# Chapter 2

# Engineering a conditional catalytic DsiRNA formation mechanism

# 2.1 Introduction

RNA is a versatile molecule responsible for many processes within a cell. It serves both as a template and as a component responsible for protein translation, RNA processing and as a regulatory element. Regulatory RNAs are non-coding RNAs (ncRNAs) that control gene expression; control of gene expression may be initiated by ribozymes, riboswitches, riboregulators, antisense and RNA interference (RNAi) [1]. In this work, RNA's ability to down-regulate gene expression through RNA interference (RNAi) [2, 3] is utilized. RNAi has the potential to silence any gene, which has made it an attractive tool to probe gene function and serve as a potential therapeutic [4–6].

RNAi is a mechanism of post transcriptional gene silencing, induced by small interfering RNAs (siRNAs) in a sequence specific manner. siRNAs are short dsRNAs 21–25 nucleotides in length, with a phosphate at the 5' end and a two-base overhang at the 3' end [7– 10]. siRNAs can be introduced directly into the cell or they may be processed in the cytoplasm from long double-stranded RNAs (dsRNAs) or short-hairpin RNAs (shRNAs) by an RNaseIII endonuclease called Dicer [11–13]. Following siRNA formation, a single strand of the siRNA, the guide strand, is incorporated into a complex of proteins known as the RNA-induced silencing complex (RISC) [14], while the second strand, the passenger strand, gets degraded [15–18]. Next, RISC uses the guide strand to find the complementary mRNA sequence via Watson-Crick base-pairing and endonucleolytically cleaves the target mRNA [10, 19]. Once activated, RISC can undergo multiple rounds of mRNA cleavage, mediating a robust response against the target gene [20].

The logic operation RNAi implements using an siRNA for gene Y is: silence gene Y. As a result, RNAi is constitutively on. This may pose a limitation on the study of essential genes as well as therapeutics. There are several approaches for spatio-temporal control of RNAi, divided into "traditional" and "engineered" approaches. Traditional approaches include targeted delivery [21] and controlled shRNA expression either by tissue-specific promotors and/or activation/inactivation of promoters by small molecules or enzymatic means [22–26] (discussed in Chapter 4). Engineered approaches rely on the use of noncoding RNAs to control RNAi activity following detection of an input signal. These noncoding RNAs harbor an RNAi effector molecule which, in its initial state, may or may not be functional. The presence of an input signal results in a conformational change of the molecule allowing RNAi to be turned on or off.

Small molecules have been used as the input signal to obtain conditional RNAi in cells [27–31]. These mechanisms use an aptamer as the input signal that controls RNAi. In addition to conditionality, small molecule activators also allow the output signal to be tuned (e.g., more ligand, stronger signal). Current small-ligand based mechanisms rely on cellular expression of the non-coding RNA and exogenous addition of the input signal.

As an alternative to small molecules, nucleic acids can be used to control the conformation of non-coding RNAs; this opens the possibility of controlling RNAi via endogenous nucleic acids such as mRNA. Several groups have attempted to achieve this goal. Xie et al. [32] have engineered a sensor that generates an siRNA in response to a 140-nt RNA in *Drosophila* embryo cell-free extract. While the system succeeds in detecting a long RNA molecule, the detection sequence differs by two nucleotides from the sense strand of the output siRNA resulting in the logic: If gene Y is detected, silence gene Y'.

With careful design, a conditional RNAi system can be engineered to implement the logic operation: If gene X is detected, silence independent gene Y.

So far, two systems [33, 34] have been designed toward this goal. While both designs detect and silence two independent sequences, they rely on the use of a short synthetic target and not on a full-length mRNA. In the first system, Kumar et al. [34] based their design on the miRNA pathway. In this pathway, a long stem-loop structure called primary-miRNA is being processed by Drosha to produce a precursor-miRNA (pre-miRNA) which can then be processed by Dicer [35, 36]. In their system, a non-coding RNA was expressed in mammalian cells; upon the transfection of a short target X, the non-coding RNA underwent a conformational change which resulted in the formation of a pri-miRNA. In the second system, Masu et al. [33] engineered a system that, when annealed in a test tube with a short synthetic RNA target X, generates a Dicer substrate. Neither system worked by detecting an endogenous target. For RNAi activation in cells, the systems were either reacted outside the cells or expressed inside the cells and triggered by addition of a chemically modified synthetic target.

In this chapter, we present a conditional RNAi mechanism, that is intended to implements the logic: *If gene X is detected, silence independent gene Y.* Upon the detection of mRNA X, the mechanism produces a Dicer substrate targeting gene Y. Conditional RNAi activation is mediated by small conditional RNAs (scRNAs) through toehold-mediated strand displacement. Activation of the mechanism is demonstrated in the presence of a short synthetic nucleic acid target and full-length mRNA in a test tube. The mechanism exhibits good ON-to-OFF ratio; in the absence of a detection target, minimal Dicer substrate is formed. The Dicer substrate can be processed by Dicer in vitro while initial and intermediate components remain intact. When generated in a test tube, the Dicer substrate leads to down-regulation of gene expression in tissue culture.

# 2.2 Mechanism

We envision a conditional RNAi mechanism based on Dicer processing. As such, the output of the mechanism, the final product, must be an RNAi effector molecule: an siRNA, shRNA or longer dsRNA. A long dsRNA was chosen since it allows more flexibility in the design. Moreover, Kim et al. [37] have demonstrated that synthetic dsRNA duplexes 25–30 nucleotides long are more potent RNAi activators than siRNAs. This enhanced potency is attributed to the fact that longer dsRNAs are Dicer substrates, directly linking siRNA production and incorporation into RISC.

Our mechanism is based on metastable hairpins. By metastable we mean that the hairpin conformation is not the global minima and it is kinetically trapped in a hairpin state. A good mechanism should have good ON and OFF states. In the OFF state the hairpins are kinetically trapped in the monomer state. In the presence of a detection target (X) the mechanism is turned ON and the hairpins are "released" from their trap and interact to form a Dicer substrate. The system components are hairpins with 3' toeholds. This way, as opposed to the mechanism presented in Appendix B, the siRNA antisense sequence is never exposed as a single-stranded region during mechanism transduction and therefore cannot interact with the silencing target prematurely.

The mechanism depicted in Figure 2.1, is an extension of the catalytic hairpin cascades pioneered by Yin et al. [38]. The mechanism reactants consist of three hairpins: the first hairpin (A) detects the detection target X, while the other two hairpins (B and C) serve to produce a Dicer substrate against the silencing target Y. In the presence of detection target X, toehold 'a<sup>\*</sup>' of hairpin A binds to 'a' of the detection target, initiating a branch migration of hairpin A, ending in the complex X·A and exposing 'c' on hairpin A (step 1, Figure 2.1). Next, toehold 'c<sup>\*</sup>' of hairpin B binds to 'c' in the single-stranded region of complex X·A, followed by a branch migration leading to the opening of hairpin B and to the formation of complex X·A·B, exposing 'x' on B (step 2, Figure 2.1). In the third step, toehold 'x<sup>\*</sup>' of hairpin C binds to 'x' in the single-stranded region of complex X·A·B, leading to a branch migration and formation of complex X·A·B·C (step 3a, Figure 2.1). Finally, entropy drives the release of complex B·C from X·A (step 3b, Figure 2.1). The formed B·C complex harbors the output signal, domains 'v-w-x-y-z', which serve as a substrate for Dicer, triggering the RNAi pathway.

The output of the mechanism (B·C in Figure 2.1) is inspired by Dicer substrate interfering RNA (DsiRNA). Typical DsiRNAs contain a 25bp stem and a two nucleotide 3' overhang in the antisense strand [37, 39, 40]. The incorporation of a 3' overhang on only one end introduces a preference for Dicer processing to start from that end since Dicer acts as a molecular ruler, measuring its cleavage site from the 3' end overhang [39, 41]. Due to design constraints, the B·C final product contains a longer stem and a 5' overhang instead of a blunt end.

A notable feature of this mechanism is catalytic production of the final Dicer substrate B·C. When complex B·C dissociates from X·A·B·C then X·A is released (step 3b, Figure 2.1). The re-emergence of complex X·A allows it to interact with a new B hairpin without the need to detect a new target molecule. This way, the detection of one target molecule (by one A hairpin) can lead to the formation of multiple Dicer substrates (B·C duplexes), limited by the amount of B and C hairpins present.

# 2.3 Design

The design follows the logic operation: If gene X is detected, silence independent gene Y. As can be seen in Figure 2.1, complete sequence independence is observed between the detection target X (sequence 'a-b-c-d') and the silencing target Y (sequence 'v-w-x-y-z'). This makes it possible to re-program the mechanism to detect and silence different genes. The sequence design space is constrained by the detection and silencing targets. The sequences are constrained by either the detection target (DsRed mRNA) or silencing target (destabilized eGFP mRNA, d2EGFP) with the exception of domain 'e'. The purpose of this domain is to allow some structural flexibility to the mRNA·A·B complex. Domain 'e' was



Figure 2.1: Conditional catalytic DsiRNA formation schematic. The detection target X is detected by hairpin A (step 1) initiating a cascade of reactions resulting in the opening of hairpins B (step 2) and C (step 3a) leading to catalytic formation of Dicer substrate B·C (step 3b). Domain dimensions are listed.

set initially as two nucleotides but it can be made longer, shorter or removed altogether. Domain 'd' in the loop of hairpin A is part of the detection feature of the hairpin. The purpose of this domain is to help hairpin A stay bound to the target by the addition of extra binding through the loop. This segment can also be removed if deemed unnecessary.

Sequences were assigned to our structures using the concentration-based multi-state design feature on NUPACK [42, 43](Wolfe, unpublished data). It was previously demonstrated in our lab that region 591–623 of DsRed is a good detection region. We therefore specified the design code to chose the detection sequence from this region. The siRNA output was selected from the full-length eGFP coding region. To assign sequences to structures, the design code locates regions in the mRNA that minimize the sum of the ensemble defect (a measurement of how far the system is from an ideal design) [43, 44]. Designed structures include the initial hairpin components (A, B, C), the final structure B·C as well as the structures produced in the first and second steps (X·A and X·A·B, respectively). Concentration based design was used to ensure that structure X·A·B dominates over dimer X·A and monomer B in a dilute solution. The sequence segment 'e' that is not constrained by the detection or silencing sequence was assigned by NUPACK to minimize the ensemble defect.

Following the initial design of hairpins on NUPACK we used NUPACK's thermodynamic analysis tools [42, 45–47] to pick the set of designed sequences that performed best according to the design specifications (starting materials form the correct structure and that only desired complexes are formed). Based on thermodynamic analysis we then modified the dimensions of hairpins B and C to improve the design. Two nucleotides were removed from the 5' end of hairpin B and the corresponding two nucleotides were removed from the toehold of hairpin C to ensure complementarity. This change made the stem of hairpin B shorter while making the toehold longer and was predicted to improve the binding of hairpin B to complex  $X_{short}$ ·A ( $X_{short}$  corresponds to the sequence of the detected region on DsRed mRNA). To prevent Dicer from cleaving the starting and intermediate structures, we made the molecules shorter than a standard Dicer substrate and/or used 2'-OMe modifications.

Following initial *in vitro* studies (data not shown) the stem of hairpin C was shortened by two nucleotides in order to obtain a longer toehold with the aim of improving conversion. We also changed the 2'-OMe modification pattern of hairpin B. In the original design the toehold of hairpin B was 2'-OMe while the rest of the hairpin was made of RNA. In the second iteration, the toehold of hairpin B was changed to RNA and five bases at the 3' end of the stem of hairpin B were changed to 2'-OMe. With this change we hoped to achieve two goals: improve conversion and reduce leakage. When the toehold of hairpin B binds to hairpin A we make 2'-OMe:RNA base-pairs rather than 2'-OMe:2'-OMe basepairs, potentially making a stronger bond which should improve our conversion. By placing 2'-OMe bases at the stem we change one end of the stem from RNA:RNA to RNA:2'-OMe, again making a tighter bond [48, 49], which should reduce leakage. Alternative modification patterns for all three hairpins were also explored (data not shown); we chose to proceed with modifications which resulted in the best ON:OFF ratios and did not lead to unwanted Dicer cleavage of reactants.

Hairpin A is made entirely of 2'-OMe, hairpin B has a stem which is partially modified with 2'-OMe and the stem of hairpin C is half RNA and half 2'-OMe. The sequences listed in Table 2.1 are the final versions used to generate the data presented in this chapter.

# 2.4 Results

#### 2.4.1 In vitro studies

#### 2.4.1.1 Study of ON:OFF properties of triggered Dicer substrate formation

A good mechanism is turned OFF in the absence of a detection target (hairpins do not interact), while forming the final product (Dicer substrate) in its ON state upon the presence of a detection target. We demonstrate the different steps of our mechanism using native polyacrylamide gel electrophoresis (Figure 2.2(a) and Figure A.1 in Appendix A). In the OFF state, a minimal amount of Dicer substrate B·C is produced. The OFF state is represented either using no target (lane 4, Figure 2.2(a)), an off-target mRNA Z (GAPDH mRNA, lane 9, Figure 2.2(a)) or the output silencing target Y (d2EGFP mRNA, lane 10, Figure 2.2(a)). The silencing target was used as an OFF state measurement since both B and C hairpins contain sequences that are complementary to d2EGFP and we wanted to verify that the output target cannot turn the mechanism ON by reversing the steps of the mechanism. In the ON state, the Dicer substrate B·C is produced. The ON state is demonstrated using both a short synthetic target ( $X_{short}$ ) and full-length DsRed mRNA (X) (lanes 7,8 of Figure 2.2(a), respectively). The amount of B·C formed in the ON and OFF states is quantified and normalized relative to production using  $X_{short}$  (Figure 2.2(a) and Figure A.2 in Appendix A).

#### 2.4.1.2 Study of the catalytic properties of the mechanism

To demonstrate the catalytic property of the mechanism we used sub-stoichiometric amounts of  $X_{short}$  to trigger the formation of complex B·C. While the consumption of hairpin A is limited by the amount of  $X_{short}$ , if the mechanism is indeed catalytic then B and C will still be consumed. As expected, the amount of B·C formed is greater than the amount of  $X_{short}$  present in the reaction; B and C are nearly consumed with as little as  $0.3 \times X_{short}$ and roughly 50% are consumed with  $0.1 \times X_{short}$  within two hours (Figure 2.2(b), left to right and Figure A.3 in Appendix A). The amount of B·C formed is quantified relative to B·C production using  $1 \times X_{short}$ .

#### 2.4.1.3 Dicer cleavage assays

The final product of our mechanism, complex  $B \cdot C$ , was designed to be processed by Dicer to produce an siRNA. We show here that indeed the Dicer substrate can be cut by Dicer while the initial and intermediate components remain uncut. Components in the absence



Figure 2.2: Conditional catalytic DsiRNA formation in a test tube. (a) Conditional Dicer substrate formation *in vitro*. ON and OFF states of the mechanism in the presence of a short synthetic target  $X_{short}$  (lane 7), DsRed mRNA target X (lane 8), no target (lane 4), GAPDH off-target mRNA Z (lane 9) and silencing off-target mRNA d2EGFP Y (lane 10). (b) Catalytic Dicer substrate formation. Sub-stoichiometric amounts of target  $X_{short}$  generate above stoichiometric amounts of B·C (target levels increase between lanes 1 through 4). All the lanes have a  $0.5\mu$ M mixture of hairpins A, B and C. Plots represent the normalized intensity of BC in each lane. Quantification was done as described in methods.



Figure 2.3: Conditional siRNA production in vitro. In the OFF state (lane 1), hairpins A, B and C are not cleaved by Dicer (lane 2). In the ON state, the presence of  $X_{short}$  leads to formation of the Dicer substrate B·C (lane 3). B·C is cleaved by Dicer to produce siRNAs (lane 4). (-/+) indicate absence/presence of Dicer.

or presence of  $X_{short}$  were subjected to an *in vitro* Dicer cleavage assay. In the OFF state, none of the hairpins are processed by Dicer (Lane 2 in Figure 2.3), whereas in the ON state, bands corresponding to an siRNA and higher molecular weight leftover products are formed while the B·C band disappears (lane 4 in Figure 2.3). None of the other intermediate complexes are processed by Dicer *in vitro* (see Figure A.4 in Appendix A).

#### 2.4.1.4 Cell studies

We examine here the functionality of our mechanism in tissue culture. The sequences of our mechanism were designed to detect DsRed and silence d2EGFP. The ON state of the mechanism can be examined in cells expressing both DsRed and d2EGFP while the OFF state can be examined in cells expressing only d2EGFP. Ideally, d2EGFP levels should be knocked down in DsRed/d2EGFP expressing cells and remain unchanged in d2EGFP cells.

We analyze our mechanism by examining the effect of each component on its own, the full mechanism, a full mechanism with a short synthetic target as well as a DsiRNA control against the same d2EGFP region. As can be seen in Figure 2.4(a), transfection of hairpin A on its own results in DsRed down-regulation and d2EGFP up-regulation. Hairpin A is designed to detect the target DsRed, the binding of hairpin A to DsRed may result in DsRed knockdown by means of antisense or another pathway. RNAi is less likely since hairpin A is entirely modified with 2'-OMe. The up-regulation of d2EGFP in response to DsRed down-regulation might be due to more translation of d2EGFP now that DsRed is not being generated. Hairpin B on its own seems to lead to some knockdown of d2EGFP. In vitro Dicer cleavage assays show that hairpin B is not cut by Dicer (Figure 2.3 and Figure A.4 in Appendix A). It is possible that the cellular environment allows hairpin B to be cut by Dicer, or silencing may be mediated without Dicer processing (see discussion). Hairpin C on its own does not significantly down-regulate DsRed or d2EGFP, as expected. When the full mechanism (A+B+C) is introduced into cells DsRed is down-regulated and surprisingly d2EGFP is highly up-regulated. Transfection of a full mechanism with a short synthetic target  $(X_{short}+A+B+C)$ , of an annealed final product B·C or of a DsiRNA did not result in d2EGFP knockdown.

When designing the mechanism, the d2EGFP silencing region was selected by the NUPACK design code to optimize the ensemble defect of the mechanism and without using prior knowledge regarding the effectiveness of the silenced region. Unfortunately, as is evident from Figure 2.4(a), this region is not amenable to RNAi. Lack of silencing is due to sequence and not transfection conditions as is exemplified by comparing DsiRNA to DsiRNA2 in Figure Figure 2.4(b).

Due to the choice of d2EGFP silencing region, the current design was not capable of leading to d2EGFP knockdown even if a Dicer substrate is formed once transfected. We therefore modified the sequence of the design to detect the same DsRed region but silence a d2EGFP region which works well according to data in the lab (for sequences see



Figure 2.4: Transfection of conditional Dicer substrate formation into HEK293 d2EGFP DsRed cells. Reverse transfection of 20pmol of each oligonucleotide or annealed B·C. Flow cytometry was used to determine fluorescence. Mean fluorescence was normalized relative to mock treated cells. Green bars represent relative d2EGFP fluorescence, red bars represent relative DsRed fluorescence. Error bars represent the standard error of the mean of three samples. (a) Transfection of mechanism components. B·C was annealed prior to transfection. (b) d2EGFP silencing via DsiRNA transfection. DsiRNA targets the same d2EGFP region as the mechanism, DsiRNA2 was used as a control. See table 2.2 for regions and sequences.

Table 2.3). The ON and OFF states of the mechanism were compared by transfecting the same mixture into cells lacking or expressing the DsRed detection target (Figure 2.5(a)and (b), respectively). In both cell lines, transfection of a pre-annealed Dicer substrate  $B2 \cdot C2$  resulted in ~80-90% d2EGFP knockdown, demonstrating that the final product is functional and that the chosen d2EGFP silencing region works well. Transfection of the three hairpins with a short synthetic target  $(X_{short}+A2+B2+C2)$  resulted in ~40% d2EGFP knockdown in d2EGFP cells and  $\sim 60\%$  d2EGFP knockdown in d2EGFP DsRed cells.  $X_{short}$  was added to the hairpins immediately before the transfection reagent was added to the mix so the reaction components were pre-incubated for a minimal time prior to transfection. Comparing the silencing efficiency of B2·C2 relative to  $X_{short}$ +A2+B2+C2 suggests that the reaction with  $X_{short}$  does not go to completion and less B2·C2 is formed. When the hairpin components of the mechanism are transfected (A2+B2+C2),  $\sim 15\%$ d2EGFP knockdown is observed in d2EGFP cells (similar to B2 transfection on its own) and  $\sim 40\%$  d2EGFP knockdown in d2EGFP DsRed cells (Figure 2.5(a) and (b), respectively). In cells expressing both d2EGFP and DsRed both B2 and C2 hairpins knockdown d2EGFP by  $\sim 20\%$  (Figure 2.5(b)).

Next, we examine whether the  $\sim 40\%$  d2EGFP knockdown observed by A2+B2+C2 transfection into cells expressing for d2EGFP and DsRed is due to the mechanism being turned ON. Due to the variability of the d2EGFP silencing efficiency between the two cell lines we chose to use the DsRed expressing cell line for this study. Unfortunately, a  $\sim 20\%$  variability in d2EGFP knockdown exists between the two transfections of the ON state (A2+B2+C2 samples in Figures 2.5(b) and (c)) making it difficult to compare the separate experiments. Still, d2EGFP appears to be down-regulated by transfection of the un-annealed B2 and C2 hairpins (sample B2 + C2 in Figure 2.5(c)). It is not clear if this is due to leakage or a cumulative effect of each hairpin silencing d2EGFP on its own.



Figure 2.5: Conditional Dicer substrate formation is not functional *in vivo*. Reverse transfection of 20pmol of each oligonucleotide or annealed B2·C2. Flow cytometry was used to determine fluorescence. Mean fluorescence was normalized relative to mock treated cells. Green bars represent relative d2EGFP fluorescence, red bars represent relative DsRed fluorescence. Error bars represent the standard error of the mean of three samples. (a) Validation of OFF state. Transfection of mechanism components into cells lacking the DsRed detection target. B2·C2 was annealed prior to transfection. (b) Validation of ON state. Transfection of mechanism components into cells expressing the DsRed detection target. B2·C2 was annealed prior to transfection of leakage controls into cells expressing DsRed and d2EGFP.

# 2.5 Discussion

In this chapter we presented a catalytic mechanism that conditionally produces a Dicer substrate upon the detection of an mRNA target. While traditional RNAi implements the logic silence gene Y, the mechanism described in this chapter implements the logic if gene X is detected then produce a Dicer substrate targeting independent gene Y. Our mechanism is comprised of three scRNA hairpins which form a signal transduction cascade in which the detection of an mRNA target X results in conformational change of the hairpins leading to formation of a Dicer substrate targeting independent gene Y.

We demonstrated conditional Dicer substrate formation in a test tube. In the absence of a detection target the hairpins do not interact and minimal Dicer substrate is formed. Upon the presence of a short synthetic target or a full-length mRNA detection target the output of the mechanism results in a Dicer substrate. The mechanism is catalytic, detection of sub-stoichiometric amounts of target result in production of Dicer substrate with observed turnover of approximately 100% with as little as  $0.3 \times$  target and roughly 50% turnover is observed with as little as  $0.1 \times$  target.

The produced Dicer substrate was inspired by DsiRNAs yet it is a non-canonical Dicer substrate. Like a DsiRNA, it has a two nucleotide 3' overhang on one end. The purpose of this overhang is to introduce a preference for Dicer processing to start from that end. Unlike a DsiRNA, its opposite end has a 5' overhang instead of a blunt end. Despite being a non-canonical substrate, it is cleaved by recombinant Dicer *in vitro*. Furthermore, when transfected into cells it results in efficient gene knockdown, comparable to that of a DsiRNA.

The silencing observed by the generated non-canonical Dicer substrate suggests that the output of a functioning mechanism should result in gene knockdown. To date, we have not been successful in achieving conditional RNAi in cells. Several factors can contribute to our mechanism being non-functional *in vivo*. For the mechanism to work, three hairpins need to be co-delivered into cells. Our current delivery strategy might be insufficient; alternative delivery methods need to be explored. One such possibility is expressing the scRNAs off a plasmid, this is further discussed in Chapter 3.

Our hairpins were designed to be small as well as chemically modified so that they are not cleaved by Dicer. *In vitro* studies show that we were successful in this goal. Yet, some silencing was observed by single hairpins in cells. It is possible that silencing is mediated by non-Dicer pathways. Other than achieving gene-knockdown, it could also be that the hairpins are bound or degraded by cellular proteins and are therefore not available to the mechanism. This is discussed in further detail in Chapter 3.

For signal transduction to occur, the mechanism relies on toehold-mediated branch migration. The toehold dimensions and/or effective concentration may not be sufficiently high. Some DsRed knockdown is observed by hairpins A and A2 (Figure 2.4 and 2.5, respectively). Both hairpins are fully modified with 2'-OMe and therefore are not expected to be enzymatically processed. Therefore, we postulate that DsRed silencing is observed via an antisense mechanism, suggesting that the hairpin is bound to the DsRed mRNA and that toehold-mediated branch migration is observed. Further studies are needed to validate this hypothesis.

The selected detection and silencing targets used are fluorescent proteins. Interestingly, in some cases when d2EGFP expression is down-regulated, DsRed expression is up-regulated and vice versa (see Figures 2.4 and 2.5). One explanation would be that the mRNA of one fluorescent protein is degraded allowing for more translation of the other fluorescent protein. However, this phenomenon is not consistent across different transfected samples. For example, in Figure 2.5(b) transfection of DsiRNA2 results in d2EGFP knockdown and DsRed up-regulation while transfection of annealed B2·C2 down-regulates d2EGFP to the same extent of sample DsiRNA2 but DsRed expression does not appear to change. The cause for this discrepancy could be activation of an immune response such as protein kinase R (PKR) by B2·C2 but not by DsiRNA2. Activation of PKR would inhibit translation [50, 51], potentially causing DsRed expression to remain unchanged. Further studies are needed to validate this hypothesis.

While still facing challenges *in vivo*, implementing conditional RNAi has profound applications as both a research tool and as a therapeutic agent. As a research tool, triggered RNAi mechanisms will allow gene Y to be silenced in a specific tissue or at a specific developmental stage by appropriately selecting gene X. Alternatively, the spatio-temporal expression of any gene can be reported visually by specifying gene Y as a fluorescent protein or a regulator of a fluorescent protein. As a therapeutic, triggered RNAi could potentially treat any disease that is encoded genetically and could benefit from down-regulation of gene expression or specific cell death such as cancer or autoimmune diseases. For example, to treat cancers, it could be possible to detect an mRNA cancer marker and silence a housekeeping gene to kill the diseased cell.

The mechanism proposed in this work produces a dsRNA substrate designed to interact with the RNAi pathway. However, it is not limited to RNAi. By altering the final product this mechanism has the potential to (conditionally) interface with biology through other means. The final product can have immunostimulatory effects through induction of proinflamatory cytokines and type I interferon via interaction with receptors such as RIG-I and TLRs etc [52–54]. The final product can also be designed to serve a double function as both immunostimulatory and gene downregualtor [55]. Furthermore, gene knockdown is not limited to eukaryotes encoding the RNAi pathway. It has recently been discovered that bacteria and archaea have nucleic acid based adaptive immune systems termed CRISPR. This system relies on small RNAs for sequence specific silencing of foreign nucleic acids [56, 57]. As understanding of this system grows it is becoming evident that CRISPR, in a similar fashion to RNAi, can be programmed as well [58, 59]. The above described mechanisms may offer the potential to conditionally knock down genes not only in eukaryotes but in bacteria and archaea as well.

## 2.6 Materials and methods

**Oligonucleotides.** Oligonucleotides were synthesized and HPLC purified by Integrated DNA Technologies (IDT). Strands were diluted to the desired concentration in  $1 \times$  duplex buffer (20 mM Hepes, pH 7.5, 100 mM Potassium Acetate). 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  $95^{\circ}$ 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  $90^{\circ}$ C for 3 minutes followed by a controlled gradual cooling at  $-1^{\circ}$ C per minute to  $23^{\circ}$ C in a PCR block.

Oligonucleotide sequences. For a list of sequences see Tables 2.1, 2.2, 2.3.

To separate  $X_{short}$  A from B·C on a 20% native polyacrylamide gel, a target longer than 27 nucleotides is needed. Therefore, a 33-nucleotides-long target was used. Three bases were added to the 5' end of the target and three bases were added to the 3' end of the target (see Table 2.1). These extra bases do not affect the properties of our mechanism (data not shown).

**Polyacrylamide gel electrophoresis**. Hairpins and  $X_{short}$  were used at  $0.5\mu$ M each, mRNA targets were used at  $1\mu$ M. Reactions were carried out for two hours at 37°C in 1× duplex buffer. 20% 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).

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

Strand	Sequence
X <sub>short</sub>	GGCAAGCUGGACAUCACCUCCCACAACGAGGAC
А	<u>UCACCUCCCACAACGCUUCAAGUCCGCCAUCUCUCGUUGUGGGAGGU</u>
	GAUGUCCAGCUU
В	UCAAGUCCGCCAUGCCCG CAACGAUGGCGGA <u>CUUGA</u> AG <mark>CGUUG</mark>
$\mathbf{C}$	<u>CGCCAUGCCCGCAACGCU</u> UCAAGUCCGCCAU <mark>CGUUG</mark> *CGGGCAUGGCG
	GACUUGAAG

Table 2.1: List of strands for triggered Dicer substrate formation mechanism. DsRed region: 592–618, d2EGFP region: 252–271. In red are sequences corresponding to DsRed, in green are sequences corresponding to EGFP, in black are random bases. 2'-OMe modifications are underlined. \* The nucleotide at the 5' end of the guide strand is part of the DsRed coding sequence. This is not part of the overall design but rather a result of trimming of hairpin C (see Section 2.3). Since silencing potency was not affected by this, we left it as is.

Strand	Sequence
DsiRNA sense	UCAAGUCCGCCAUGCCCGCAACGAU
DsiRNA antisense	AUCGUUGCGGGCAUGGCGGACUUGAAG
DsiRNA2 sense	UACGGCAAGCUGACCCUGAAGUCUC
DsiRNA2 antisense	GAGACUUCAGGGUCAGCUUGCCGUACA

Table 2.2: List of DsiRNA sequences. DsiRNA targets region 252–271 of d2EGFP. DsiRNA2 targets region 118–140 of d2EGFP.

Strand	Sequence
X <sub>short</sub>	GGCAAGCUGGACAUCACCUCCCACAACGAGGAC
A2	<u>UCACCUCCCACAACGCUGACCCUGAAGUUCCACUCGUUGUGGGAGGU</u>
	GAUGUCCAGCUU
B2	GACCCUGAAGUUCAUCUG <mark>CAACG</mark> GAACUUCA <u>GGGUC</u> AG <mark>CGUUG</mark>
C2	AAGUUCAUCUGCAACGCUGAACCCUGAAGUUCCGUUG*CAGAUGAACUU
	CAGGGUCAG

Table 2.3: List of strands for triggered Dicer substrate formation mechanism 2. DsRed region: 592–618, d2EGFP region: 127–148. In red are sequences corresponding to DsRed, in green are sequences corresponding to EGFP, in black are random bases. 2'-OMe modifications are underlined. \* The nucleotide at the 5' end of the guide strand is part of the DsRed coding sequence. This is not part of the overall design but rather a result of trimming of hairpin C (see Section 2.3). Since silencing potency was not affected by this, we left it as is.

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

Turbo Dicer Enzyme kit (Genlantis) according to the manufacturer with some modifications. Reactions were preformed at  $0.5\mu$ M in 10  $\mu$ L using 0.5 unit of turbo Dicer. Hairpins were snap cooled prior to Dicer reaction. Dicer, target and reactants were all mixed at the same time (i.e., the reactants were not pre-incubated with their target prior to addition of Dicer). Dicer 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 polyacrylamide gel electrophoresis.

Cell lines and transfections. HEK293 d2EGFP cells were a generous gift from Dr. Chase Beisel. The destabilized EGFP sequence comes from pd2EGFP-1 plasmid (Clonetech, PT3205-5 catalog #6008-1). HEK293 d2EGFP DsRed cells were generated by a stable transfection of pDsRed2-1-C1 (Clonetech, PT3603-5 catalog #632407) into HEK293 d2EGFP cells. Cells were maintained at 37°C 5% CO<sub>2</sub> in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Transfections were carried out at the specified oligonucleotide concentrations using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the reverse transfection protocol.  $1-2*10^5$  cells were plated per well of a 24-well plate. Cell counts and viability were determined using the Countess automated cell counter according to the manufacturer (Invitrogen).

Flow cytometry. 24–48 hours post transfection samples were trypsinized for 5 minutes at 37°C, the trypsin was then quenched with DMEM growth media supplemented with 10% FBS. Samples were run on a BD Accuri C6 flow cytometer (BD Biosciences). Healthy cells were gated according to their scatter using untreated cells. Results represent the normalized mean and error bars represent the standard deviation of the mean of three transfected samples.

pTnT-DsRed construction. The DsRed mRNA coding sequence was amplified off

pDsRed2-C1 (Clonetech, catalog #632407) using Taq DNA polymerase. The forward and reverse primers included the MluI and NotI restriction sites (respectively) for directional cloning. The DsRed mRNA coding sequence was cloned into pTnT vector (Promega, catalog #L5610) between the MluI and NotI restriction sites. The construct was verified by sequencing.

#### DsRed mRNA sequence

1ATGGCCTCCTCCGAGAACGTCATCACCGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGG61ACCGTGAACGGCCACGAGTTCGAGATCGAGGGCGGGCCCCAGGGCCGCCCCTACGAGGGG121CACAACACCGTGAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATC181CTGTCCCCCCAGTTCCAGTACGGCTCCAAGGTGTACGTGAAGCACCCCGCCGACATCCCC241GACTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCACCCGCGCGACATCCAG301GACGGCGGCGTGGCGACCGTGAACTTCCCCTCCTCCCTGCAGGACGGCGCTTCATCTAC361AAGGTGAAGTTCATCGGCGTGAACTTCCCCTCCCGACGGCCCCGTGATGCAGAAGAAGACC421ATGGGCTGGGAGGCCTCCACCGAGCGCCGTACCCCCGGGAGTGAAGGGCGAG481ACCCACAAGGCCCTGAAGCTGAAGGACGCCGGCCACTACCTGGTGGAGTTCAAGTCCATC541TACATGGCCAAGAAGCCCGTGCAGCTGCCCGCGCACTACTACGTGGACGCCAAGCTGGAC541ACCCACCAGGAGAAGCCCGTGCAGCTGCCCGCGCACTACTACGTGGACGCCAAGCTGGAC541TACATGGCCAAGAAGCCCGTGCAGCTGCCCGCGAGCAGTACGAGCGCACCGAGGGCCGC661CACCACCTGTTCCTGAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCG721GGCCCGGGATCCACCGGATCTAGATAATAGATGCAATAGATAA

**pGEM-T easy-d2EGFP construction.** The d2EGFP mRNA coding sequence was cloned from cells expressing d2EGFP (generous gift from Dr. Beisel) based on the pd2EGFP-1 (Clontech, catalog #6008-1) sequence and cloned into pGEM-T easy vector (Promega, catalog #A1360)

#### d2EGFP mRNA sequence

1 ATGGTGAGCA AGGGCGAGGA GCTGTTCACC GGGGTGGTGC CCATCCTGGT CGAGCTGGAC

61GGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTAC121GGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGCCCGTGCC181CTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAG241CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGGGCACCATCTC301TTCAAGGACGACGGCAACTACAAGACCGCCGCCGAGGTGAAGTTCGAGGGCGACACCCTG361GTGAACCGCATCGAGGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCAC421AAGCTGGAGTACAACTACAACAGCCACACGTCTATATCATGGCCGACAAGCAGAAGAAT481GGCATCAAGGTGAACTTCAAGATCCGCCACACAGCGCAGGAGCAGCACACC541GACCACTACCAGCAGGACGACCCCGGGGATCACTCTCGGCAAGAAGCGCGATCACATGGTC661CTGCTGGAGTTCGTGACCGCGGACGGGGATACTCTCGGCAAGGACGAGCTGTACAAGAAG721CTTAGCCAGGAGAGCGGGATGGACCGTCACCCTGCAGCCTGTGCTTCTGCTAGGATCAAT841GTGTAG

**mRNA** *in vitro* transcription. pGEMTeasy-GAPDH was a gift from Lisa Hochrein. Plasmids were linearized by restriction digestion. DsRed was in vitro transcribed using T7-Scribe Standard RNA IVT Kit (CELLSCRIPT) according to the manufacturer. d2EGFP and GAPDH were in vitro transcribed using SP6-Scribe Standard RNA IVT Kit (CELLSCRIPT) according to the manufacturer. Transcribed mRNA was purified using RNeasy Protect Mini Kit (Qiagen) according to the manufacturer. mRNA concentration was determined using A<sub>260</sub> absorbance on a NanoDrop8000 (Thermo Scientific).

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