Appendix A

Supplementary information for Chapter 2

A.1 Stepping gel

Each step of the conditional catalytic Dicer substrate formation mechanism was validated by comparing conversion to anneal on native polyacrylamide gel electrophoresis.

- Detection target X_{short}, hairpin A, hairpin B, and hairpin C are run individually as reactant size markers (lanes 1–4, Figure A.1).
- Step 1: Detection target X_{short} and hairpin A interact to form a band that corresponds to product X_{short}·A (lane 5, Figure A.1). As expected, this product migrates at about the same speed as the annealed product X_{short}·A (lane 6, Figure A.1).
- Step 2: Detection target X_{short}, hairpin A, and hairpin B interact to form a band that corresponds to product X_{short}·A·B (lane 7, Figure A.1). As expected, this product migrates at about the same speed as the annealed product X_{short}·A·B (lane 8, Figure A.1).
- Step 3: Detection target X_{short}, hairpin A, hairpin B, and hairpin C interact to form bands that correspond to products X_{short}·A, B·C and X_{short}·A·B·C (lane 9, Figure

A.1). As expected, similar products are observed in the annealed reaction (lane 10, Figure A.1).

- In the absence of a detection target (OFF state), the hairpins are metastable and minimal B·C is formed (lane 11, Figure A.1). When all three hairpins are annealed, a product corresponding to B·C is formed (lane 12, Figure A.1).

- In the absence of a detection target, hairpins B and C are metastable (lane 13, Figure A.1). When the two hairpins are annealed, a product corresponding to B·C is formed (lane 14, Figure A.1). Lane 14 also serves as a size marker for the expected final product B·C.

- Not all of B and C are consumed in the anneal, possibly because the basepairs in the hairpins form more easily than intermolecular basepairs.

A.2 Quantification of ON and OFF states

Three independent experiments were carried out to examine the ON and OFF states of conditional Dicer substrate formation (Figure A.2). The amount of Dicer substrate B·C formed was quantified relative to detection target X_{short}. The mechanism is turned ON by either a short synthetic target or DsRed mRNA (lanes X_{short} and X, respectively, in Figure A.2). Minimal B·C is produced in the OFF states. The OFF state was examined in the absence of a detection target, in the presence of an off-target mRNA GAPDH or in the presence of the silencing target d2EGFP mRNA (lanes "no target", Z and Y, respectively in Figure A.2).

A.3 Quantification of catalytic property

Three independent experiments were carried out to examine the catalytic property of conditional Dicer substrate formation. The amount of Dicer substrate B·C formed was quantified

relative to 1× detection target X_{short}. Minimal B·C is produced in the absence of a detection target, whereas as little as 0.1× X_{short} is sufficient to convert roughly 50% of hairpins B and C. Conversion with 0.3× X_{short} is equivalent to conversion with 1× X_{short} (Figure A.3).

A.4 *In vitro* Dicer assay

Validation that only the final product, B·C, of the conditional catalytic DsiRNA formation mechanism is processed by Dicer. Each step of the reaction was subjected to incubation in Dicer reaction conditions in the presence '+' lanes or absence '−' lanes of Dicer. For a detailed description of each step see section A.1. Complex B·C is formed in the presence of detection target X_{short} and hairpin A (lanes 13, 14, Figure A.4(a)), or when hairpins B and C are pre-annealed (lanes 15, 16, Figure A.4(a)). Minimal B·C is formed in the absence of X_{short} (lanes 7, 8, Figure A.4(a)). Native and denaturing polyacrylamide gel electrophoresis demonstrate that the B·C complex is processed by Dicer to form an siRNA (lanes 8, 14, 16, Figure A.4(a) and (b), respectively). X_{short}·A is not fully denatured under these conditions as evidenced by lanes 9 through 14 of Figure A.4(b).

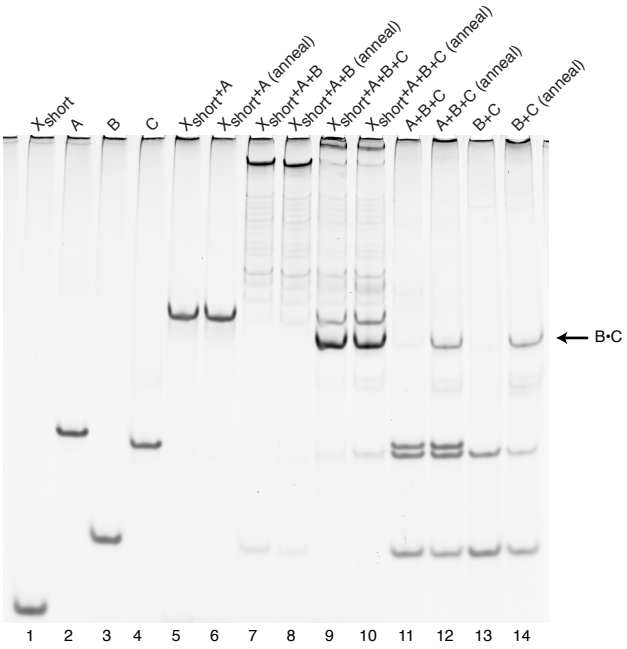

Figure A.1: **Stepping gel for the conditional catalytic DsiRNA formation mechanism.** Native polyacrylamide gel electrophoresis demonstrating each step of the reaction depicted in Figure 2.1. A 0.5μM reaction master mix was prepared for each duplicate. The master mix was then split into two separate reactions: 2-hour incubation at 37°C and an anneal. Reactions were run on 20% polyacrylamide gel at 200 volts for 9.5 hours in 1×TBE followed by SYBRGold staining.

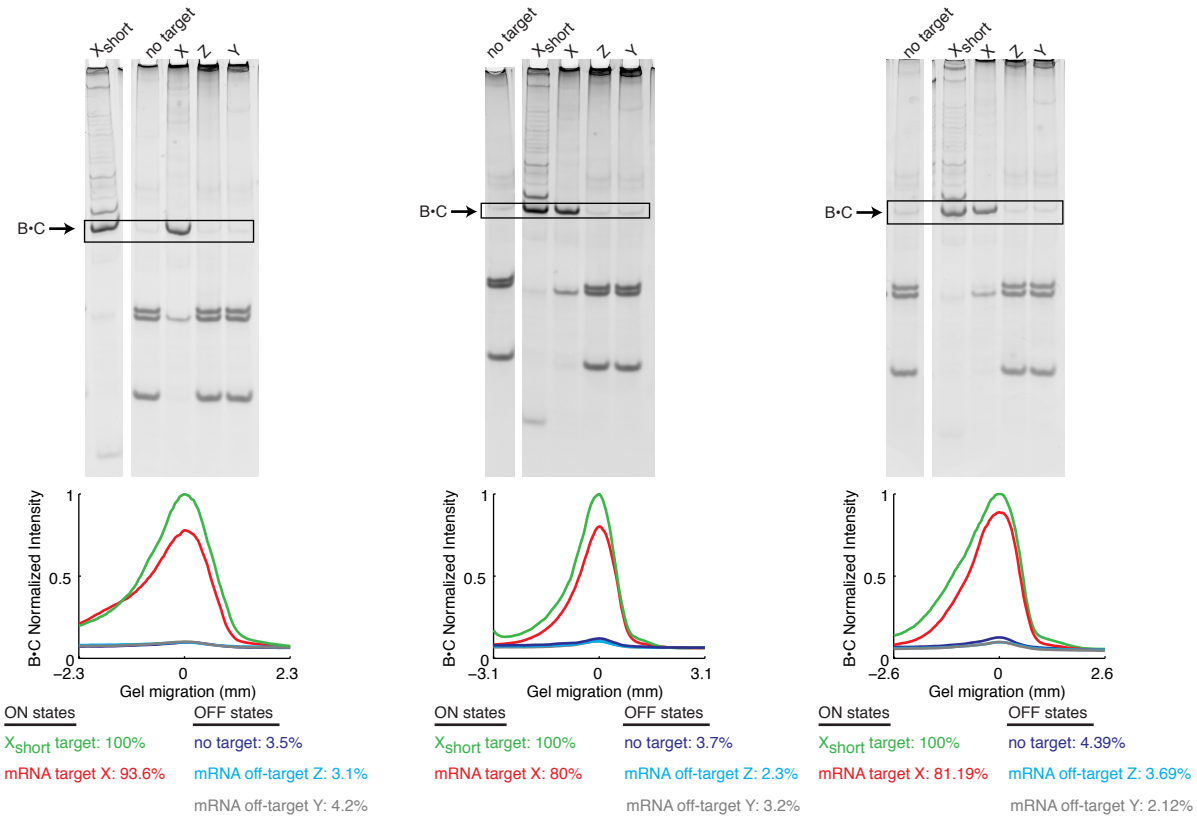

Figure A.2: **Quantification of B·C final product formation in ON and OFF states.** ON states (X_{short}, DsRed mRNA target X) and OFF states (no target, GAPDH mRNA target Z and silencing d2EGFP target Y) of the mechanism. Plots represent the normalized intensity of B·C in each lane across three separate experiments. Quantification was done as described in methods section of Chapter 2. A 0.5μM reaction master mix containing A, B and C was prepared. The master mix was then split into the separate reactions and targets were added for a 2-hour incubation at 37°C. Reactions were run on 20% polyacrylamide gel at 200 volts for 10.5 hours in 1×TBE followed by SYBRGold staining. Images were edited to remove irrelevant lanes, missing lanes are represented as a white space.

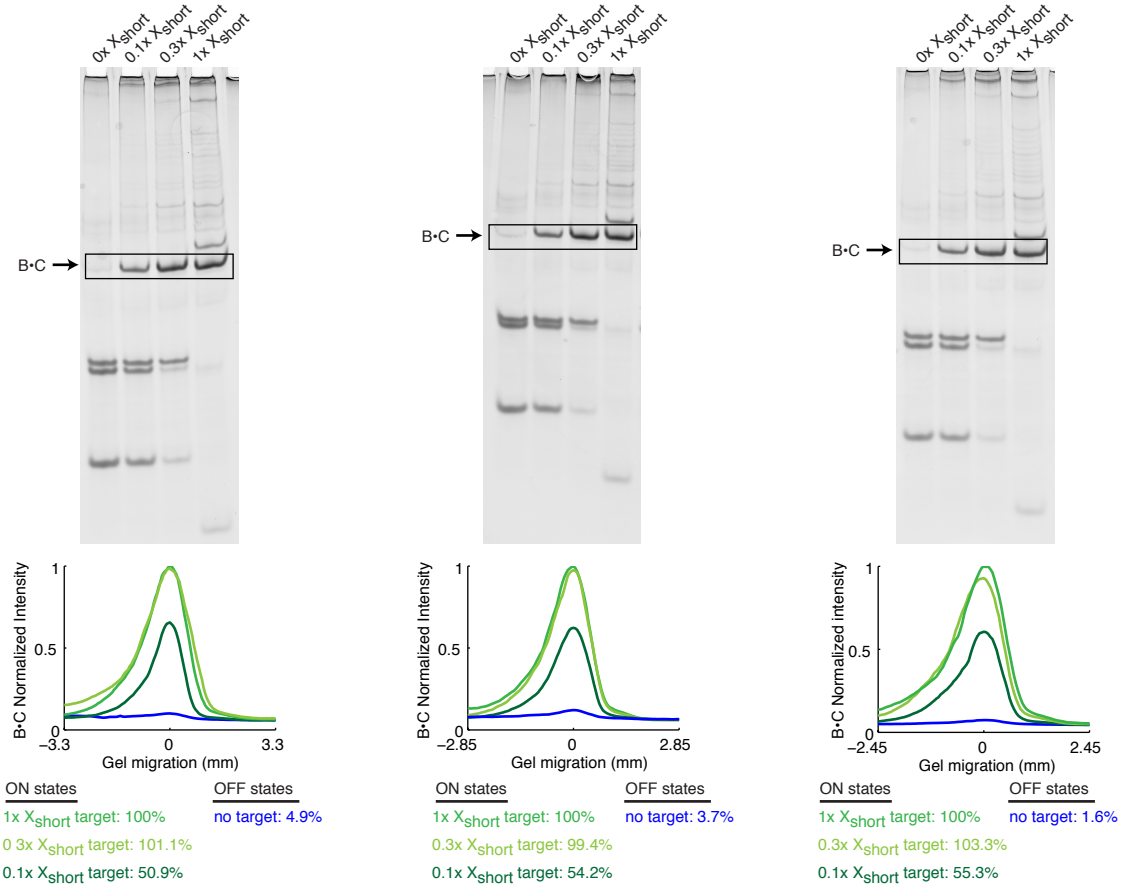

Figure A.3: **Quantification of catalytic B·C final product formation.** Sub-stoichiometric amounts of target X_{short} generate above stoichiometric amounts of B·C. Plots represent the normalized intensity of B·C in each lane across three separate experiments. Quantification was done as described in methods section of Chapter 2. A 0.5μM reaction master mix containing A, B and C was prepared. The master mix was then split into the separate reactions and targets were added for a 2-hour incubation at 37°C. Reactions were run on 20% polyacrylamide gel at 200 volts for 10.5 hours in 1×TBE followed by SYBRGold staining.

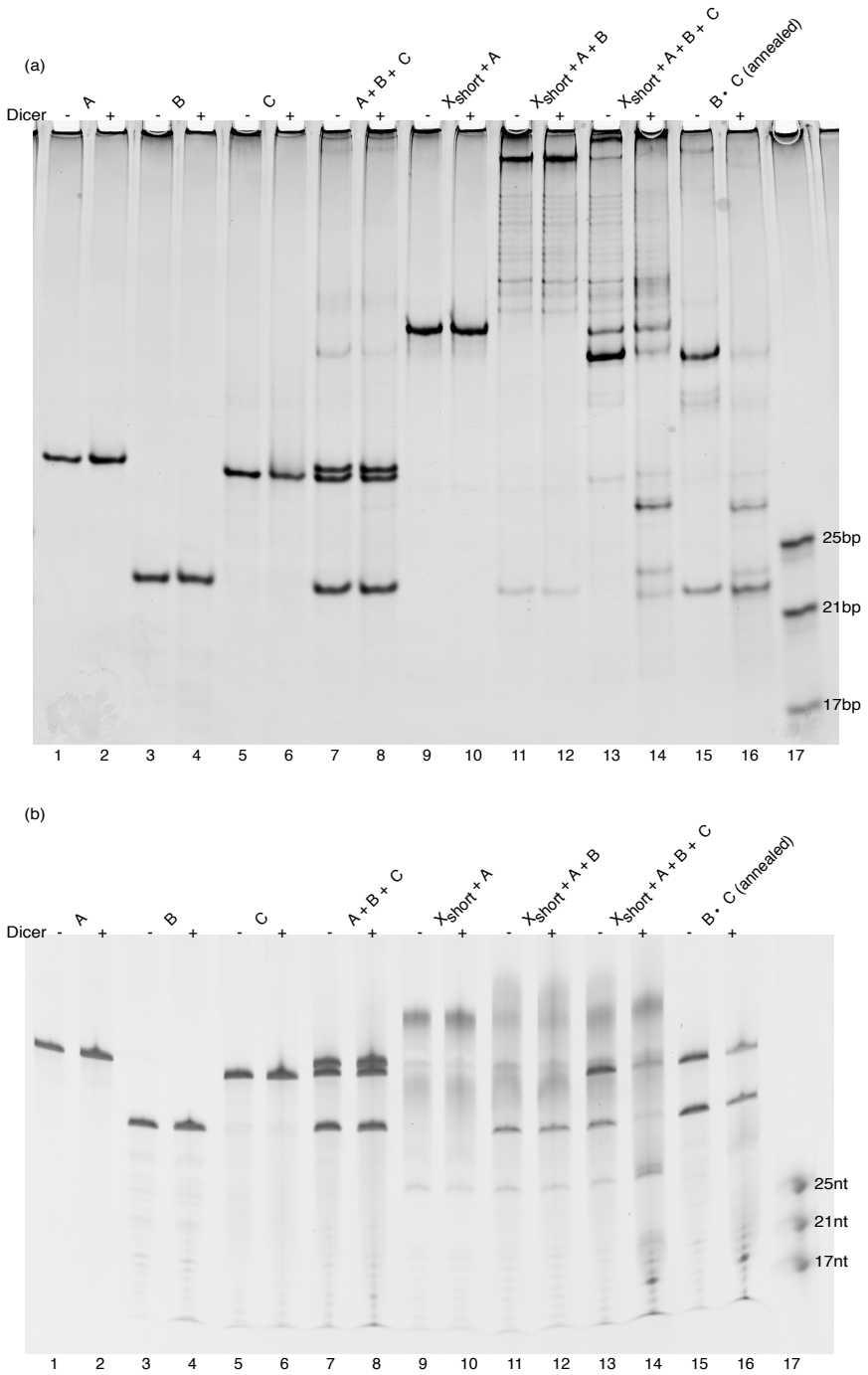

Figure A.4: **Dicer processing gel for the conditional catalytic DsiRNA formation mechanism.** 0.5μM reaction master mix was prepared for each duplicate. The master mix was then split into two separate reactions, with and without Dicer. Reactions were incubated for 2 hours at 37°C. (a) Native polyacrylamide gel electrophoresis. 4μl of each reaction were run on 20% polyacrylamide gel at 200 volts for 10.5 hours in 1×TBE followed by SYBRGold staining. (b) Denaturing polyacrylamide gel electrophoresis. 14% denaturing polycacylamide gel (with formamide) was pre-run at 15W 1×TBE for 1 hour. 3μl of each reaction were run at 15W for 1 hour in 1×TBE followed by SYBRGold staining.