## Chapter 3

# Characterization of cellular modes of failure for conditional Dicer substrate formation in tissue culture

### 3.1 Introduction

In Chapter 2 we presented the design of a conditional catalytic RNAi mechanism that intended to implement the logic operation: *If gene X is detected, then silence independent gene Y.* We demonstrated that minimal Dicer substrate was formed in the absence of X while in the presence of either a short synthetic or a full-length mRNA target X the Dicer substrate for Y was generated. While the generated Dicer substrate is non-canonical, it is functional. Dicer can process the substrate *in vitro* and when transfected into cells as a pre-made complex, knockdown of Y was observed. Despite being able to design a system that is functional *in vitro*, the mechanism does not lead to gene silencing in tissue culture. The mode of failure may be due to a poor choice of sequences, a mechanistic flaw, branch migration not occurring in cells, a problem in delivery, protein sequestration and/or degradation of components or a combination thereof.

In our design process, both the hairpins as well as intermediate components were de-

signed to not be processed by Dicer. This was done by keeping hairpin stem dimensions smaller than standard shRNAs [1] and/or by using 2'-OMe chemical modifications so that the duplexes are not cleaved by Dicer. However, recent findings suggest that molecules shorter than standard siRNAs and shRNAs can also lead to gene silencing. Asymmetric interfering RNA (aiRNA) duplexes with a full antisense strand and a 15bp sense strand can lead to gene knockdown via RNAi [2–4] as can short siRNA with a 16bp duplex and two base overhangs [3]. RNAi can also be achieved by segmenting the sense strand of siR-NAs into two short sequences in order to generate a small internally segmented interfering RNA (sisiRNA) [5]. shRNAs with shorter stems (16–19bp, termed sshRNA) are not processed by Dicer *in vitro*, however they do maintain their gene silencing properties [6]. It is speculated that a nuclease other than Dicer is responsible for cleavage of these sshRNAs. Supporting this hypothesis, Ago2 has been recently found to catalyze the maturation of pre-miRNA-451 [7–9]. The secondary structure of pre-miRNA-451 is similar to that of an sshRNA; it contains a 17bp stem and is not processed by Dicer.

This chapter aims to characterize the reason why triggered RNAi is not observed in tissue culture. To overcome delivery issues we constructed plasmids to express hairpins or a subset of our mechanism. The fact that many non-canonical substrates can lead to efficient RNAi raises the question whether our molecules also interact with the RNAi pathway in a non-desired manner. We examine the fate of some of our hairpins inside the cells. We address the question of whether the hairpins are being processed using northern blots and of whether they can lead to Ago2-mediated silencing using 5' rapid amplification of complementary DNA (cDNA) ends (5' RACE).

#### 3.2 Results

We tested multiple designs for a conditional Dicer substrate formation mechanism in test tube as well as in tissue culture. While the work in Chapter 2 was done using a 3'-toehold-based mechanism, the work presented in this chapter uses hairpins both from a

5'-toehold-based mechanism (see Appendix B) as well as a 3'-toehold-based mechanism. To address the issues of poor choice of sequences and/or a mechanistic flaw we tried different designs using different subsequences of DsRed and d2EGFP, as well as testing different dimensions of toeholds, stems and loops. None of these variations resulted in a functional mechanism in tissue culture (data not shown).

## 3.2.1 Examination of toehold mediated branch migration in tissue culture and combatting delivery

The conditional Dicer substrate formation mechanism is complex. Three hairpins must enter the same cell, detect an endogenous mRNA target as well as interact with one another. We next tried to reduce the complexity of the mechanism by using the minimal components necessary to induce RNAi. The minimal requirements to form a Dicer substrate are the single stranded region of hairpin M1.B (termed M1.B<sub>short</sub>) bound to hairpin M1.C (Figure 3.1(a), highlighted parts). If transfection of M1.B<sub>short</sub> together with hairpin M1.C will lead to down-regulation of d2EGFP in cells, this is an indication that branch migration may be occurring within cells. Figure 3.1(b) demonstrates that transfection of either component on its own results in minimal down-regulation of d2EGFP; this is despite the fact that  $M1.B_{short}$  is the antisense sequence to d2EGFP. When transfected together (in the same vesicle), M1.B<sub>short</sub> and M1.C can interact during the transfection process but may not necessarily interact inside the cells. To verify, the transfection was split into two: one complex contained only  $M1.B_{short}$  while the second complex contained only M1.C, both complexes were added to the cells simultaneously. Split transfection of  $M1.B_{short}$  and M1.Cleads to approximately 50% knockdown of d2EGFP while transfection of the annealed duplex or a control DsiRNA results in approximately 90% knockdown. No increase in down-regulation was observed when the transfection concentration is increased  $\sim$ 4-fold. It is possible that, despite forming a separate transfection complex for each strand, the  $M1.B_{short}$  vesicles and the M1.C vesicles fuse during the transfection processes resulting in binding of M1.B<sub>short</sub> and M1.C outside of the cells and not in the cells. To rule this out, hairpin M1.C was transfected on its own and three hours later an additional transfection of M1.B<sub>short</sub> was done (Figure 3.1(b), 83nM C, then B<sub>short</sub>). Similar levels of d2EGFP knockdown were observed compared to a split transfection. This data is suggestive of the opening of hairpin M1.C by M1.B<sub>short</sub> inside cells. The cells were not washed prior to the second transfection and so it is still possible that some vesicles containing M1.C remained outside the cells and that those vesicles fused with the B<sub>short</sub> vesicles prior to entering the cells. We therefore sought to express both strands in cells; expression of the components rules out their interaction during transfection.

Strands were cloned into pSilencer plasmid (Ambion) under the control of an H1 promoter between the BamHI and HindIII sites. Co-delivery of pSilencer-M1. $B_{short}$  with hairpin M1.C or co-delivery of pSilencer-M1.B<sub>short</sub> and pSilencer-M1.C did not result in d2EGFP knockdown (data not shown). To overcome the need to deliver two plasmids into the same cell we next sought to express both strands off the same plasmid.  $M1.B_{short}$  was cloned into pSilencer under the control of a U6 promoter between the BamHI and HindIII sites. A shorter version of  $M1.B_{short}$  without domain 'b' (see Figure 3.1(a)) was used. Domain 'b' is the two nucleotide overhang of the Dicer substrate, this overhang will be introduced from the termination sequence of Pol III polymerase and is therefore unnecessary. The U6 promoter:  $M1.B_{short}$  cassette was amplified off the plasmid and cloned between the NarI sites in pSilencer H1 promoter: M1.C plasmid. Expression of the simplified system did not lead to d2EGFP knockdown. The observed reduction in d2EGFP levels appears to be due to expression of hairpin M1.C, this is contradictory to data obtained from transfecting hairpin M1.C (Figure 3.1(b) and (c)). Northern blot analysis was done to confirm the expression of M1.C and M1. $B_{short}$ , however no signal was obtained (data not shown). Future studies are needed to determine whether this is due to a low expression level or an unoptimized blotting protocol. Expression of a GFP shRNA in combination with a DsRed shRNA into d2EGFP DsRed expressing cells resulted in knockdown of both proteins as was

determined by flow cytometry, suggesting that a different plasmid expressing two hairpins is functional (data not shown).

An additional attempt to combat insufficient delivery of hairpins and/or degradation was made by generating an expression plasmid that can transcribe three different inserts under separate promoters. Such a plasmid will enable a single cell to transcribe the three hairpins of the mechanism while a continuous production of hairpins would compensate for hairpin degradation. Each insert was cloned using a different set of restriction sites allowing for convenient exchange of inserts (Figure 3.2(a), for a list of constructed plasmids, refer to the Materials and methods section). Transfection of a plasmid expressing a full conditional Dicer substrate formation mechanism (sample A B C in Figure 3.2(b)) did not lead to downregulation of d2EGFP. The observed reduced expression of d2EGFP appears to be due to expression of hairpin M1.C as can be seen by sample hairpin C and two negative control shRNAs in Figure 3.2(b). The negative control shRNA does not lead to d2EGFP downregulation as can be seen by transfection of a plasmid expressing three negative control shRNAs (Figure 3.2(b)). To examine whether the plasmid expresses all components, a northern blot was performed. The data suggests that only hairpin M1.C is expressed in significant levels in cells. An extremely faint band was detected for hairpin M1.B and no signal was detected for hairpin M1.A.

Both U6 and H1 promoters are transcribed by RNA polymerase III and are therefore competing for resources. Work comparing the efficiency of U6 versus the H1 promoters for lentiviral delivery of shRNAs shows that the U6 promoter is superior to H1 in both tissue culture and *in vivo* [10]. In the expression plasmid created, hairpin M1.C is under the control of a U6 promoter whereas hairpins M1.A and M1.B are under the control of an H1 promoter. This could result in the observed variation of hairpin expression. To further pursue the expression of a full mechanism, the plasmid should be changed so that all hairpins are under the control of the same promoter with the goal of achieving a similar expression level for all hairpins.



Figure 3.1: **d2EGFP knockdown via a simplified triggered Dicer substrate for**mation mechanism (M1). (a) Schematic representation of the simplified mechanism. Highlighted regions represent the transfected strands. (b) Relative d2EGFP fluorescence 26 hours post transfection. Transfections were done in triplicate using 20nM final concentration of each strand unless otherwise specified. (c) Plasmid expression of a simplified mechanism. Relative d2EGFP fluorescence 48 hours post transfection. M1.B<sub>short</sub>+M1.C and negative shRNA+GFP shRNA are plasmids expressing two different strands from the same plasmid. One sample per transfection was analyzed.



Figure 3.2: Expression of three hairpins from one plasmid, M1 mechanism. (a) Schematic representation of the expression construct. Black arrows represent promoters and their direction. Colored lines represent three different inserts. Restriction enzymes used for the cloning of each hairpin are listed. Drawing is not to scale. (b) Relative d2EGFP and DsRed fluorescence 48 hours post triple-expression plasmid transfection. Transfections were done in triplicate, error bars represent the standard deviation of the mean. Negative shRNA sequence was adapted from the pSilencer negative control (Ambion). (c) Northern blot analysis for the expression plasmid was transfected into HEK293 d2EGFP cells. Total RNA was extracted using Trizol 24 hours post transfection,  $30\mu$ g total RNA were run per blot. Two pmol of synthetic M1.A and M1.B hairpins and five pmol of M1.C hairpin were blotted as controls. Blotting conditions were according to the *mir*Vana miRNA blotting procedure (see Materials and methods in Chapter 5) using 40nM of biotin labeled DNA probes. Probes are the reverse complement of each hairpin. Detection was carried out using a biotin chromogenic detection kit (Thermo Scientific) according to the manufacturer.

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#### 3.2.2 Study of hairpin degradation in tissue culture

Expression of hairpin M1.C in cells leads to d2EGFP knockdown which indicates that the hairpin may get cleaved inside the cells. Hairpin M1.C has an 18bp stem, a 5' eight-base toehold and a 16-nucleotide loop with some secondary structure (Figure 3.3(a)). While it has a shorter stem than a canonical Dicer substrate (as well as a non-canonical 5' toehold) the secondary structure of the loop may be considered as part of the stem. Indeed, this hairpin can be processed by Dicer in vitro (see Appendix B Figure B.1)b)). We used 2'-OMe blot analysis to determine whether hairpin M1.C is being cut inside the cells. Northern blot confirms that a fraction of the hairpin is being cut 10 hours post transfection (Figure 3.3, C hairpin). Chemical modification of hairpin M1.C with 2'-OMe reduced cleavage as expected (Figure 3.3, C2 hairpin and Appendix B Figure B.1(c)). It is yet to be determined whether the observed lower band in Figure 3.3(c) (C2 hairpin) is due to some cleavage or impurity in the IDT synthesis. Cleavage products were observed both via SYBRGold staining pre-transfer and using a 2'O-Me blot (Figure 3.3(b) and (c), respectively). Not all of the cleavage product bands are detected by blotting, this is probably due to the choice of a full complement probe which has a hairpin secondary structure. To see if loop size and secondary structure matter for cleavage we changed the loop of hairpin C to four nucleotides (Figure 3.3, C3); the shorter loop did not affect cleavage.

#### 3.2.3 Silencing by mechanism hairpin C is mediated by RNAi

In Chapter 2 we presented a system based on hairpins with 3' toeholds. This section focuses on studies done with hairpin M2.C from a 3' toehold system. This hairpin is shorter than the hairpin in Chapter 2. Hairpin M2.C is predicated to have a 15bp stem and a six-nucleotide 3' toehold (Figure 3.4(a), M2.C) and is therefore not a conventional Dicer substrate. However, *in vitro* Dicer assays show that this hairpin can be somewhat cleaved. To reduce Dicer processing hairpin M2.C was modified with 2'O-Me across the 5' end of the stem and halfway into the loop (Figure 3.4(a), M2.C2). This modification pattern



Figure 3.3: Hairpin C of mechanism M1 is partially degraded in tissue culture. (a) Predicted minimum free energy secondary structure of hairpins analyzed by NUPACK. Highlighted bases represent 2'O-Me modifications. (b) Pre-transfer SYBRGold staining of 2'O-Me blot gels. 50nM of each hairpin was transfected into HEK293A cells, transfections were done in duplicate. Total RNA was extracted using Trizol 10 hours post transfection, all of the total RNA collected from two transfections was run per blot. One pmol of synthetic M1.C hairpin was blotted as a control. (c) Northern blot analysis for the degradation of hairpin C in cells. Blotting conditions were according to the *mir*Vana miRNA blotting procedure (see Materials and methods in chapter 5) using 20nM of biotin labeled DNA probe (hairpin C reverse complement). Detection was carried out using a biotin chromogenic detection kit (Thermo Scientific) according to the manufacturer.

abrogated Dicer cleavage *in vitro* (Figure 3.4(b)). Mechanism M2 targets d2EGFP region 597–615. This region is not a good silencing target, as can be seen by the siRNA transfection in Figure 3.4(c). Nevertheless, hairpin M2.C can lead to d2EGFP knockdown in similar levels to an siRNA. As expected, M2.C2, which is chemically modified, exhibits reduced d2EGFP knockdown. Supporting evidence comes from 2'O-Me blot analysis demonstrating that hairpin C was digested to an siRNA-like size in tissue culture. While the chosen probe and/or blotting conditions are not sufficient for detection of full-length M2.C and M2.C2, a band corresponding an siRNA in size is observed in cells transfected with M2.C. Again, hairpin M2.C2 does not appear to be cleaved (Figure 3.4(d)).

Finally, we use 5' rapid amplification of cDNA ends (5' RACE) to examine whether knockdown is mediated by the RNAi pathway. Ago2 has a defined cleavage site and is expected cut the target mRNA between bases nine and ten relative to the 5' end of the mRNA (10–11 nt downstream from the 5' end of the guide strand) [11, 12]. If M2.C silence via Ago2, the d2EGFP mRNA is expected to be cleaved between nucleotides 605 and 606. Figure 3.4(e) demonstrates the mRNA cut sites obtained by 5' RACE from cells transfected with M2.C or M2.C2. Indeed, cells transfected with M2.C mostly display a cleavage pattern around the predicted site (Figure 3.4(e)). It is possible that the reason why a uniform cleavage point is not observed is due to the non-canonical structure of M2.C. It was difficult to obtain 5' RACE data for M2.C2 cleavage. This is most likely due to the fact that it results in low knockdown levels. Still, some data mapped to the siRNA region, however not to the expected site (Figure 3.4(e)).

## 3.3 Discussion

We have discussed in this chapter several reasons why conditional Dicer substrate formation is not functional in a cell. Issues such as delivery, the ability to carry out toehold-mediated branch migration, hairpin processing in cells and mode of d2EGFP down-regulation were explored. Initial studies of toehold-mediated branch migration using a minimal system



Figure 3.4: Hairpin C of mechanism M2 leads to RNAi mediated knockdown. (a) Predicted minimum free energy secondary structure of hairpins analyzed by NUPACK. Highlighted bases represent 2'O-Me modifications. (b) Hairpin M2.C but not 2'O-Me chemically modified M2.C2 is cut by Dicer *in vitro* (for methods see Materials and methods section in Chapter 2). (c) Relative d2EGFP knockdown following hairpin or siRNA transfectoin. Mean fluorescence represents data from 3–4 independent experiments, error bars represent the standard deviation of the mean. (d) Northern blot analysis for the degradation of hairpin C in cells. Blotting conditions were according to the *mir*Vana miRNA blotting procedure (see Materials and methods in Chapter 5) using 20nM of biotin labeled 2'O-Me probe (5'-AmGmCmCmAmCmUmAmCmUmGmAmGmCmAmCmCmCmAmG-3'). Detection was carried our using a biotin chromogenic detection kit (Thermo Scientific) according to the manufacturer. (e) Observed d2EGFP knockdown is mediated by the RNAi pathway. 5' RACE data obtained from transfection of a M2.C, M2.C2 and siRNA demonstrating the cleavage point on d2EGFP.

were promising, suggesting that toehold-mediated branch migration is likely occurring. Attempts to express such a system resulted in silencing by hairpin M1.C, contradictory to transfection of a synthetic hairpin (Figure 3.1(b) and (c)). Similar results were obtained when trying to express a full mechanism Figure 3.2(b)). In addition, expression of a full system resulted in the sole expression of hairpin C. This is most likely due to choice of promoters. U6 promoter, which controls the expression of hairpin C, has a stronger expression than the H1 promoter [10], which controls the expression of hairpins A and B. For future expression of a full mechanism, the same promoter must be used to express all three hairpins.

Due to the observed silencing by hairpin C, we next focused on examining the fate of hairpin C once transfected into cells. Hairpin C was studied for both a 5'- and a 3'toehold mechanism (Figures 3.3 and 3.4, respectively). Despite being an sshRNA, hairpin C can be processed by Dicer *in vitro*, and is cleaved *in vivo*; when chemically modified with 2'O-Me, processing is negated. Hairpin C appears to down-regulate d2EGFP by an RNAi-dependent mechanism of action as based on 5' RACE data.

The short stem of hairpin C is shorter than that of a conventional shRNA, as for an sshRNA. These hairpins are thought to be processed by an enzyme other than Dicer, possibly Ago2, in cells [6–9]. The activity of sshRNAs appears dependent on their designation. Hairpins with the guide strand upstream (5') to the loop region are designated as Lefthand shRNAs (L shRNA) and those with the guide strand downstream to the loop (3') are designated as Right-hand (R shRNA) [6]. For R shRNAs it appears that endonucle-olytic cleavage of the loop region is required for functional RNAi [13]. When the loop region was changed to 2'O-Me, the potency of the same sshRNA has decreased. However, when 2'O-Me and phosphorothioate (PS) modifications were placed on the passenger arm across from Ago2's slicer activity, the silencing ability of R sshRNAs was not significantly affected. Conversely, L shRNAs do not depend on loop cleavage, but rather depend on slicing activity (stem cleavage) [13]. Both types of hairpin C (5'- and 3'-toehold) are con-

sidered to be R type hairpins. Processing of the loop may explain the observed silencing of M2.C2, whereas M1.C2, whose entire loop is 2'O-Me, does not down-regulate d2EGFP (Figure 3.4, data not shown for M1.C2). It would be interesting to see whether changing hairpin M2.C2 to an RNA loop would improve its silencing capabilities.

To conclude, the work presented in this chapter suggests that at least part of the transfected hairpins undergo unwanted processing. Similar analysis must be done for hairpins A and B as well. It may be beneficial to alter their sequence in order to observe d2EGFP knockdown and mRNA cleavage. Further studies are needed to determine whether hairpin processing in cells is mediated by Dicer, Ago2 or another protein.

#### **3.4** Materials and methods

Cell lines. 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  $37^{\circ}$ C 5% CO<sub>2</sub> in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen).

**Oligo transfections**. Transfections were carried out at the specified oligonucleotide concentrations using HiPerFect transfection reagent (Qiagen) according to the Fast-Forward 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).

**Plasmid transfections**. Transfections were done using Lipofectamine2000 (Invitrogen) according to the manufacturer. Cell counts were determined as described above.

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.

Construction of a three hairpin expression plasmid. A custom plasmid (pIDTSmart backbone) was synthesized by IDT containing the following insert: NotI restriction site - H1 promoter - SacI restriction site - GFP shRNA - polIII termination sequence -KpnI restriction site. The expression cassette was amplified using the following primers and cloned into a pSilencer 2.1 U6 backbone between the EcoRI site:

 $3^\prime$  this primer adds the EcoRI and BglII restriction sites to the amplicon

pIDTSMART GFP shRNA rev: 5'-AGCTAGGAATTCATCGATGGTACCTTCCAAAAAAGACCCTG-

 $3^\prime$  this primer adds the EcoRI and ClaI restriction sites to the amplicon

A second custom plasmid (pIDTSmart backbone) was synthesized by IDT containing the following insert: BglII restriction site - H1 promoter - SpeI restriction site - GFP shRNA - polIII termination sequence - EcoRV restriction site - NotI restriction site. The expression cassette was amplified using the following primers and cloned into a pSilencer 2.1 U6 H1 promoter backbone (see above) between the BglII and NotI sites:

pIDTSMARTv2 for for: 5'-GATTCTGAATTCAGATCTGTCAGGCTATGGCGCG-3' this primer adds the EcoRI restriction site to the amplicon

pIDTSMARTv2 rev: 5'-ATGACAGAATTCGATATCTTCCAAAAAAGACCC-3' this primer adds the EcoRI restriction site to the amplicon

All plasmids were verified to contain the correct sequence via sequencing (Laragen).

Plasmid	U6: BamHI HindIII	H1: SacI KpnI	H1: SpeI EcoRV
$3 \times negative shRNA$	negative shRNA	negative shRNA	negative shRNA
M1.A $2 \times \text{negative shRNA}$	negative shRNA	negative shRNA	M1.A
M1.B $2 \times \text{negative shRNA}$	negative shRNA	M1.B	negative shRNA
M1.C $2 \times \text{negative shRNA}$	M1.C	negative shRNA	negative shRNA
M1.A M1.B negative shRNA	negative shRNA	M1.B	M1.A
M1.B M1.C negative shRNA	M1.C	M1.B	negative shRNA
M1.A M1.C negative shRNA	M1.C	negative shRNA	M1.A
M1.A M1.B M1.C	M1.C	M1.B	M1.A

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#### Expression plasmid hairpin sequences

M1.A: 5'-CUCGAUCUCGAACUCGUGGCUGGUCAGCUUGCCGUACACGAGUUCG-3' M1.B: 5'-CGAACUCGUGUACGGCAAGCUGACCGAGACUUCAGGGUCAGCUUGC CGUACA-3'

M1.C: 5'-UACGGCAAGCUGACCCUGAAGUCUCGGUCAGCUUGCCGUACACGAG ACUUCAGGGUCAGC-3'

Negative shRNA: 5'-GUCAGGCUAUCGCGUAUCGUUCAAGAGACGAUACGCGAUA GCCUGAC-3'

5' RACE. 30 pmol C hairpin were snap cooled and transfected into HEK293 d2EGFP cells using RNAiMAX reverse transfection protocol (Invitrogen). 48 hours post transfection the were samples were trypsinized for 5 minutes at 37°C, the trypsin was then quenched with DMEM growth media supplemented with 10% FBS. Triplicates were combined and total RNA was extracted using ZR RNA MiniPrep kit (Zymo research) with an In-column DNaseI digestion according to the manufacturer or RNA spin mini (GE healthcare) according to the manufacturer.  $1.5\mu$ g total RNA were ligated to 20pmol of GeneRacer RNA oligo (5'-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3'): total RNA and oligo were combined in a total volume of  $10\mu$ l, heated to 65°C for 5 minutes followed by a 2 minute incubation on ice. Ligation was carried out for 1 hour at 37°C using 20 units of RNA ligase 1 (NEB), 40 units RiboGuard (Epicenter), 1mM ATP 10% PEG-8000 in a total volume of  $20\mu$ l. Ligation products were then purified using RNeasy mini kit (Qiagen) according to the manufacturer. cDNA was transcribed using Super-ScriptIII (Invitrogen) according to the manufacturer using d2EGFP 839 rev primer (5'-TTGATCCTAGCAGAAGCACAGGCT-3'). The cDNA was amplified using OneTaq hot start 2× master mix with standard buffer (NEB) using touch-down PCR and the following primers: GeneRacer 5' primer (5'-CGACTGGAGCACGAGGACACTGA-3') and d2EGFP 822 reverse primer (5'-ACAGGCTGCAGGGTGACGGTCCAT-3'). PCR program: 94°C for 2 minutes, 5 cycles of 94°C for 30 seconds and 1 minute at 72°C, 5 cycles of 94°C for 30 seconds and 1 minute at 70°C, 20 cycles of 94°C for 30 seconds, 66°C for 30 seconds, 68°C for 1 minute, followed by a 5 minute incubation at 68°C. A second nested PCR reaction was carried out using the following primers: GeneRacer 5' nested primer (5'-GGACACTGACATGGACTGAAGGAGTA-3') and d2EGFP 728 reverse primer (5'-TGGCTAAGCTTCTTGTACAGCTCG-3'). PCR conditions are as listed above with the following modification: for the 20 cycles annealing was done at 68°C. PCR products were sequenced by Laragen.

#### Sequences used:

M1.B<sub>short</sub>: 5'-ACCGAGACUUCAGGGUCAGCUUGCCGUACA-3'

M1.C: 5'-UACGGCAAGCUGACCCUGAAGUCUCGGUCAGCUUGCCGUACACGAGACUU CAGGGUCAGC-3'

M1.C2: 5'-mUmAmCmGmGmCmAmAGCUGACCCUGAAGUmCmUmCmGmGmUmCmA mGmCmUmUmGmCmCmGmUmAmCmAmCmGmAmGmAmCmUmUmCmAmGmGmG mUmCmAmGmC-3'

M1.C3: 5'-UACGGCAAGCUGACCCUGAAGUCUCGGUCACGAGACUUCAGGGUCAGC-3'

M2.C: 5'-UACCUGAGCACCCAGCCACUACCUCUGGGUGCUCAGGUAGUGGCU-3' M2.c2: 5'-mUmAmCmCmUmGmAmGmCmAmCmCmCmAmGmCmAmCUACCUC UGGGUGCUCAGGUAGUGGCU-3'

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