Chapter 4

Engineering a conditional shRNA transcription mechanism

4.1 Introduction

As introduced in Chapter 2, RNAi can be induced by siRNAs, dsRNAs, and shRNAs. Once in cells, these molecules turn RNAi on. The need for spatio-temporal induction of RNAi has led to the development of methods to conditionally activate RNAi. In this chapter, we present a conditional shRNA transcription mechanism. We engineered an inactive promoter upstream to an shRNA coding sequence. Upon the presence of an input nucleic acid detection molecule, the promoter changes conformation into an active state, thus driving the expression of an shRNA.

shRNAs can be transfected directly into cells, however, it is common practice to incorporate them into vectors and have them transcribed in cells under the control of polymerase II (pol II) or polymerase III (pol III) promoters [1–4]. Once transcribed, the shRNA is exported from the nucleus to the cytoplasm by Exportin-5 and processed by Dicer into an siRNA [2, 5, 6]. Pol III promoters such as the U6 and H1 promoters are ideal for the production of shRNAs as they naturally transcribe small RNA transcripts lacking a $5'$ cap and a polyadenylation tail [1]. In addition, their transcription initiation point and termination signal (4–6 thymidines) are well defined, ensuring expression of an shRNA with an exact

sequence and without added bases, a property critical for siRNA generation [7, 8]. Pol II promoter-driven shRNAs can be expressed in a tissue specific matter and are transcribed as micro-RNA precursors that have a 5′ cap and polyadenylation tail that must be processed [9, 10].

Constitutively expressed shRNAs have the advantage of mediating prolonged silencing phenotypes compared to chemically synthesized siRNAs, yet they limit the analysis of genes essential for cell survival, cell cycle regulation and cell development. Another disadvantage of constitutive shRNA expression is the risk of off-target effects due to saturation of endogenous silencing pathways [11, 12]. Therefore, a conditional shRNA expression system may be favorable. To this end, tissue specific promoters [10, 13, 14] or inducible promoters [8, 10] can be used. Inducible promoters can be either reversible or irreversible. Reversible induction of shRNA expression systems are most commonly based on tetracycline or ecdysone [10, 15–19]. Irreversible systems are based on the removal of a genetic element that inhibits or promotes transcription and are mostly based on Cre-loxP or Flp recombination [10, 20–22].

Recently, the use of non-coding RNAs to control shRNAs has been developed [23–27]. In this approach, non-coding RNAs containing an shRNA sequence and an aptamer are expressed either in an on (shRNA accessible to RNAi machinery) or in an off (shRNA inaccessible to RNAi machinery) conformation. Addition of a small molecule induces a conformational change in the non-coding RNA which results in turning RNAi from an on state to an off state (or vice versa).

With the exception of tissue specific promoters, a limitation of the approaches described so far is that they rely on the addition of an external molecule (small ligand or an enzyme). An advantage for enzymes is that they can be co-expressed with the shRNA or genetically encoded into the cells. An advantage for the use of a ligand is that ligand concentration can tune the level of shRNA expression. A disadvantage is that the need to add an inducer in addition to an expression construct complicates the system.

An alternative approach to achieve conditional shRNA transcription is to rely on nucleic acids as the inducer molecule. Similar to aptamer-based approaches, nucleic acids can lead to structural changes in conformation of a nucleic acid molecule thus making it active or inactive. Nucleic acid triggers offer a further advantage over aptamers in that they can be programmable, whereas aptamers cannot. An important aspect of a nucleic acid-based approach is that the inducer can be an endogenous nucleic acid target. This overcomes the need to deliver or express an inducer. By choosing the inducer appropriately (e.g., mRNA target expressed only in a subset of cells), spatio-temporal control of expression can be achieved.

We present a method to control shRNA transcription based on nucleic acids. Similar to the mechanism in Chapter 2, this mechanism follows the logic operation: *If gene X is detected, silence independent gene Y*. This mechanism is fundamentally different from the mechanism described in Chapter 2 because in this design, the Dicer substrate (shRNA) is made by enzymatic means and not solely based on strand displacement cascades. To achieve such a design, the transduction molecules must be made of DNA, whereas the design described in Chapter 2 is made of RNA and 2′-OMe. The concept behind this mechanism is that a promoter is active only when a sufficient number of base pairs are formed. Kim et al. [28] have previously demonstrated the use of conditional hybridization of a double-stranded DNA promoter in order to obtain conditional *in vitro* transcription. Here, conditional shRNA transcription is mediated by small conditional DNAs (scDNAs) through toehold-mediated strand displacement. Activation of the mechanism is demonstrated in the presence of a short synthetic nucleic acid target in a test tube. The mechanism exhibits good ON-to-OFF ratio; in the absence of a detection target, minimal shRNA is transcribed. The transcribed shRNA can be processed by Dicer *in vitro* as well as lead to d2EGFP knockdown in tissue culture.

4.2 Mechanism and Design

4.2.1 Mechanism

We have designed a conditional RNAi mechanism based on conditional shRNA transcription. The concept behind this mechanism is that a promoter is active only when a sufficient number of base pairs are present. In the absence of a detection target X (OFF state), the hairpins maintain their secondary structure and should not interact with one another. In the presence of a detection target X (ON state), interaction between the hairpins occurs, a functional promoter is formed and an shRNA targeting gene Y is transcribed.

Our mechanism is comprised of two metastable DNA hairpins kinetically trapped in the hairpin state. The presence of a detection target X starts a cascade of events that allows the hairpins to reach a thermodynamic equilubrium that otherwise cannot be reached in short time scales. The system (Figure 4.1) has the following domains: a target recognition sequence ('b*-a*'), a disrupted promoter sequence ('p1-p2'), an shRNA coding sequence ('z-y*-z*') and a transcription termination sequence ('t'). In the presence of a detection target, toehold 'b*' of hairpin A can bind to the target leading to a branch migration in which complex X·A is formed (Step 1). Next, toehold 'p1*' of hairpin B nucleates with segment 'p1' of hairpin A resulting in a branch migration in which complex X·A·B is formed (Step 2). Complex X·A·B now has a full dsDNA promoter followed by an shRNA coding sequence which can be transcribed (Step 3). This mechanism is catalytic in the sense that one template can be transcribed into multiple shRNAs.

4.2.2 Design

The design follows the logic operation: *If gene X is detected, silence independent gene Y.* As can be seen in Figure 4.1, complete sequence independence is observed between the detection target X (sequence 'a-b') and the silencing target Y (sequence 'z'). This allows one to re-program the mechanism to detect and silence different genes. The design space

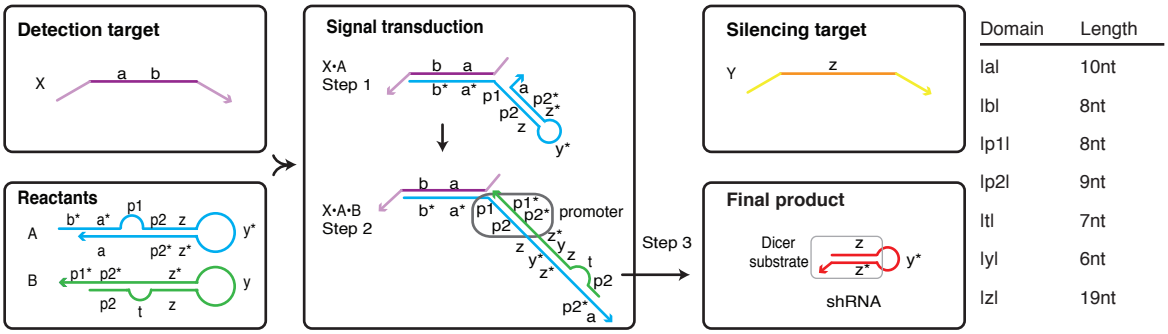

Figure 4.1: **Conditional shRNA transcription mechanism schematic.** The detection target X is recognized by A (Step 1), leading to the binding and opening of B (Step 2), which in turn forms an intact promoter and drives the transcription of a shRNA against the silencing target Y (Step 3).

is constrained for this mechanism. All sequences but the loop of the shRNA (domain 'y') are constrained by the detection target, silencing target, termination signal and promoter sequence. Some flexibility exists in the choice of dimensions for the shRNA (stem and loop size); typically, shRNA with 19–29bp stems separated by a 4–9 nucleotide loop are used [1, 29, 30]. We have chosen to use a 19bp stem with a six nucleotide loop. Careful consideration had to be put into the length specification of 'p1'. It needs to be long enough to make 'p2-p2*' a non-functional promoter, yet short enough in order to minimize the interaction between hairpins A in B in the absence of a detection target (OFF state, also termed 'leakage'). Leakage is also controlled by the length choice of domain 'a': the longer 'a', the less favorable it is for hairpin A to bind to hairpin B in the absence of a detection target. Sequences were assigned to our structures using the design feature on NUPACK [31, 32]. Sequences were specified for the promoter choice (discussed below) and for the shRNA. The shRNA sequence targets a destabilized version of enhanced green fluorescent protein (d2EGFP) and was adapted from an siRNA used by Ohert et al. [33]. To allow more flexibility in the design, for the work presented here, the target sequence was unspecified and the sequence was assigned by NUPACK to minimize the ensemble defect (a measurement of how far the system is from an ideal system)[32, 34]. We then

used the thermodynamic analysis tool on NUPACK [31, 35–37] to handpick the set of designed sequences that performed best based on further analysis (starting materials form the correct structure and that only desired complexes are formed).

4.2.3 Promoter choice

A suitable promoter for use in triggered shRNA transcription should have a defined transcription start site and preferably a transcription stop site. The promoter should be minimally active when not in full duplex form. Ideally, it should also be short for design and synthesis purposes. Natural candidates were the short and well-characterized bacteriophage T3 [38], SP6 [39] and T7 [40] promoters as well as the mammalian H1 promoter [41]. In this work we use the T7 and H1 promoters (for work pertaining to the H1 promoter see appendix C).

T7 RNA polymerase is not present in mammalian cells and therefore a T7-promoter based mechanism is less likely to interfere with endogenous pathways. However, this also means that T7 RNA polymerase needs to be introduced into mammalian cells, which is a disadvantage. The T7 promoter is extremely short, requiring only 17 nucleotides and has a well-defined transcription start site, both of which are a great advantage. Considering its short length, we postulated that by making half of the promoter single stranded, the promoters' function will be disrupted. Some termination of transcription is achieved by the synthesis of an RNA hairpin followed by a stretch of uridine (U) residues [42–44]. We have therefore added a poly-U termination sequence after the shRNA (domain 't', Figure 4.1). Another major advantage is the availability of enzyme for *in vitro* transcription. The use of T7 RNA polymerase *in vitro* to transcribe the strands of an siRNA as well as shRNAs to mediate RNAi has been well documented [2, 8, 45]. Conditional transcription has also been demonstrated *in vitro* by controlling the activity of the promoter using nucleic acid hybridization [28, 46]. Several groups have demonstrated that mammalian cells expressing the T7 RNA polymerase enzyme can trigger RNAi when presented with an

siRNA or shRNA under the control of a T7 promoter [47–49]. This strategy could be made conditional by expressing T7 RNA polymerase under the control of an inducible promoter.

4.3 Results

4.3.1 *In vitro* studies

The mechanism has two parts: conditional transcription template formation and shRNA transcription. For both parts, our aim was to verify that in the absence of a detection target, the OFF state is preserved, and that the presence of a detection target switches the mechanism to its ON state (complex X·A·B is formed and an shRNA is transcribed). Native polyacrylamide gel electrophoresis demonstrates that minimal A·B duplex fomation is observed in the OFF state, while a higher order complex X_{short}·A·B is formed in the presence of a short synthetic DNA target X_{short} (lanes 3 and 5 (respectively) in Figure 4.2(a) and Figure C.1(a) in Appendix C). The system also converts with X_{short} that is made of RNA as is demonstrated in Figure C.1(b) in Appendix C. Radioactive *in vitro* transcription was used to verify that the T7 promoter is off when the target is absent and that it can transcribe an shRNA in the presence of a detection target. In the OFF state, minimal shRNA is produced (3.2%) while in the ON state an shRNA is formed (lanes 1 and 3 in Figure 4.2(b) and (c)); the shRNA transcribed can be cut into an siRNA by Dicer as demonstrated in Figure 4.2(b) lanes 2 and 4. Minimal transcription is observed from the reactants or intermediates in the absence of a target (Figure C.2 in Appendix C). The transcribed shRNA contains the expected shRNA sequence as can be seen by northern blot using a probe against the expected shRNA sequence (Figure 4.2(d)).

4.3.2 *In vivo* studies

So far we have demonstrated the conditional transcription of an shRNA that harbors a sequence targeting d2EGFP. The termination signal for T7 RNA polymerase is not very

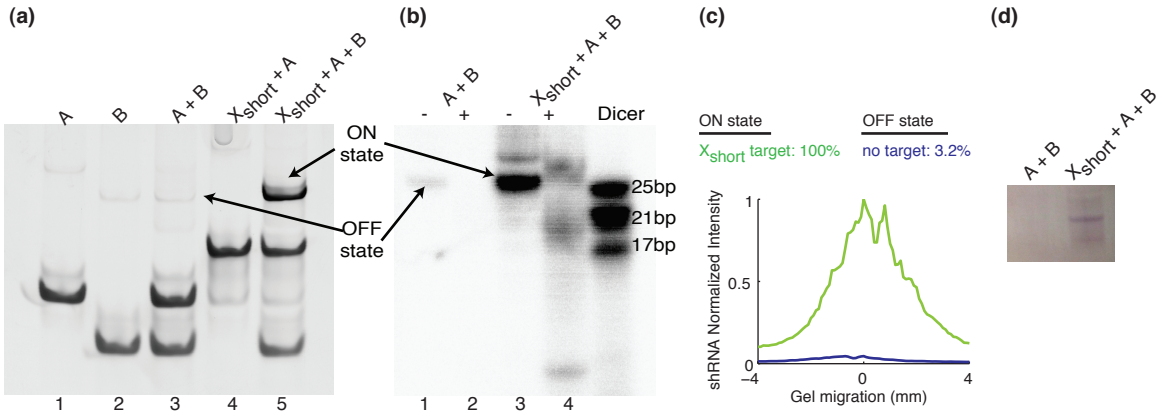

Figure 4.2: **Conditional shRNA transcription. (a)** Transcription template formation *in vitro*. In the OFF state, a minimal amount of A·B is produced (lane 3). In the ON state, the transcription template X_{short}·A·B is produced (lane 5). **(b)** Conditional shRNA transcription and siRNA production *in vitro*. Radioactive shRNA transcription followed by Dicer processing. In the OFF state, a minimal amount of shRNA is transcribed (lane 1). In the ON state, a significant amount of shRNA is transcribed (lane 3). The shRNA is cleaved by Dicer to produce an siRNA (lanes 2,4). (−/+) indicates the absence/presence of Dicer. **(c)** ON:OFF shRNA transcripiton ratio. The shRNA band in panel (b) (− dicer lanes) was quantified and band intensity plotted. **(d)** Northern blot analysis demonstrating that the transcribed shRNA binds to the expected shRNA probe sequence.

strong and therefore the transcribed shRNA can have varying lengths of a $3'$ overhang (up to 16 nucleotides for a run-off transcript). Studies suggest that for shRNAs with $3'$ overhangs longer than three nucleotides, Dicer no longer uses the "$3'$ end rule" to determine the cleavage site but rather cleaves the shRNA in a similar pattern as if it contained a three-nucleotide overhang [50]. Since the transcribed shRNA might not have a canonical shRNA overhang, we next tested whether it can down-regulate d2EGFP. Figure 4.3 demonstrates that a 20nM transfection of *in vitro* transcribed shRNA into HEK293 d2EGFP cells can down-regulate d2EGFP. This suggests that if the mechanism functions in cells, then a functional shRNA will be produced.

To test the functionality of our system in tissue culture, we needed cells that have the RNAi machinery and that express T7 RNA polymerase. We generated a stable HEK293 d2EGFP cell line expressing a cytoplasmic version of T7 RNA polymerase (see Materials and methods). The expression of T7 RNA polymerase was confirmed by western blot and compared to a T7 RNA polymerase enzyme solution (Figure 4.4(a)). The *in vivo* activity of the T7 RNA polymerase was demonstrated by reverse transcription PCR (RTPCR) following the transfection of a plasmid (pT7CAT [51]) containing the chloramphenicol acetyltransferase (CAT) gene flanked by T7 promoter and termination signals. Figure 4.4(b) demonstrates that the CAT gene is being transcribed in cells transfected with the pT7CAT plasmid that express the T7 RNA polymerase mRNA (Figure 4.4(c)).

Next, we examined if, under the control of a T7 promotor, a linear DNA template containing an shRNA against d2EGFP can lead to d2EGFP down-regulation. Multiple attempts using various transfection reagents (Lipofectamine2000 (Invitrogen), Trans-IT-Oligo (Mirus)) as well as different amounts of DNA, the use of a carrier plasmid, and scrambled shRNA and promoter sequences were used. No significant down regulation of d2EGFP that can be attributed to RNAi was observed (data not shown). It appeared that when mild d2EGFP knockdown was observed it was sequence independent and/or due to toxicity during the transfection. Due to the lack of a proper positive control we did

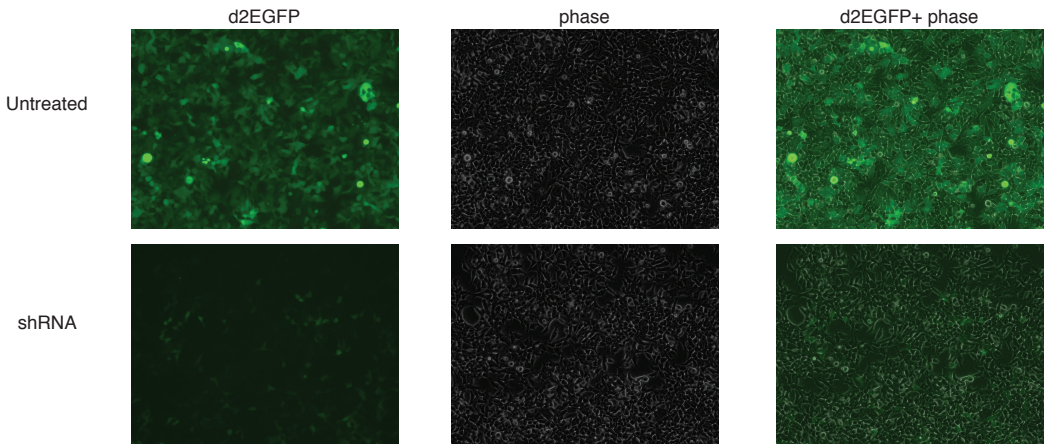

Figure 4.3: ***In vitro* transcribed shRNA down-regulates d2EGFP.** 20nM of *in vitro* transcribed shRNA were reverse-transfected (HiPerFect, Qiagen) into HEK293 d2EGFP cells. Transcribed shRNA was purified using a MasterPure RNA purification kit (Epicentere) according to the manufacturer. Images were taken with an inverted fluorescent microscope 48 hours post transfection.

not pursue the examination of the conditional shRNA transcription mechanism in tissue culture.

4.4 Discussion

In this chapter we introduced a conditional shRNA transcription mechanism based on T7 RNA polymerase. The mechanism implements the logic: *If gene X mRNA is detected, then produce an shRNA specific to independent gene Y.* While following the same logic operation as the mechanism presented in Chapter 2, conditional shRNA transcription is fundamentally different. This mechanism is based on DNA and relies on cellular machinery for shRNA transcription. This mechanism is potentially cheaper, more nuclease resistant, and is programable using different promoters. As such, the mechanism was re-programmed to use an H1 pol III promoter as demonstrated in Appendix C.

For the T7-promoter-based system, we showed that in a test tube, the mechanism

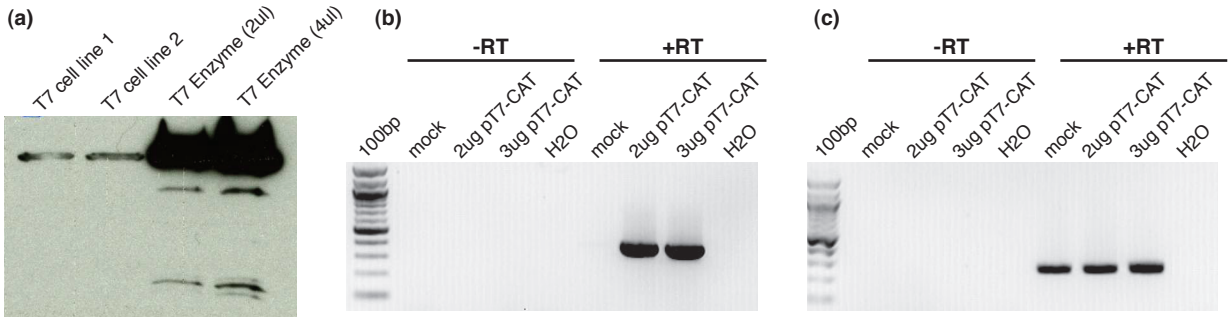

Figure 4.4: **T7 RNA expression and functionality in HEK293 d2EGFP cell line.** **(a)** Western blot of T7 RNA polymerase from protein extracted from HEK293 d2EGFP T7 cell line. Lanes 1 and 2: protein extracted from two cell line clones. Lanes 3 and 4: T7 RNA polymerase enzyme solution (Epicentere). **(b)** RTPCR using CAT primers following 2μg or 3μg pT7CAT plasmid transfection into HEK293d2EGFP T7 cell line. **(c)** RTPCR using T7 RNA polymerase primers following 2μg or 3μg pT7CAT plasmid transfection into HEK293d2EGFP T7 cell line.

conditionally transcribes an shRNA and that the shRNA is cleaved by Dicer to produce an siRNA. The mechanism exhibits a good ON-to-OFF ratio; minimal shRNA is transcribed in the absence of a detection target, while the presence of a detection target leads to significant shRNA transcription. We also demonstrated that the transcribed shRNA is functional, when introduced into cells expressing d2EGFP, the fluorescence intensity is knocked down.

T7 RNA polymerase is not endogenous to mammalian cells. We have therefore generated a stable cell line expressing d2EGFP and T7 RNA polymerase to test conditional shRNA transcription *in vivo*. However, we were not able to down-regulate d2EGFP via T7 transcription of shRNA from a linear DNA template and therefore conditional shRNA transcription has not been attempted. RNAi knockdown using linear DNA templates containing either a T7, H1 or U6 promoter has been previously demonstrated [49, 52, 53] suggesting this approach should work with further optimization. It is possible that the expression of T7 RNA polymerase is not high enough. T7 RNA polymerase is expressed biscistronically with an antibiotic selection marker, increasing the selection pressure will

select for cells expressing more enzyme. Further optimization of the transfection of short dsDNA oligonucleotides may also be needed.

Some modifications to the design may be needed to obtain efficient conditional shRNA transcription *in vivo*. In the current design, the transcription template is not released from the bound target. To achieve transcription while bound to a target mRNA, it may be necessary to add a spacer sequence between the promoter region and the mRNA binding site to accommodate the RNA polymerase; we have yet to explore this case. The immunostimulatory effects of a T7-RNA-polymerase-transcribed shRNA also need to be addressed. The transcription product of T7 RNA polymerase contains a $5'$ triphosphate. It was recently discovered that triphosphates induce type I interferon through activation of retinoic acid-inducible protein 1 (RIG-1) [54, 55]. Consequently, siRNAs and shRNAs transcribed by T7 RNA polymerase may induce an interferon response [56]. Some work suggests that the presence of $3'$ overhangs [57, 58] or that additional guanine residues at the $5'$ end or a $5'$ overhang abrogate interferon induction [58, 59]. For the transcribed shRNA, it should be verified that knockdown is due to RNAi and not interferon. While our design already contains a $3'$ overhang, modifications may be needed at the $5'$ end sequence if interferon induction is observed.

The proposed mechanism can be extended from RNAi to other cellular pathways that interact with nucleic acids. Many bacteria and archaea also use RNA to guide the destruction of foreign genetic elements [60, 61]. In response to viral or plasmid challenge, short nucleic acid elements from the invader are integrated into the host genome into a clustered regularly interspaced short palindromic repeat (CRISPR) [62–64]. The CRISPR loci is transcribed and the transcript is processed into short CRISPR-derived RNAs (crRNAs) [65] which guide the destruction of complementary genetic elements [64, 65]. Three types of CRISPR systems exist, which differ in the crRNA precursor (single-stranded vs. double-stranded RNA) [66, 67] and in whether they target DNA or RNA for destruction [68]. It has been demonstrated *in vitro* that engineered crRNAs can direct the cleavage of

a selected target [69, 70]. While the CRISPR mechanism is quite distinct from RNAi, the use of complementary nucleic acids for gene silencing suggests that the described mechanism can be adjusted to trigger gene silencing in bacteria as well by transcribing a crRNA precursor.

4.5 Materials and methods

Strand sequences. The hairpins and target for this mechanism are DNA. The T7 promoter sequence used is TAATACGACTCACTATA**G**, where **G** is the first base incorporated into the transcript. The shRNA sequence is GCAGCACGACUUCUUCAAGAGCUGACUUGAAGAAGUCGUGCUGCUU where the last two U's are incorporated from the transcription termination signal. The detection target used for this design is a random sequence, the transcribed shRNA targets nucleotides 240–258 on eGFP.

Strand	Sequence
X	ATAAGCCCTCATCCAACT
A	AGTTGGATGAGGGCTTATTAATACGACTCACTATAGCAGCACGACTT CTTCAAGAGCTGACTTGAAGAAGTCGTGCTGCTATAGTGAGATAAG CCCTC
B	CTCACTATAAAAAAAAGCAGCACGACTTCTTCAAGTCAGCTCTTGAA GAAGTCGTGCTGCTATAGTGAGTCGTATTA

Table 4.1: List of strands for T7-promoter-based shRNA transcripiton.

Oligonucleotides. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT). DNA hairpins were synthesized as two pieces (each PAGE purified by IDT) which were then ligated to produce the full hairpin. Ligation was performed using T4 DNA ligase (New England Biolabs) and were purified using denaturing polyacrylamide gel electrophoresis followed by an ethanol precipitation.

Oligonucleotide concentrations were determined and adjusted using A_{260} absorbance on a NanoDrop8000 (Thermo Scientific). Further adjustments were performed by incubating different ratios of individual strands for 2 hours at 37°C followed by gel electrophoresis until correct stoichiometry was obtained.

Hairpins were snap-cooled by heating them to 95°C for 90 seconds followed by a 30 second incubation on ice and room temperature incubation of at least 30 minutes. Complexes were annealed by heating to 90°C for 3 minutes followed by a controlled gradual cooling at -1°C per minute to 23°C in a PCR block.

Polyacrylamide gel electrophoresis. Reactants were incubated at 0.5μM each for two hours at 37°C in 1× SPSC buffer (50mM Na_2HPO_4, 0.5M NaCl, pH7.5). Native polyacrylamide gels were cast and run in 1× TBE (Tris-Borate-EDTA) at 200V. Denaturing polyacrylamide gels were cast and run in 1× TBE at 500V unless otherwise specified. Denaturing gels were pre-run at 500V for 1–2hr (unless otherwise specified). Gels were stained in 1× SYBR Gold (Life Technologies) for 10 minutes at room temperature and imaged using an FLA-5100 imaging system (Fuji Photo Film).

***In vitro* transcription**. Oligonucleotides were snap cooled prior to transcription. Transcription was carried out using the T7-Scribe standard RNA IVT kit (CELLSCRIPT) according to the manufacturer, 2pmol of each DNA strand were included per 20μl reaction. For radioactive transcription only 50nmole of UTP was used in combination with 3–4μl of [$\alpha-^{32}$P] UTP (10mCi/ml, MP Biomedicals). Transcription reactions were incubated for 3 hours at 37°C followed by 20 minutes of DnaseI treatment at 37°C. Transcription products were recovered by Organic extraction / Chromatography / Ethanol precipitation. The reaction volume was adjusted to 200μl using RNase-free water and extracted using 1:1 (v/v) TE-saturated phenol/chloroform. Unincorporated NTP's were removed from the aqueous phase by spin column chromatography using NucAway spin columns (Life Technologies) according to the manufacturer. Ethanol precipitation was done by incubation on ice for 15 minutes in 1:10 (v/v) of 3M sodium acetate and 2.5× (v/v) 95% EtOH. The RNA was pelleted followed a 70% EtOH wash. The pellet was dried and resuspended in 1× duplex buffer. Counts were measured on a Beckman LS-5000TD Liquid Scintillation Counter.

5′ end labeling. microRNA and siRNA markers (New England Biolabs) were 5′ end labeled with [$\gamma-^{32}$P] ATP (10mCi/ml, MP Biomedicals) using T4 polynucleotide kinase (New England Biolabs). Unincorporated [$\gamma-^{32}$P] ATP were removed by spin column chromatography using Illustra MicroSpin G-25 columns (GE Healthcare) according to the manufacturer.

***In vitro* Dicer assay.** Dicer reactions were performed using the Recombinant Human

Turbo Dicer Enzyme kit (Genlantis) according to the manufacturer with some modifications. The volume corresponding to 10,000–20,000 cpm of labeled shRNA from"ON" reaction X·A·B was used for the Dicer reactions (i.e., if 2μl of shRNA from X·A·B transcription reaction correspond to 20,000 cpm then 2μl were used from the other reactions as well). One Dicer unit per 20,000 cpm was used. Hairpins were snap cooled prior to Dicer reaction. Reactions were carried out for 2 hours at 37°C, reactions were stopped by the addition of the appropriate loading dye. siRNA formation was determined by EMSA. Radioactive gels were exposed overnight onto an image plate (Fujifilm type BAS-MS) and scanned using an FLA-5100 imaging system (Fuji Photo Film).

Quantification and band intensity plots. Multi Gauge ver2.0 (Fujifilm) software was used for quantification and intensity plot data. Bands were quantified using the "Quant Measure mode." Data points for band intensity plots were gathered using the profile feature. ON-to-OFF ratio was determined by setting the ON ratio with a short target to 100%.

Northern blot. See miRNA blotting procedure with Ultrahyb-Oliog in materials and methods of Chapter 5. Biotin chromogenic detection (Thermo Scientific) according to the manufacturer. Probe: 5′-biotin-AAG CAG CAC GAC TTC TTC AAG TCA GCT CTT GAA GAA GTC GTG CTG C -3′

HEK293 d2EGFP-T7 RNA polymerase cell line generation. HEK293 d2EGFP cells were a generous gift from Dr. Chase Beisel. T7 RNA polymerase was cloned from pT7POL26 (Gentaur) into pIREShyg3 vector (clontech) by Keyclone Technologies to generate pIREShyg-T7 pol. pIREShyg-T7 pol plasmid was linearized with Bstz17I (NEB) followed by nucleotide removal using QIAquick nucleotide removal kit (Qiagen). 400ng of linearized plasmid were transfected into 293 d2EGFP cells using Attractene transfection reagent (Qiagen) according to the manufacturer. Transfected cells were selected in DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin 125μg/ml Hygromycin B. Cells were grown at 37°C 5% CO_2.

Western blot. Protein was extracted from cells using CellLytic M (Sigma-Aldrich) ac-

cording to the manufacturer. Bradford reagent (Sigma Aldrich) was used to determine the amount of protein according to the manufacturer. 36μg of protein were denatured by heating to 95°C for five minutes and separated on a 10% TRIS-HCL SDS polyacyrlamide gel (Bio-Rad). Protein was transferred to a nitrocellulose membrane using semi dry transfer (Owl separation systems). The membrane was blocked overnight at 4°C in 1× TBST (10mM Tris, 150mM NaCl, 0.05%(v/v) Tween20, pH 7.5) 5% non-fat milk and 2% BSA. Following blocking the membrane was washed 3 times in 1× TBST and then blotted in 1× TBST with 1:1,000 mouse anti T7 RNA polymerase monoclonal antibody (Novagen catalog #70566-3). The membrane was then washed 3 times in 1× TBST and incubated in 1× TBST containing 1:2000 Anti-mouse IgG HRP-linked antibody (Cell Signaling catalog #7076). The membrane was washed in 1× TBST and signal was developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer.

Plasmid transfection for reverse transcription PCR. The pT7CAT plasmid was a generous gift from Dr. Bernard Moss (NIH). Transfections were carried out using Lipofectamine2000 (Invitrogen) according to the manufacturer. RNA was extracted 24 hours post transfection using Trizol (Invitrogen) followed by a DnaseI treatment (NEB) according to the manufacturer. EDTA was added to the sample to a 0.5M final concentration and the Dnase was heat inactivated by a 10 minute incubation at 4°C. cDNA was generated using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems catalog #4368814) according to the manufacturer. The cDNA was then PCR amplified using the Taq PCR Core kit (Qiagen catalog #201223) with the following program: 3 minute incubation at 94°C followed by 30 cycles of 0.5 minute at 94°C, 0.5 minute at 60°C, 1 minute at 72°C, an additional 10 minute incubation at 72°C was added at the end. CAT primers: Forward 5′-ATTCACATTCTTGCCCGCCTGATG-3′ Reverse 5′-GGAAGCCATCACAAACGGCATGAT-3′

T7 polymerase primers: Forward 5′-AACTCCCGATGAAACCGGAAGACA-3′ Reverse

5′-ACCTTGCGGGTTGAACATTGACAC-3′

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