoter + 5' 1101 UTR (Δ cas9)	Δ cas9 Δ 1104-1101::1101 promoter +1101 5' UTR + <i>gfp</i> *+kan ^r	This study
1101 promoter only (WT)	Δ 1104-1101::1101 promoter + <i>gfp</i> *+kan ^r	This study
1101 promoter only (Δ cas9)	Δ cas9 Δ 1104-1101::1101 promoter + <i>gfp</i> *+kan ^r	This study
Synthetic promoter + 5' 1101 UTR (WT)	Δ 1104-1101::p146 +1101 5' UTR + <i>gfp</i> *+kan ^r	This study
Synthetic promoter + 5' 1101 UTR (Δ cas9)	Δ cas9 Δ 1104-1101::p146 +1101 5' UTR + <i>gfp</i> *+kan ^r	This study
Synthetic promoter only (WT)	Δ 1104-1101::p146 + <i>gfp</i> *+kan ^r	This study
Synthetic promoter only (Δ cas9)	Δ cas9 Δ 1104-1101::p146 + <i>gfp</i> *+kan ^r	This study
Lac promoter + 5' 1104 UTR (WT)	Δ 1104-1101::lac promoter+1101 5' UTR + <i>gfp</i> *+kan ^r	This study
Lac promoter + 5' 1101 UTR (Δ cas9)	Δ cas9 Δ 1104-1101::lac promoter+1101 5' UTR + <i>gfp</i> *+kan ^r	This study
Lac promoter only (WT)	Δ 1104-1101::lac promoter+ <i>gfp</i> *+kan ^r	This study
Lac promoter only (Δ cas9)	Δ cas9 Δ 1104-1101::lac promoter+ <i>gfp</i> *+kan ^r	This study
1104 promoter + 5' 1104 UTR + TGA PAM (WT)	Δ 1104-1101::1104 promoter +1104 5' UTR (TGG toTGA PAM mutation) + <i>gfp</i> *+kan ^r	This study
1104 promoter + 5' 1104 UTR + TGA PAM (Δ cas9)	Δ cas9 Δ 1104-1101::1104 promoter +1104 5' UTR (TGG toTGA PAM mutation) + <i>gfp</i> *+kan ^r	This study
1104 promoter + 5' 1104 UTR + TAG PAM (WT)	Δ 1104-1101::1104 promoter +1104 5' UTR (TGG toTAG PAM mutation) + <i>gfp</i> *+kan ^r	This study
1104 promoter + 5' 1104 UTR + TAG PAM (Δ cas9)	Δ cas9 Δ 1104-1101::1104 promoter +1104 5' UTR (TGG toTAG PAM mutation) + <i>gfp</i> *+kan ^r	This study

1104 promoter + 5' 1104 UTR + TAA PAM (WT)	Δ1104-1101::1104 promoter +1104 5' UTR (TGG toTAA PAM mutation) + <i>gfp</i> *+kan ^r	This study
1104 promoter + 5' 1104 UTR + TAA PAM (Δ <i>cas9</i>)	Δ <i>cas9</i> Δ1104-1101::1104 promoter +1104 5' UTR (TGG toTAA PAM mutation) + <i>gfp</i> *+kan ^r	This study
1101 promoter + 5' 1101 UTR + TAA PAM (WT)	Δ1104-1101::1101 promoter +1101 5' UTR (TGG toTAA PAM mutation) + <i>gfp</i> *+kan ^r	This study
1101 promoter + 5' 1101 UTR + TAA PAM (Δ <i>cas9</i>)	Δ <i>cas9</i> Δ1104-1101::1101 promoter +1101 5' UTR (TGG toTAA PAM mutation) + <i>gfp</i> *+kan ^r	This study
Complementarity to scaRNA tail fusion constructs		
Δ1104-1101::p146 + (8, 11, 15, 20 bp scaRNA complementarity + PAM)+ <i>gfp</i> *+kan ^r		
0bp (synthetic promoter only construct)	Δ1104-1101::p146 + <i>gfp</i> *+kan ^r	This study
8 bp complementarity	Δ1104-1101::p146 + (agctgtaatgg)+ <i>gfp</i> *+kan ^r	This study
11 bp complementarity	Δ1104-1101::p146 + (attagctgtaatgg)+ <i>gfp</i> *+kan ^r	This study
15 bp complementarity	Δ1104-1101::p146 + (aaagattagctgtaatgg)+ <i>gfp</i> *+kan ^r	This study
20 bp complementarity	Δ1104-1101::p146 + (atgaaaaagattagctgtaatgg)+ <i>gfp</i> *+kan ^r	This study
Distance from transcriptional start site fusion constructs		
Δ1104-1101::p146 + (0, 5, 10, 20 bp + 11bp scaRNA complementarity + PAM)+ <i>gfp</i> *+kan ^r		
0 bp distance	11 bp complementarity construct	This study
5 bp distance	Δ1104-1101::p146 + (ttagt + 11bp complementarity +PAM)+ <i>gfp</i> *+kan ^r	This study
10 bp distance	Δ1104-1101::p146 + (tttacttagt + 11bp complementarity +PAM)+ <i>gfp</i> *+kan ^r	This study
20 bp distance	Δ1104-1101::p146 + (agaaatccaattacttagt+ 11bp complementarity +PAM)+ <i>gfp</i> *+kan ^r	This study
1104-1101 locus mutants		
Δ1104-1101	Δ1104-1101	This study
Δ1104-1101 Δ <i>cas9</i>	Δ1104-1101 Δ <i>cas9</i>	This study
Δ1104 Δ <i>cas9</i>	Δ1104 Δ <i>cas9</i>	This study
Δ1103 Δ <i>cas9</i>	Δ1103 Δ <i>cas9</i>	(Jones et al., 2012)
Δ1102 Δ <i>cas9</i>	Δ1102 Δ <i>cas9</i>	This study
Δ1101 Δ <i>cas9</i>	Δ1101 Δ <i>cas9</i>	This study
Δ1104-1101 + 1102 Δ <i>cas9</i> (1102 complement)	Δ1104-1101 ::1102 Δ <i>cas9</i>	This study
Δ1104-1102 Δ <i>cas9</i> (1101 functional complement)	Δ1104-1102 Δ <i>cas9</i>	This study
scaRNA tail reprogrammed for 0544-0545		
Reprogrammed scaRNA sequence : 5' agcaaaugaaaa <u>guuuuuauacuuucaguuu</u> caguuguuagauuuuugguauquacu (bold: interacts with 0544-0545 intergenic region, underlined: interacts with tracrRNA)		
Δ544 (<i>naxD</i>)	Δ544	(Llewellyn et al., 2012)
Reprogrammed scaRNA (WT)	scaRNA Reprogrammed for 0544-0545	This study
Reprogrammed scaRNA (Δ <i>cas9</i>)	scaRNA Reprogrammed for 0544-0545 Δ <i>cas9</i>	This study
Plasmids with scaRNA complementarity between promoter and gfp		
pBAV1K-T5-GFP derived plasmids. T5/lac promoter region driving <i>gfp</i> was replaced with p146 + (0, 8, 11, 15, 20 bp scaRNA complementarity +PAM)		
0 bp complementarity	pBAV1K-T5-GFP :: p146 + (PAM)	This study
8 bp complementarity	pBAV1K-T5-GFP:: p146 + (agctgtaatgg)	This study
11 bp complementarity	pBAV1K-T5-GFP :: p146 + (attagctgtaatgg)	This study
15 bp complementarity	pBAV1K-T5-GFP :: p146 + (aaagattagctgtaatgg)	This study
20 bp complementarity	pBAV1K-T5-GFP :: p146 + (atgaaaaagattagctgtaatgg)	This study
Plasmids with Cas9 crRNA spacer		
Spacer + PAM	pBAV1K-T5-GFP :: spacer +PAM	(Price et al., 2015)

Spacer + no PAM	pBAV1K-T5-GFP :: spacer only	(Price et al., 2015)
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^a kan^r (Gallagher et al., 2008)

^b *gfp** (bases 170-499) from pBav vector (Bryksin and Matsumura, 2010)

^c p146 (McWhinnie and Nano, 2014)

^d *Lac* promoter from pBav vector (+1 operator removed) (Bryksin and Matsumura, 2010)

Table S2. Oligos

Name	Sequence 5'-3'	F/R ^a	Usage	Reference
<u>Northern blot (NB) assays</u>				
OLEC4268	CCAACAGCCTGCCTGCCACT	R	NB FTN_1101	This study
OLEC4269	GTCTCCGTACTGTAAC TTGC	R	NB FTN_RS05660	This study
OLEC4274	ACCCAAC TACCATCGCCACA	R	NB FTN_1103	This study
OLEC4276	TGGCAGCCAAAATGCGGTAG	R	NB FTN_1104	This study
OLEC4323	ACCTACAGACCCTTTACGCC	R	NB 16S rRNA	This study
OLEC2861	CTAACAGTAGTTTACCAAATAATTCAGCAACTGAAAC	R	NB crRNA	This study
OLEC2866	ATTACAGAGCATTAATTATTTGGTACATTTATAATTT	R	NB tracrRNA	This study
OLEC2864	AACACAAGTACATACCAAATAATCTAACAACTGAAAC	R	NB scRNA	This study
OLEC5225	ACCTACTTTCACCTGGGCAA	R	NB 5S rRNA	This study
<u>RNA production</u>				
OLEC4211	TAATACGACTCACTATA	F	IVT T7 promoter	This study
OLEC4407	AAAAATAAGTAGGTCTAAAAGTGAATTTTCTAGCTACTTTAAA CTAACACAAGTACATACCAAATAATCTAACAACTATAGTGA GTCGTATTA	R	IVT T7 scaRNA template with OLEC4211	This study
OLEC3089	GAAATTAATACGACTCACTATAGGATAACTCAATTTGTA AAAA AGTTTCAGTTGCTGAATTATTTGGTAAAC	F	IVT T7 crRNA speM spacer	(Fonfara et al., 2014)
OLEC3090	GTTTACCAAATAATTCAGCAACTGAACTTTTTTACAAATTGA GTTATCCTATAGTGAGTCGTATTAATTTT	R	IVT T7 crRNA speM spacer	(Fonfara et al., 2014)
OLEC3102	GAAATTAATACGACTCACTATAGGGTACCAAATAATTAATGCT CTG	F	IVT T7-tracrRNA (processed)	(Fonfara et al., 2014)
OLEC3103	GTTATTCAGACGTGTCAAACAG	R	IVT T7-tracrRNA	(Fonfara et al., 2014)
OLEC4649	TAATACGACTCACTATAGGATCTAAAATTATAAATGTACAAA TAATTAATGC	F	IVT T7-tracrRNA (unprocessed)	This study
OLEC4683	AAAAATAAGTAGGTCTAAAAGTGAATTTTCTAGCTACTTTAAA CTAACACAAGTACATACCAAATAATCTAACAACTGAACTTAC AGCTAATCTTTTTTCATTGCTCCTATAGTGAGTCGTATTA	R	IVT T7-scaRNA (long) with OLEC4211	This study
<u>DNA Electrophoretic Mobility Shift Assays (EMSAs)</u>				
OLEC9025	TAAATACCAATTGACATATATAAATGATTCTGATATAAATTAG ATAAGGGAGGGCCAACACTTGTCCTACTCTGACG	F	DNA EMSA 0 bp complementarity)	This study
OLEC9026	CGTCAGAGTAGTGACAAGTGTTGGCCCTCCCTTATCTAATTTA TATCAGAATCATTTATATATGTCAATTGGTATTTA	R	DNA EMSA 0 bp complementarity	This study
OLEC9027	TAAATACCAATTGACATATATAAATGATTCTGATATAAATTAG ATAAGGGAGCTGTAATGGGGCCAACACTTGTCCTACTCTG ACG	F	DNA EMSA 8 bp complementarity	This study
OLEC9028	CGTCAGAGTAGTGACAAGTGTTGGCCCCATTACAGCTCCCT TATCTAATTTATATCAGAATCATTATATATGTCAATTGGTATT TA	R	DNA EMSA 8 bp complementarity	This study
OLEC9029	TAAATACCAATTGACATATATAAATGATTCTGATATAAATTAG ATAAGGGATTAGCTGTAATGGGGCCAACACTTGTCCTACTC TGACG	F	DNA EMSA 11 bp complementarity	This study
OLEC9030	CGTCAGAGTAGTGACAAGTGTTGGCCCCATTACAGCTAATC CCTTATCTAATTTATATCAGAATCATTTATATATGTCAATTGGT ATTTA	R	DNA EMSA 11 bp complementarity	This study
OLEC9031	TAAATACCAATTGACATATATAAATGATTCTGATATAAATTAG ATAAGGGAAAGATTAGCTGTAATGGGGCCAACACTTGTCCT ACTCTGACG	F	DNA EMSA 15 bp complementarity	This study

Name	Sequence 5'-3'	F/R ^a	Usage	Reference
OLEC9032	CGTCAGAGTAGTGACAAGTGTTGGCCCCATTACAGCTAATCTTCCCTTATCTAATTTATATCAGAATCATTTATATATGTCAATTGGTATTTA	R	DNA EMSA 15 bp complementarity	This study
OLEC9033	TAAATACCAATTGACATATATAAATGATTCTGATATAAATTAGATAAGGGATGAAAAAGATTAGCTGTAATGGGGCCAACACTTGTCACTACTCTGACG	F	DNA EMSA 20 bp complementarity	This study
OLEC9034	CGTCAGAGTAGTGACAAGTGTTGGCCCCATTACAGCTAATCTTTTCATCCCTTATCTAATTTATATCAGAATCATTTATATATGTCAATTGGTATTTA	R	DNA EMSA 20 bp complementarity	This study
OLEC9035	TAAATACCAATTGACATATATAAATGATTCTGATATAAATTAGATAAGGGTTAGTATTAGCTGTAATGGGGCCAACACTTGTCTACTCTGACG	F	DNA EMSA 11 bp complementarity 5 bp from TSS	This study
OLEC9036	CGTCAGAGTAGTGACAAGTGTTGGCCCCATTACAGCTAATAC TAACCTTATCTAATTTATATCAGAATCATTTATATATGTCAATTGGTATTTA	R	DNA EMSA 11 bp complementarity 5 bp from TSS	This study
OLEC9037	TAAATACCAATTGACATATATAAATGATTCTGATATAAATTAGATAAGGGTTTACTTAGTATTAGCTGTAATGGGGCCAACACTTGTCACTACTCTGACG	F	DNA EMSA 11 bp complementarity 10 bp from TSS	This study
OLEC9038	CGTCAGAGTAGTGACAAGTGTTGGCCCCATTACAGCTAATAC TAAGTAAACCTTATCTAATTTATATCAGAATCATTTATATATGTCAATTGGTATTTA	R	DNA EMSA 11 bp complementarity 10 bp from TSS	This study
OLEC9039	TAAATACCAATTGACATATATAAATGATTCTGATATAAATTAGATAAGGGAGAAATCCAATTTACTTAGTATTAGCTGTAATGGGGCCAACACTTGTCACTACTCTGACG	F	DNA EMSA 11 bp complementarity 20 bp from TSS	This study
OLEC9040	CGTCAGAGTAGTGACAAGTGTTGGCCCCATTACAGCTAATAC TAAGTAAATTGGATTTCTCCCTTATCTAATTTATATCAGAATCATTTATATATGTCAATTGGTATTTA	R	DNA EMSA 11 bp complementarity 20 bp from TSS	This study

^a F, forward primer; R, reverse primer.

Table S3. Primers used in study

#	Name	Sequence 5'-3'	Usage
1	1104_rt_fwd	CAGAGTCAAACCTCCGCTGCG	qRT-PCR
2	1104_rt_rev	TGGCAGCCAAAATGCGGTAG	qRT-PCR
3	1103_rt_fwd	ATGGTGGGCAGTCTAGCGCA	qRT-PCR
4	1103_rt_rev	ACCCAACCTCACCATCGCCACA	qRT-PCR
5	1102_rt_fwd	GAAGCTTTTGATCGCGATTATAGTA	qRT-PCR
6	1102_rt_rev	TGTAACCTTGCCAATATAGCAACACT	qRT-PCR
7	1101_rt_fwd	TGAACAAGCAGATACGCCATGGGT	qRT-PCR
8	1101_rt_rev	CCAACAGCCTGCCTGCCACT	qRT-PCR
9	HelC_RT_Fwd	GGGATGTCGCCTTTTGATTTTC	qRT-PCR housekeeping gene
10	HelC_RT_Rev	CTCTTTTGTCCTTGTGCTTGC	qRT-PCR housekeeping gene
11	Pbav_gfp_Fwd	GTGCCATGCCCGAAGGTTAT	qRT-PCR
12	Pbav_gfp_Rev	GCGTCTTGTAGTTCCCGTCA	qRT-PCR
13	Pbav_kan_Fwd	CTTCGGGCTTTTCCGTCTTT	qRT-PCR
14	Pbav_kan_Rev	GCCGATGTGATTGCGAAAA	qRT-PCR
15	1104pro+tss_fwd	AGACAACTTTTGATAGGCGTTG	qRT-PCR Cas9-FLAG pulldown
16	1104pro+tss_rev	CCAATAACGCAGCACTAGAAAT	qRT-PCR Cas9-FLAG pulldown
17	1101pro+tss_fwd	GAAGGTTGTGATTGGCGCAG	qRT-PCR Cas9-FLAG pulldown
18	1101pro+tss_rev	TTGCTAATAGTACCGTGCCAGA	qRT-PCR Cas9-FLAG pulldown
19	0544_RT_fwd	GATTGGGGCGCGCTACCTGA	qRT-PCR
20	0544_RT_Rev	TGAGCGGATATAGGTCTGCTTAGTGT	qRT-PCR

#	Name	Sequence 5'-3'	Usage
21	0545_RT_fwd	TCAGAGGACTTTTTCATGG	qRT-PCR
22	0545_RT_Rev	GATCTGCAACCTCAAATGGT	qRT-PCR
1104 promoter + 5' 1104 UTR			
23	4-5 Larm_F_04pro fusions	GAATATTAGCGTAATTGCAGCTG	cloning
24	4 Larm_R_04Pro04UTR GFP	GAGTAGTGACAAGTGTGGCCATATTTAATACCTCCATTACAGC	cloning
25	4 GFP_F_04Pro04UTR GFP	GCTGTAATGGAGGTATTAATATGGCCAACACTTGTCACTACTC	cloning
26	2-4 Gfp_R_UvrDpGFPkan	GCTTATCGATACCGTCGACCTCACATTGTGTGAGTTATAGTTGTAC	cloning
27	2-4 Kan_F_UvrDpGFPkan	GTACAACTATAACTCACACAATGTGAGGTGACGGTATCGATAAGC	cloning
28	1-4 Kan_R_Kan1100arm	CCACTAAAGTTAAATTCGACAAGCATAGCTGCAGGATCGATATC	cloning
29	1-4 Rarm_F_Kan1100arm	GATATCGATCCTGCAGCTATGCTTGTGCGAAATTTAACTTTAGTGG	cloning
30	1-4 Rarm_R_Kan1100arm	CCATTGATAGGTGATTTAAATG	cloning
31	4-5 seq_F_1104 pro fusions	CTATTATGAATTAATCTATC	Mutant confirmation
32	4-5 ampli_F_1104pro fusions	CCAAGAATTGCATAAAGTGCTG	Mutant confirmation
33	1-5 amp4seq_R_1100inter nal	CTGGATATGGTGTACACTCATC	Mutant confirmation
1104 promoter only Use primers: 23, 26-33			
34	5 Larm_R_04UTR onlyGFP	GAGTAGTGACAAGTGTGGCCAGCAAAAAAGTATAACATAATAAT	cloning
35	5 GFP_F_04UTR onlyGFP	ATTATTATGTTATACTTTTTTGTGCTGGCCAACACTTGTCACTACTC	cloning
1101 promoter only Use primers: 23, 26-30, 44-46			
36	1-3 Larm_F_UvrDpro	GTATTCCTTGGATCTCAAGTGG	cloning
37	7-8 Larm_R 1101p	GAATTACAATACCATATGGTAGAGTTAGTTGAAATAATCTCTCAAAT	cloning
38	7-8 1101pro_only_Fwd	ATTTGAGAGATTATTTCAACTAACTCTACCATATGGTATTGTAATTC	cloning
39	7 1101pro_only_R_gfp	GAGTAGTGACAAGTGTGGCCAGCAAAAAATTATAACAAAAAATTC	cloning
40	7 Gfp_F_1101pGfp_shor t	GCTGGCCAACACTTGTCACTACTC	cloning
41	7b Gfp_F_1101pGfp_long	CAATTTTTTTGTTATAATTTTTTGTGCTGGCCAACACTTGTCACTACTC	cloning
1101 promoter + 5' 1101 UTR Use primers: 36-38, 26-30			
42	8 1101proUTR_R_gfp	GAGTAGTGACAAGTGTGGCCATTATCATCCTTATTTTTTTATTA	cloning
43	8 Gfp_F_1101pUTRGfp	TAATAAAAAATAAGGATGATAATGGCCAACACTTGTCACTACTC	cloning
44	1-3 seq_F_UvrDpro	GTAAGCAAATATTAGAATACTG	Mutant confirmation
45	1-3 amp4seq_F_UvrDpro	GCTCTTGTGGATATGCATTTGC	Mutant confirmation
46	1-5 amp4seq_R_1100inter al	CTGGATATGGTGTACACTCATC	Mutant confirmation
Lac promoter + 5' 1104 UTR Use primers: 36, 26-30, 44-46			
47	9-10 Larm_R_lacPro	TCCGCTCACAAAGCAAATAAATTTTTATGATTCCTCTTTAGTTGAA ATAATCTCTCAA	cloning
48	9 04UTRGFP_F_fused2 Lac	TTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAAGCTTTAT ATAAATTAGCTG	cloning

#	Name	Sequence 5'-3'	Usage
Lac promoter only Use primers: 36,47, 26-30, 44-46			
49	10 GFP_Fwd_ fused2lacpro	TTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAAGGCCAA CACTTGCTCACTAC	cloning
Lac promoter + 5' 1101 UTR Use primers: 36,47, 26-30, 44-46			
50	11_01utrGfp_F1_ fused2lacP	CAAGCTATTACTTAGTAATTAGCTGTAATGGAGAAATCCAAATG	cloning
51	11_01utrGfp_F2_ fused2lacP	ATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAAGCTATTTAC TTAGTAATTAGC	cloning
Synthetic promoter (p146) only Use primers: 36, 26-30, 44-46			
52	12-14 LarmR_146p	TATATCAGAATCATTTATATATGTCAATTGGTATTTATTAGTTGAAATA ATCTCTCAAAT	cloning
53	12 GFP_F (fused to 146p)	GACATATATAAATGATTCTGATATAAATTAGATAAGGGAGGCCAACCA CTTGCTCACTACTC	cloning
All fusion constructs with synthetic promoter p146: Amplify from "Synthetic promoter only" template DNA For PCR fragments: 36 (Fragment1_Forward), 30 (Fragment2_Rev) For amplifying and sequencing mutants: 44-46 Additional primers needed for each mutant are listed by # under the name of the construct			
Synthetic promoter + 5' 1104 UTR Use primers: 52			
54	13 04GFP_F (fused to 146p)	ATATATAAATGATTCTGATATAAATTAGATAAGGGAGCTTTATATAAA TTAGCTGTAATG	cloning
Synthetic promoter + 5' 1101 UTR Use primers: 52			
55	14 01GFP_F (fused to 146p)	CATATATAAATGATTCTGATATAAATTAGATAAGGGAGCTATTTACTT AGTAATTAGCTG	cloning
Synthetic promoter + 8bp complementarity to scaRNA tail			
56	26 L arm R_146pro8sca	GTTGGCCCCATTACAGCTCCCTTATCTAATTTATATCAG	cloning
57	26 GFP_F_146pro8sca	GATAAGGGAGCTGTAATGGGGCCAACACTTGTCCTACTC	cloning
Synthetic promoter + 11bp complementarity to scaRNA tail			
58	25 L arm R_146pro11sca	GTTGGCCCCATTACAGCTAATCCCTTATCTAATTTATATCAG	cloning
59	25 GFP_F_146pro11sca	GATAAGGGATTAGCTGTAATGGGGCCAACACTTGTCCTACTC	cloning
Synthetic promoter + 15bp complementarity to scaRNA tail			
60	27 L arm R_146pro15sca	GTTGGCCCCATTACAGCTAATCTTTCCCTTATCTAATTTATATCAG	cloning
61	27 GFP_F_146pro15sca	GATAAGGGAAAGATTAGCTGTAATGGGGCCAACACTTGTCCTACT C	cloning
Synthetic promoter + 20bp complementarity to scaRNA tail			
62	31 L arm R_146pro20sca	GTTGGCCCCATTACAGCTAATCTTTTCCCTTATCTAATTTATAT CAG	cloning
63	31 GFP_F_146pro20sca	GATAAGGGATGAAAAAGATTAGCTGTAATGGGGCCAACACTTGTC CTACTC	cloning
Synthetic promoter + 5bp from TSS (11bp complementarity to scaRNA tail)			
64	28 L arm R_146pro5bp11sca	GTTGGCCCCATTACAGCTAATACTAACCCTTATCTAATTTATATCAG	cloning
65	28 GFP_F_146pro5bp11s ca	GATAAGGGTTAGTATTAGCTGTAATGGGGCCAACACTTGTCCTACT C	cloning
Synthetic promoter + 10bp from TSS (11bp complementarity to scaRNA tail)			
66	29 L arm R_146pro10bp11sca	CCATTACAGCTAATACTAAGTAAACCCTTATCTAATTTATATCAG	cloning
67	29 GFP_F_146pro10bp11 sca	TTACTTAGTATTAGCTGTAATGGGGCCAACACTTGTCCTACTC	cloning

#	Name	Sequence 5'-3'	Usage
<u>Synthetic promoter + 20bp from TSS (11bp complementarity to scaRNA tail)</u>			
68	30 L arm R_146pro20bp11sca	CCATTACAGCTAATACTAAGTAAATTGGATTTCTCCCTTATCTAATTT ATATCAG	cloning
69	30 GFP_F_146pro20bp11 sca	AGAAATCCAATTTACTTAGTATTAGCTGTAATGGGGCCAACACTTGT CACTACTC	cloning
<u>For 1104 PAM mutants:</u> For PCR fragments: 23 (Fragment1_Forward), 30 (Fragment2_Rev) For amplifying and sequencing mutants: 31-33 Template: "1104 promoter+5' 1104 UTR" construct			
<u>1104 promoter + 5' 1104 UTR + TAA PAM</u>			
70	15 Larm_R_04Pro04UTR GFP	AGAGTAGTGACAAGTGTGGCCATATTTAATACCTTTATTACAGC	cloning
71	15 GFP_F_04Pro04UTR GFP	GCTGTAATAAAGGTATTAATATGGCCAACACTTGTCACTACTC	cloning
<u>1104 promoter + 5' 1104 UTR + TAG PAM</u>			
72	16 Larm_R_04Pro04UTR GFP	GAGTAGTGACAAGTGTGGCCATATTTAATACCTCTATTACAGC	cloning
73	16 GFP_F_04Pro04UTR GFP	GCTGTAATAGAGGTATTAATATGGCCAACACTTGTCACTACTC	cloning
<u>1104 promoter + 5' 1104 UTR + TGA PAM</u>			
74	17 Larm_R_04Pro04UTR GFP	GAGTAGTGACAAGTGTGGCCATATTTAATACCTTCATTACAGC	cloning
75	17 GFP_F_04Pro04UTR GFP	GCTGTAATGAAGGTATTAATATGGCCAACACTTGTCACTACTC	cloning
<u>1101 promoter + 5' 1101 UTR + TAA PAM</u> For PCR fragments: 26 (Fragment1_Forward), 30 (Fragment2_Rev) For amplifying and sequencing mutants: 44-46 Template: "1101 promoter+5' 1101 UTR" construct			
76	46 Larm_R_01Pro01UTR GFP	CATTTGGATTTCTTTATTACAGCTAATTAC	cloning
77	46 GFP_F_01Pro01UTR GFP	GTAATTAGCTGTAATAAAGAAATCCAAATG	cloning
<u>scaRNA tail reprogrammed for 0544-0545</u>			
78	scaReprog_LarmF	CAACACTCAAAGATAGGTAAC	cloning
79	scaRprg_0544/0545_1 5bp_LRev	GTATTTTATACTTTCAGTTTCAGTTGTTAGATTATTTGG	cloning
80	scaRprg_0544/0545_1 5bp_scaF	GAAACTGAAAGTATAAAATACTTTTCATTTGCTTTGATAATTC	cloning
81	scaReprog_scaRev	GCTTATCGATACCGTCGACCTCGCTTTATACTACTTTATTTTAGC	cloning
82	scaReprog_KanF	GCTAAAATAAAGTAGTATAAAGCGAGGTGACGGTATCGATAAGC	cloning
83	scaReprog_KanRev	CGCGTTTATAGCAACCTATATGCATAGCTGCAGGATCGATATC	cloning
84	scaReprog_RarmF	GATATCGATCCTGCAGCTATGCATATAGGGTTGCTATAAACGCG	cloning
85	scaReprog_RarmR	GGTTACGCTTGGTAAATGATGAG	cloning
86	scaReprog_ampliF	GTCAAATATTGGTCGAGTTATC	Mutant confirmation
87	scaReprog_ampliR	GGCACTATCATAAACAGATACC	Mutant confirmation
88	scaReprog_seqF	GTAATCAAGCTTGCCATTGTC	Mutant confirmation
89	scaReprog_seqRev	GTTTGTCTATTTCTGAAAGTAG	Mutant confirmation

#	Name	Sequence 5'-3'	Usage
<u>1104-1101 locus mutants</u>			
<u>Δ1104:Kan^r</u>			
90	1104-FRT-F1	GAA CCA ACA CTG CGA GGT G	cloning
91	1104-FRT-arm1Rev	CTTCGCTTATCGATACCGTCGACCTCATATTTAATACCTCCATTACA GC	cloning
92	1104-FRT-Kan-Fwd	GCTGTAATGGAGGTATTAATATGAGGTCGACGGTATCGATAAGCG AAG	cloning
93	1104-FRT-Kan-Rev	CATATTTTCATATTTTCAATAATCCCCTACAACCAAGCATAGCTGCAG GATCGATATCG	cloning
94	1104-FRT-arm2Fwd	CGATATCGATCCTGCAGCTATGCTTGTTGTAGGGGATTATTGAAAA ATATGAAATATG	cloning
95	1104-FRT-R1	CTCACCATCGCCACACTACG	cloning
96	1104-FRT-CheckFwd	GCAGCTACTTGTAGGTCTGATG	Mutant confirmation
97	1104-FRT-CheckRev	GTCAGCCTTGACGAAATGGC	Mutant confirmation
<u>Δ1102:Kan^r</u>			
98	new02(05660)_LarmF	CATCAGCTGGAACAAGTTTGGC	cloning
99	new02(05660)_LarmR ev	CGATATCGATCCTGCAGCTATGCACCCCTATATTGAGAAGTTATTAC	cloning
100	new02(05660)_kanfwd	GTAATAACTTCTCAATATAGGGGTGCATAGCTGCAGGATCGATATC G	cloning
101	new02(05660)_KanRe v	GTAGAGTATAGCTTGATACTATTAAGAGGTCGACGGTATCGATAAG C	cloning
102	new02(05660)_RFwd	GCTTATCGATACCGTCGACCTCTTAATAGTATCAAGCTATACTCTAC	cloning
103	new02(05660)_RarmR ev	CTTACCTAACATATTAGTGTAGC	Mutant confirmation
104	new1102del_SeqRev	CTTCAGAATTATCAGCCAATTCAG	Mutant confirmation
<u>Δ1101:Kan^r</u>			
105	1101_Larm_F	CTGTAATAACTTCTCAATATAG	cloning
106	1101_Larm_R	GCTTATCGATACCGTCGACCTCCCTATTTTTTTATTATTATTAACAT TTG	cloning
107	1101_Kan_F	CAAATGTTAATAATAATAAAAAAATAAGGGAGGTCGACGGTATCGAT AAGC	cloning
108	1101_Kan_R	CCACTAAAGTTAAATTTTCGACAATGCATAGCTGCAGGATCGATATC	cloning
109	1101_Rarm_F	GATATCGATCCTGCAGCTATGCATTGTGAAATTTAACTTTAGTGG	cloning
110	1101_Rarm_R	CGTTAAATCCATGGAGTGAACC	cloning
<u>Δ1104-1101:Kan^r</u>			
111	1104-01 del 5' FWD	GATAATTGCACTGTTTATAGATCCTAACAAG	cloning
112	1104-01 del 5' Rev	GCT TAT CGA TAC CGT CGA CCT CCAT ATA TTT AAT ACC TCC ATT ACA GC	cloning
113	1101-04delKanFwd	GCTGTAATGGAGGTATTAATATATGAGGTCGACGGTATCGATAA GC	cloning
114	1101-04delKanRev	CTATCCACTAAAGTTAAATTTTCGACAGCATAGCTGCAGGATCG	cloning
115	1104-01 del 3' FWD	CGA TCC TGC AGC TAT GCTGT CGA AAT TTA ACT TTA GTG GAT AG	cloning
116	1104-01 del 3' Rev	GGA GTG AAC CAT GTT TTT TGA TAA TC	cloning
117	1104-01del conf Fwd	TTG CAG CTG GAA CTT TGC C	Mutant confirmation
118	1104-01del conf Rev	GTC ACA CCC TCT TCT GTT AGC	Mutant confirmation
<u>Δ1102 complement:Kan^r</u>			
119	02comp_LarmF	CCTAACAAGTAAGCAAATATTAG	cloning
120	02comp_LarmR (02jxn)	ATTATTATCATATTATCCATATATTTAATACCTCCATTACAGC	cloning
121	02comp_1102Fwd (Larmjxn)	GCTGTAATGGAGGTATTAATATATGATAATATGATAATAATAAAAA AG	cloning
122	02comp_1102Rev(kan jxn)	CGCTTATCGATACCGTCGACCTCCTAAGACATCTGTATACCAAG	cloning
123	02comp_Kanfwd(02jxn)	CTTGGTATACAGATGTCTTAGGAGGTCGACGGTATCGATAAGCG	cloning

#	Name	Sequence 5'-3'	Usage
124	02comp_KanRev	CCACTAAAGTTAAATTTTCGACAAGCATAGCTGCAGGATCGATATCG	cloning
125	02comp_RarmF	CGATATCGATCCTGCAGCTATGCTTGTGCGAAATTTAACTTTAGTGG	cloning
126	02comp_RarmR	CGTTAAATCCATGGAGTGAACC	cloning
127	02comp_ampliFwd	CTCATTACCGTTAGGACTTTAG	Mutant confirmation
128	02comp_ampliRev	CCGTAGCTGGATATGGTGTAC	Mutant confirmation
129	02comp_seqFwd	CATATAGCTGATGAGCCTAACG	Mutant confirmation
130	02comp_seqRev	GATCAAGTCTTAGAATGACATG	Mutant confirmation
<u>Δ1104-1102:Kan^r</u> Primers: 90-92, 104, 101-102			
<u>Plasmids with scaRNA complementarity between promoter and gfp</u>			
131	33-39 Pbav_p146_Rev_Universal	CCCTTATCTAATTTATATCAGAATCATTTATATATGTCAATTGGTATTTATAAATTTTTATGATTTCTCTAG	cloning
132	33_Pbav_p146_20sca_F	CTGATATAAATTAGATAAGGGATGAAAAAGATTAGCTGTAATGGATGCGTAAAGGAGAAGAAGCTTTTC	cloning
133	34_Pbav_p146_15sca_F	GATATAAATTAGATAAGGGAAAGATTAGCTGTAATGGATGCGTAAAGGAGAAGAAGCTT	cloning
134	36_Pbav_p146_11sca_Fwd	CTGATATAAATTAGATAAGGGATTAGCTGTAATGGATGCGTAAAGGAGAAGAAGCTTTTC	cloning
135	37_Pbav_p146_8sca_Fwd	CTGATATAAATTAGATAAGGGAGCTGTAATGGATGCGTAAAGGAGAAGAAGCTTTTC	cloning
136	39_Pbav_p146_0sca_Fwd	GATATAAATTAGATAAGGGTGGATGCGTAAAGGAGAAGAAGCTTTTC	cloning
137	Pbav_RC_seq primer	GCACTCTTGAAAAAGTCATAC	cloning
138	33-39 first round rev	CATTTATATATGTCAATTGGTATTTATAAATTTTTATGATTTCTCTAG	cloning
139	33-39 2nd round rev	CCCTTATCTAATTTATATCAGAATCATTTATATATGTCAATTG	cloning
140	33_Pbav_p146_20sca_F1	GATGAAAAAGATTAGCTGTAATGGATGCGTAAAGGAGAAGAAGCTTTTC	cloning
141	33_Pbav_p146_20sca_F2	GATATAAATTAGATAAGGGATGAAAAAGATTAGCTGTAATGGATGC	cloning
<u>1104-1101 gene junction primers</u>			
141	1104_F	CAGAGTCAAACCTCCGCTGCG	qPCR
142	1104_R	TGGCAGCCAAAATGCGGTAG	qPCR
143	1104-1103_F	CAATTACGTGGAATTTACCAGC	qPCR
144	1104-1103_R	CTAACATCATTACACTGATCTAC	qPCR
145	1102-1101TSS_F	CTGTAATAACTTCTCAATATAG	qPCR
146	1102-1101TSS_R	CGTTATCTTCATATTATCATCC	qPCR
147	1101_F	CCTGATATCGTTGATACTGG	qPCR
148	1101_R	GCCAACAGCCTGCCTGCCACT	qPCR
149	1103-1102_F	GGTAGTAGCGTCCATGTATTTTC	qPCR
150	1103-1102_R	GGTAGAGTATAGCTTGATAC	qPCR
151	1102_F	GCTGTAATGGAGGTATTAAATATATGGATAATATGATAATAATAAAAAAG	qPCR
152	1102_R	CGCTTATCGATACCGTCGACCTCCTAAGACATCTGTATACCAAG	qPCR
153	1103_F	CTTGAGTTGGCTAATGCTCAGG	qPCR
154	1103_R	GTATTACTACCAACTGGTAGG	qPCR

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