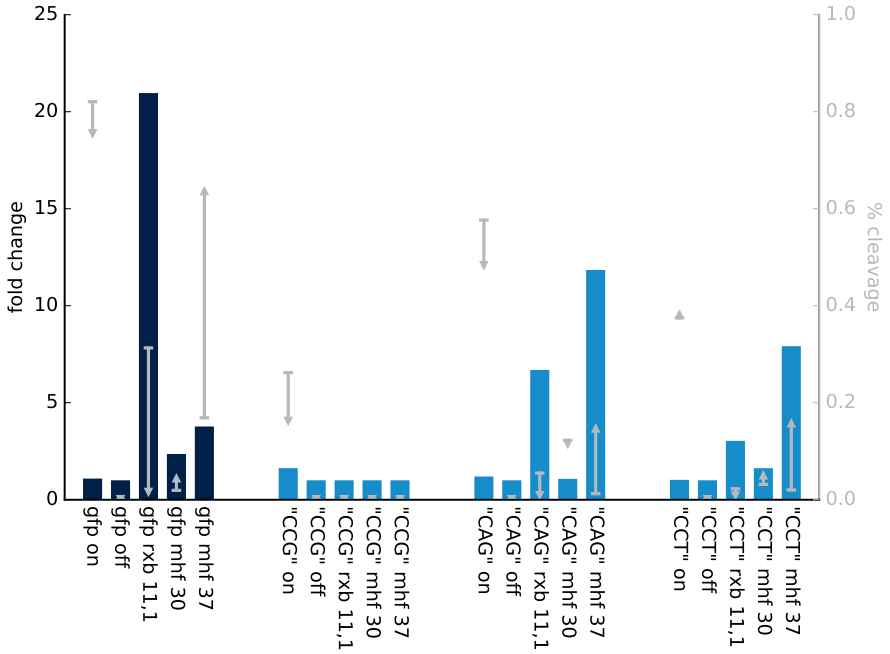

Figure 1: Results from the *in vitro* Cas9 cleavage experiment with our inducible sgRNAs in the context of four different spacers. The "on" and "off" constructs are the positive and negative controls, respectively. The negative control has two mutations in the nexus domain that render it non-functional. The dark blue bars represent the "gfp" spacer we use in our bacterial CRISPRi assay. All three of our designs work well with this spacer in that assay. The light blue bars represent the three spacers you gave us to test. Each is labeled by its first three nucleotides. "CCG" is the spacer that's already been tested in mammalian cells; "CAG" and "CCT" are the two that haven't. The grey axis on the right corresponds to the arrows. The tail of each arrow shows what percent of the DNA was cleaved without theophylline, and the head shows what percent was cleaved with.

Name	Sequence
gfp	`CATCTAATTCAACAAGAATT`
"CCG"	`CCGGCAAGCTGCCCGTGCCC`
"CAG"	`CAGGGTCAGCTTGCCGTAGG`
"CCT"	`CCTCGAACTTCACCTCGGCG`

Table 1: The spacer sequences tested in this experiment.

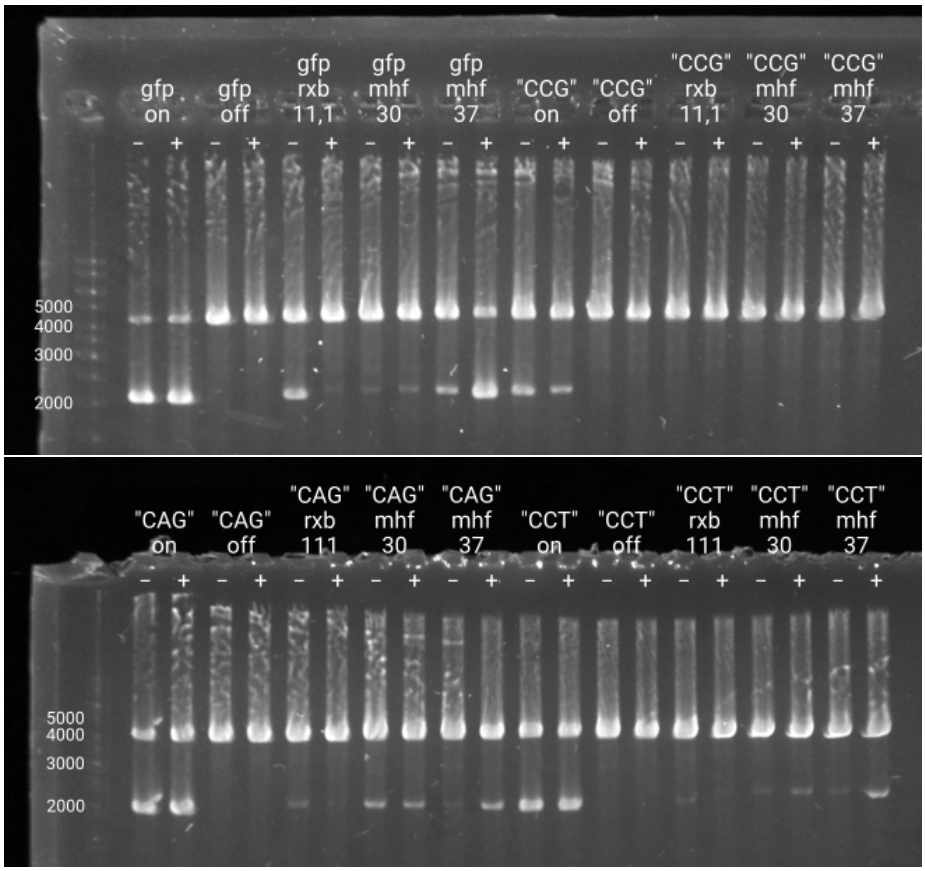

Figure 2: Raw data from the *in vitro* Cas9 cleavage experiment. Each reaction contained 30 nM Cas9, 150 nM *in vitro* transcribed sgRNA, 3 nM target DNA, and either water (in the lanes labeled "-") or 10 mM theophylline (in the lanes labeled "+"). The reactions were incubated at 37°C for 1 h, then quenched by the addition of RNase A and proteinase K. The target DNA is 4 kb and the cleavage site is roughly in the middle, so the 4 kb and 2 kb bands are the uncleaved and cleaved DNA respectively.

Discussion

All three of the designs tested here ("rxb 11,1", "mhf 30", and "mhf 37") work equally well in our bacterial CRISPRi assay, but "mhf 30" seems to work distinctly worse than the others in this cleavage assay. We're not sure why this is.

We detected no cleavage for any of the designs with the "CCG" spacer, with or without theophylline. We used `RNAfold`, an RNA secondary structure prediction

program, to look for base-pairing between the "CCG" spacer and the sgRNA that could explain the lack of activity, but no such base-pairing was predicted. The positive control for this spacer also has relatively weak cleavage, so it may be that this spacer just doesn't work that well in our assay and that we didn't detect any cleavage because the dynamic range is too small.

In contrast, we observed reasonable activity for the "rxb 11,1" and "mhf 37" designs with the "CAG" and "CCT" spacers. We believe that this activity is promising enough to justify testing these designs with these spacers in the mammalian cell assay. Even for these guides, however, it's worth noting that the positive controls only achieved about 50% cleavage.

The conditions we used in this assay probably don't correlate at all to the conditions in the mammalian cell assay. We had a 10-fold excess of Cas9 over target DNA, an 5-fold excess of sgRNA over Cas9, and a 66-fold excess of theophylline over sgRNA. In other words, we expect that every interaction in our system was saturated.