Appendix C

Supplementary information for Chapter 4

C.1 Stepping gel

Each step of the conditional shRNA transcription mechanism was validated by comparing conversion to anneal on native polyacrylamide gel electrophoresis. Both DNA and RNA X_{short} targets were used (Figure C.1 (a) and (b), respectively). The order of the lanes is identical across both gels.

- Detection target X_{short}, hairpin A, and hairpin B are run individually as reactant size markers (lanes 1–3, Figure C.1).
- Step 1: Detection target X_{short} and hairpin A interact to form a band that corresponds to product X_{short}·A (lane 4, Figure C.1). As expected, this product migrates at about the same speed as the annealed product X_{short}·A (lane 5, Figure C.1).
- Step 2: Detection target X_{short}, hairpin A, and hairpin B interact to form a band that corresponds to product X_{short}·A·B (lane 6, Figure C.1). As expected, this product migrates at about the same speed as the annealed product X_{short}·A·B (lane 7, Figure C.1).

- In the absence of a detection target, the hairpins are metastable and minimal A·B is formed (lane 8, Figure C.1). When the two hairpins are annealed, a product corresponding to A·B is formed (lane 9, Figure C.1).

C.1.1 Conditional shRNA transcription and Dicer gel

Transcription products for each step of the conditional shRNA transcription mechanism were examined by radioactive *in vitro* transcription (odd lanes ('−' Dicer) in Figure C.2). An *in vitro* Dicer cleavage assay demonstrates that the products are cleaved to generate the expected siRNA (even lanes ('+' Dicer) in Figure C.2).

- The disrupted T7 promoter is inactive. Minimal transcription is observed from either hairpin A or B (lanes 1 and 3, respectively, Figure C.2).

- Step 1: Some transcription is observed from X_{short}·A (lane 5, Figure C.2). The product is larger than the expected shRNA and is minimally present in the presence of hairpin B (lane 5 vs. lane 9, Figure C.2).

- Step 2: The major transcription product of X_{short}·A·B corresponds to an shRNA in size (lane, 9 Figure C.2). As expected, this product is cleaved by Dicer to generate an siRNA (lane 10, Figure C.2).

- In the absence of a detection target (OFF state), the hairpins are metastable and minimal shRNA is transcribed (lane 7, Figure C.2).

C.1.2 Quantification of ON-to-OFF ratios

The ON and OFF ratios of conditional shRNA transcription were determined by quantifying the shRNA transcription product. As expected, minimal transcription is observed in the absence of a detection target X_{short} (Figure C.3).

C.2 Design of a triggered shRNA transcription mechanism using the H1 promoter

Promoter choice

The second promoter considered for conditional shRNA transcription was the mammalian H1 promoter; discussed below are its advantages and disadvantages. The H1 promoter is a mammalian RNA polymerase III (Pol III) promoter that naturally drives the expression of small RNAs and is commonly used to express shRNAs [1–4]. It is relatively small (100bp), and has well-defined transcription and termination sites [5, 6]. Transcription is terminated when Pol III encounters a run of four or five thymidines; we have therefore added a poly-T termination sequence after the hairpin (domain 't' in Figure 4.1). Another advantage of the H1 promoter is that it is endogenously present in cells (unlike T7 RNA polymerase). However, this raises the possibility of interfering with endogenous pathways which is a disadvantage. Other concerns were the promoter size and the lack of *in vitro* assays for transcription. Despite being short, relative to other promoters, 99bp are significantly longer than molecules previously engineered in the lab and we were not sure whether we would be able to engineer them to undergo toehold-mediated branch migration.

Design

The design follows the same logic operation as the T7-promoter-based system described in Chapter 4. However, whereas the T7-promoter-based design is metastable, the H1-promoter-based system is designed to be stable. Meaning, the secondary structure of the starting components (hairpins A and B) is the thermodynamic minimum and therefore it is not favorable for hairpins A and B to interact. We also added cooperative and stronger binding to this design. Segment 'a*' was added to the toehold of hairpin B, this segment also binds the detection target X. Therefore, both hairpin A and hairpin B bind to the target, further stabilizing their interaction (Figure C.4). Sequence design and thermodynamic analysis was performed as discussed in Chapter 4. The transcribed shRNA is designed to

down-regulate d2EGFP.

Hairpin construction

A challenge present in this design, in comparison to the T7 promoter design, is the size of the hairpins. Despite using a relatively short promoter, the promoter itself is 99 nucleotides long. In addition, the shRNA and target detection sequences need to be added. The total length of hairpin A is 290 nucleotides, and of hairpin B 256 nucleotides. In order to construct these hairpins, each hairpin was ordered as four segments which were annealed together by heating to 95°C for 2 minutes followed by a controlled gradual cooling at -1°C per minute to 23°C in a PCR block in 1× T4 DNA ligase buffer (NEB). After the anneal, T4 DNA ligase was added to the reaction and ligation was carried out overnight at 16°C followed by a denaturing PAGE purification and ethanol precipitation. The full length of the hairpins was verified by using fluorescently labeled probes that bind to the $5'$ or $3'$ end of each hairpin (data not shown). Helper strands (strands that bind to the hairpin to assist in binding the probe) were used to facilitate binding to the $3'$ end of hairpin A and $5'$ end of hairpin B, helper strands were used.

***In vitro* studies**

Each step of the conditional shRNA transcription mechanism was validated by comparing conversion to anneal on agarose gel electrophoresis.

- Detection target X_{short}, hairpin A, and hairpin B were run individually as reactant size markers; X_{short} cannot be visualized under these conditons (lanes 1–3, Figure C.5).

- Step 1: Detection target X_{short} and hairpin A interact to form a band that corresponds to product X_{short}·A (lane 4, Figure C.5). As expected, this product migrates at about the same speed as the annealed product X_{short}·A (lane 5, Figure C.5). Hairpin B cannot bind to X_{short} without cooperativity from hairpin A (lanes 6,7 Figure C.5).

- Step 2: Detection target X_{short}, hairpin A, and hairpin B interact to form a band that corresponds to product X_{short}·A·B (lane 8, Figure C.5). As expected, this product migrates at about the same speed as the annealed product X_{short}·A·B (lane 9, Figure C.5).

- In the absence of a detection target (OFF state), the hairpins are stable and minimal A·B is formed. As expected from stable hairpins, when annealed, minimal product corresponding to A·B is formed (lanes 9 and 10, respectively, Figure C.5). It is yet to be determined whether the observed higher products are due to A or B dimers or to the product A·B.

The components of this mechanism are significantly larger than previously studied hairpins in the lab, requiring a branch migration of over 100 bases. Not only were we able to design a system composed of large components that performs well (good ON and OFF states), but this is also a first demonstration of long branch migration *in vitro*.

To the best of our knowledge, no *in vitro* Pol III transcription system is available. To obtain Pol III transcription *in vitro*, we tried to use HeLaScribe Nuclear Extract in vitro Transcription system (Promega). However, no transcription was observed. The kit is specifically designed to use with Pol II promoters and therefore may not contain Pol III or may not have appropriate conditions for Pol III transcriptions. Due to the presence of RNA Pol III in cells we chose to proceed to *in vivo* studies.

***In vivo* studies**

The H1 promoter is being transcribed by the mammalian RNA Pol III; therefore, transfection of our mechanism is expected to down-regulate d2EGFP. As described in Chapter 4, the two parts of the mechanism are transcription template formation and shRNA transcription. Our first goal was to examine whether the final product can be transcribed and lead to RNAi-mediated d2EGFP knockdown. This was tested by transfection of an annealed final product (X_{short}·A·B) into d2EGFP-expressing cells. As controls, plasmid

transfection and a linear PCR fragment of the H1 promoter driving the expression of an shRNA targeting the same d2EGFP region (PCR amplification off a plasmid, see Materials and methods) were used. To facilitate the transfection of short linear DNA fragments, pOri-e1 plasmid (generous gift from Fred Tan) was used as carrier DNA. The selected shRNA target down-regulates d2EGFP as can be seen by the pSilencer transfection in Figure C.6. The annealed mechanism does not lead to d2EGFP knockdown (Figure C.6, Annealed). Transfection of a short linear DNA fragment (Figure C.6, PCR) is able to knockdown d2EGFP about 70%, suggesting that the transfection conditions are suitable. This suggests that the annealed structure is not being transcribed, possibly due to steric hindrance making the H1 promoter inaccessible to Pol III. A spacer sequence between the target binding site and the H1 promoter might be needed in order to fit RNA Pol III and to achieve transcription of the X_{short}·A·B template. Further studies are needed in order to achieve transcription of an annealed mechanism in tissue culture. Once resolved, the full mechanism (transcription template formation followed by shRNA transcription) can be studied. Optimization will most likely be needed in determining the appropriate dimensions for promoter segments 'p1' and 'p2'. Indeed, preliminary data of hairpin A transfection into d2EGFP-expressing cells suggests that the current segmentation may result in d2EGFP knockdown (data not shown).

Conclusion

We have designed a stable conditional shRNA transcription system based on the mammalian H1 promoter. The use of the H1 promoter requires that the system components (hairpins) be significantly larger than previously studied. Using large hairpins we were able to engineer conditional hybridization cascades that performed according to design. The hairpins did not interact in the absence of a detection target, while the presence of a detection target resulted in the expected final product formation. Due to the lack of an established Pol III *in vitro* transcription system we were unable to test transcriptional activity of our mechanism. Transfection of short PCR fragments containing a d2EGFP

shRNA under the control of the H1 promoter resulted in approximately 70% d2EGFP knockdown, whereas an annealed reaction had no effect on d2EGFP expression. Additional work is required to obtain correct dimensions that enable RNA Pol III to transcribe the H1 promoter while bound to a template, as well as make the H1 promoter inactive in its initial, partially unpaired, state of each hairpin.

In comparison to the T7-promoter-based system presented in Chapter 4, the study of this mechanism is more challenging. The need for large hairpins requires a difficult ligation of several templates making small modifications to the system cumbersome. The lack of an *in vitro* transcription system is also a drawback, making it difficult to decouple transcription and transfection. The endogenous presence of RNA Pol III in mammalian cells is both an advantage and a disadvantage. The advantage is that a special cell line does not need to be created to study the system, while the disadvantage is that our mechanism might interfere with endogenous processes making it more difficult to interpret results. However, from a practical standpoint, the use of the H1-promoter-based system is more appropriate both for studying gene function via conditional RNAi and for therapeutic applications.

C.3 Materials and methods

Strand sequences. The hairpins and detection target for this mechanism are DNA. The H1 promoter sequence used (Table C.1) was adapted from pSilencer3.1 plasmid (Ambion). The d2EGFP shRNA target sequence (Table C.1) was adapted from Beisel et al.[7] (corresponding to bases 128–150). The shRNA $3'$ overhang is a UU incorporated from the transcription termination signal. The target used as the input for this design is a random sequence. See Table C.2 for full sequences.

Cloning of pSilencer d2EGFP shRNA. The shRNA coding sequence corresponding to the same d2EGFP region as the mechanism (see above) was cloned into pSilencer3.1 H1 Hygro (Ambion) according to the manufacturer using the recommended loop sequence.

Domain	Size
shRNA target	GAC CCT GAA GTT CAT CTG CAC C
H1 promoter	AAT TCA TAT TTG CAT GTC GCT ATG TGT TCT GGG AAA TCA CCA TAA ACG TGA AAT GTC TTT GGA TTT GGG AAT CTT ATA AGT TCT GTA TGA GAC CAC TCG

Table C.1: List of strands for H1-promoter-based shRNA transcripiton.

Strand	Size
X	GCT ATA ACG CAT AAT CAC CTC ATA ACA GTT CAA TCT CCC
A	GGG AGA TTG AAC TGT TAT GAG GTG ATT ATA ATT CAT ATT TGC ATG TCG CTA TGT GTT CTG GGA AAT CAC CAT AAA CGT GAA ATG TCT TTG GAT TTG GGA ATC TTA TAA GTT CTG TAT GAG ACC ACT CGG GTG CAG ATG AAC TTC AGG GTC CTC ACA GAC CCT GAA GTT CAT CTG CAC CCG AGT GGT CTC ATA CAG AAC TTA TAA GAT TCC CAA ATC CAA AGA CAT TTC ACG TTT ATG GTG ATT TCC CAG AAC ACA TAG CGA CAT GCA AAA TAA TCA CCT CAT AAC AGT TC
B	TTT GCA TGT CGC TAT GTG TTC TGG GAA ATC ACC ATA AAC GTG AAA TGT CTT TGG ATT TGG GAA TCT TAT AAG TTC TGT ATG AGA CCA CTC GAA AAA AGG TGC AGA TGA ACT TCA GGG TCT GTG AGG ACC CTG AAG TTC ATC TGC ACC CGA GTG GTC TCA TAC AGA ACT TAT AAG ATT CCC AAA TCC AAA GAC ATT TCA CGT TTA TGG TGA TTT CCC AGA ACA CAT AGC GAC ATG CAA ATA TGA ATT GCG TTA TAG C

Table C.2: List of strands for H1-promoter-based shRNA transcripiton.

Briefly, shRNA template oligonucleotides were diluted to 1μg/μl and annealed in DNA annealing solution by heating to 90°C for 3 minutes and incubating for one hour at 37°C. The annealed template was then diluted to 8ng/μl and ligated into the pSilencer vector. Clones were verified by sequencing.

Strand	Sequence
5′	GAT CCG CCC TGA AGT TCA TCT GCA CTT CAA GAG AGT GCA GAT GAA CTT CAG GGT CTT TTT TGG AAA
3′	AGC TTT TCC AAA AAA GAC CCT GAA GTT CAT CTG CAC TCT CTT GAA GTG CAG ATG AAC TTC AGG GCG

Note: The shRNA sequence should read 'GACCC...' but instead is 'GCCC...' (missing A in position 130 of d2EGFP). This does not affect the silencing ability of the shRNA.

Table C.3: DNA templates for cloning d2EGFP shRNA into pSilencer plasmid.

Linear DNA template from pSilencer d2EGFP shRNA. A short PCR fragment containing the H1 promoter and d2EGFP shRNA was PCR amplified off the pSilencer d2EGFP shRNA plasmid using the following primers and conditions:

5′ primer, m13(-20) forward: 5′-GTA AAA CGA CGG CCA GT-3′

3′ primer, pSilencer sequencing rev: 5′-GAG TTA GCT CAC TCA TTA GGC -3′

PCR conditions: annealing temperature of 55°C, protocol according to AccuPrime PFX supermix (Invitrogen). The PCR product was either gel purified from an agarose gel using the QIAquick gel extraction kit (Qiagen) or using the QIAquick PCR purification kit according to the manufacturer.

Bibliography

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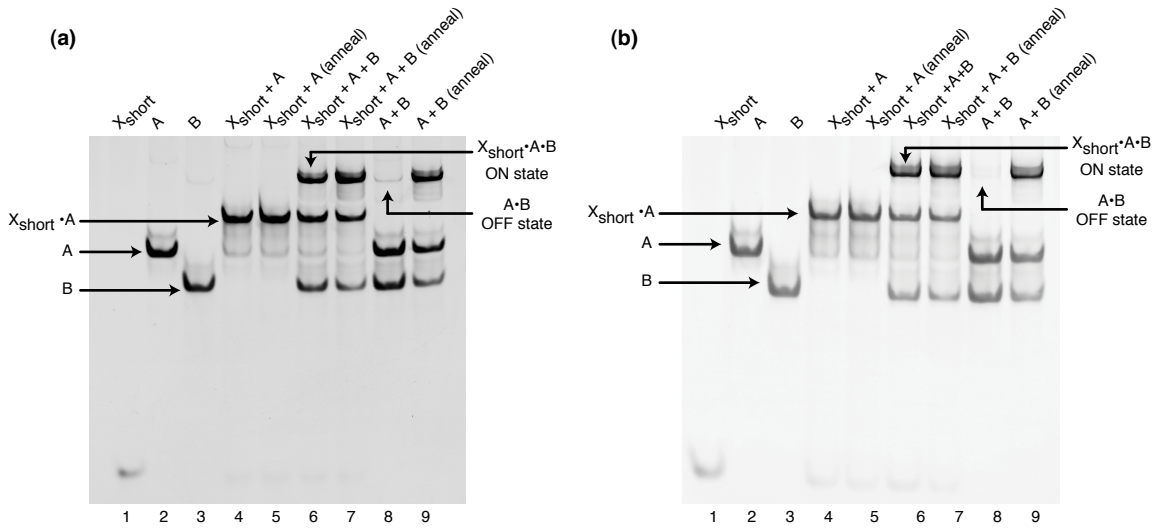

Figure C.1: **Conditional shRNA transcription template formation.** Native polyacrylamide gel electrophoresis demonstrating each step of the reaction depicted in Figure 4.2. (a) DNA detection target X_{short}. (b) RNA detection target X_{short}. A 0.5μM reaction master mix was prepared for each duplicate. The master mix was then split into two separate reactions: 2-hour incubation at 37°C and an anneal. Reactions were run on 10% polyacrylamide gel at 200 volts for 1.5 hours in 1×TBE followed by SYBRGold staining.

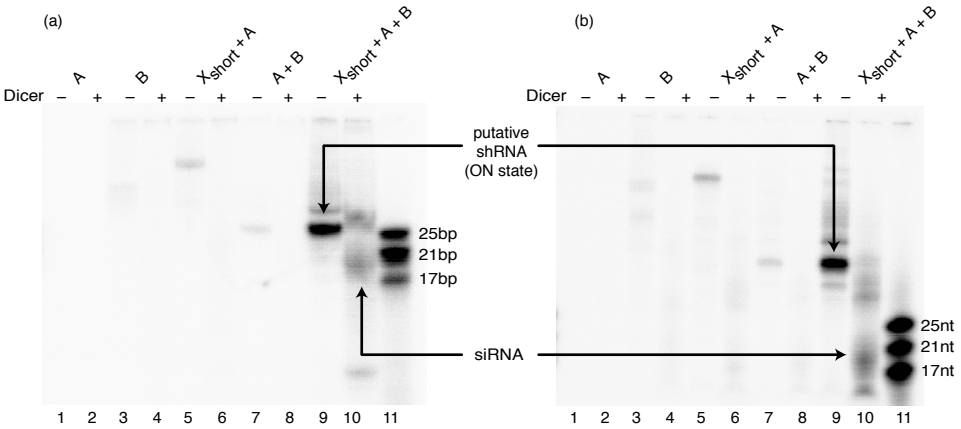

Figure C.2: **Conditional shRNA transcription and Dicer processing.** Gel electrophoresis demonstrating the transcription product of each step of the reaction depicted in Figure 4.1 of Chapter 4. Odd lanes (−) were not subjected to Dicer processing, while even lanes (+) were cleaved by Dicer. Each reaction was split into two, one half was run on a native gel and the other on a denaturing gel. (a) 20% native polyacrylamide gel electrophoresis, 250 volts for four hours. (b) 15% denaturing polyacrylamide gel electrophoresis, 500 volts for 1.5 hours.

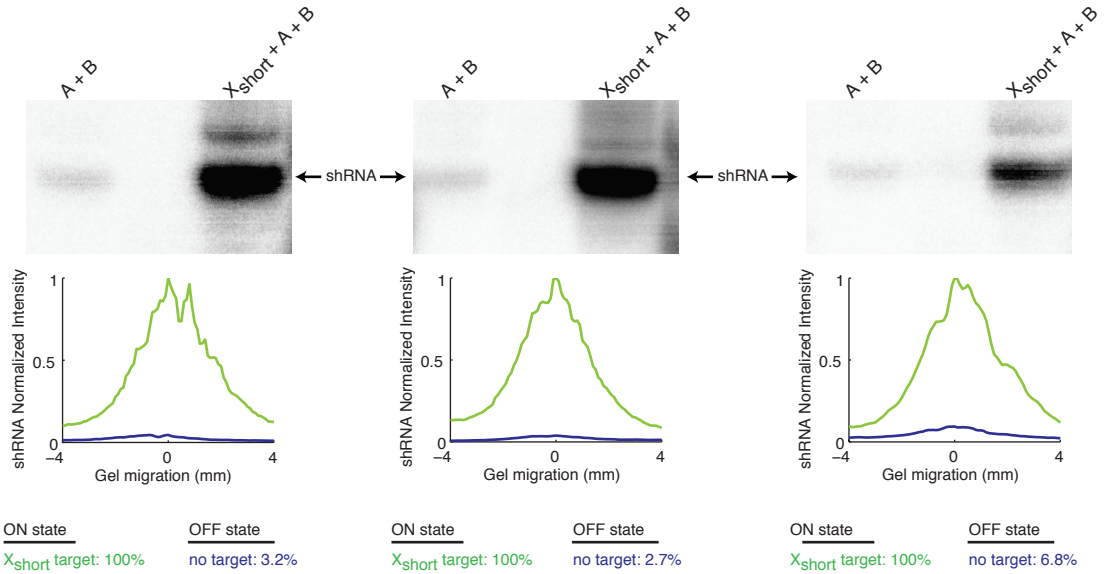

Figure C.3: **Quantification of transcribed shRNA product.** Radioactive *in vitro* transcription in the presence or absence of detection target X_{short}. The shRNA band in three separate experiments was quantified and the band intensity was plotted. For methods see the Materials and methods section in Chapter 4.

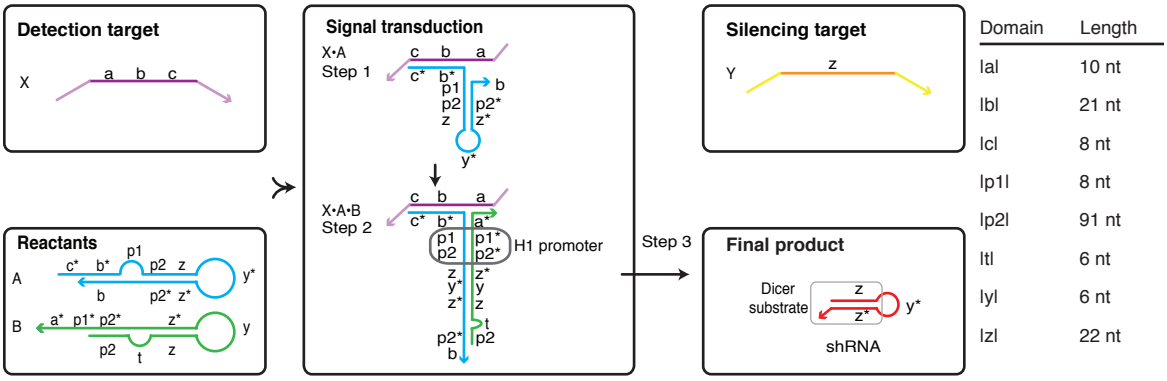

Figure C.4: **Conditional shRNA transcription mechanism based on H1 promoter schematic.** The detection target X is recognized by A (step 1), leading to the binding and opening of B (step 2), which in turn forms an intact promoter and drives the transcription of an shRNA against the silencing target Y (step 3).

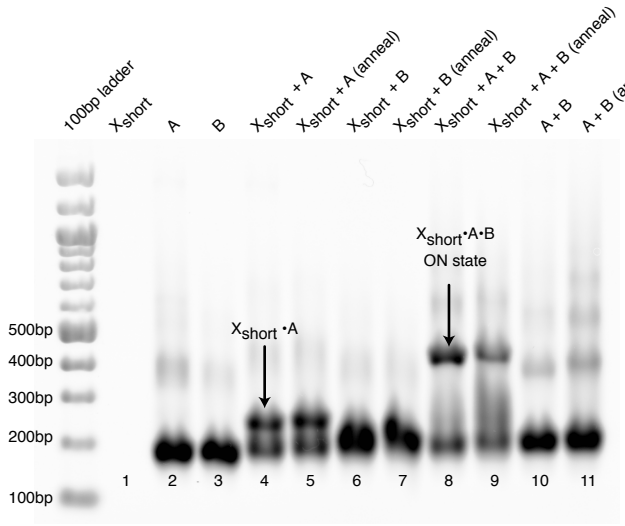

Figure C.5: **Conditional shRNA transcription template formation with H1 promoter.** Agarose gel electrophoresis demonstrating each step of the reaction depicted in Figure C.4. 0.5μM reactions were incubated for 2.75 hours at 37°C or annealed by heating to 95°C for 5 minutes followed by gradual cooling (-1°C/minute) to 23°C. Reactions were run on 1% agarose gel, SYBRGold was pre-added to the loading dye.

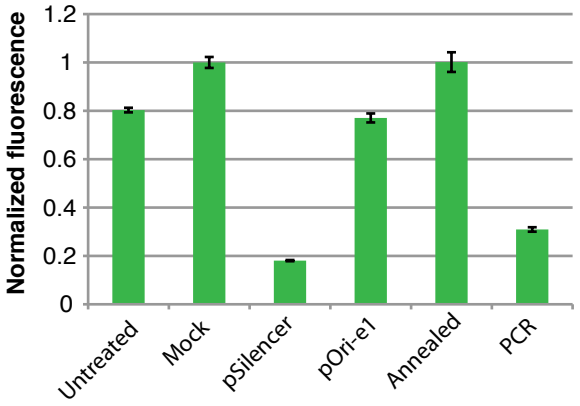

Figure C.6: **Transfection of an annealed H1-promoter-based mechanism does not lead to d2EGFP knockdown.** HEK293 d2EGFP cells were transfected with pSilencer d2EGFP plasmid (pSilencer), pOri-e1 (pOri), pOri-e1 with an annealed mechanism (Annealed) or pOri-e1 with a linear DNA template containing the H1 promoter and shRNA PCR amplified from pSilencer (PCR). Transfection was done in a 24-well plate formate according to the Lipofectamine2000 protocol (Invitrogen) using 800ng plasmid DNA and 180ng linear DNA. 44 hours post transfection, cells were analyzed on a flow cytometer. Transfections were normalized relative to a mock transfection.