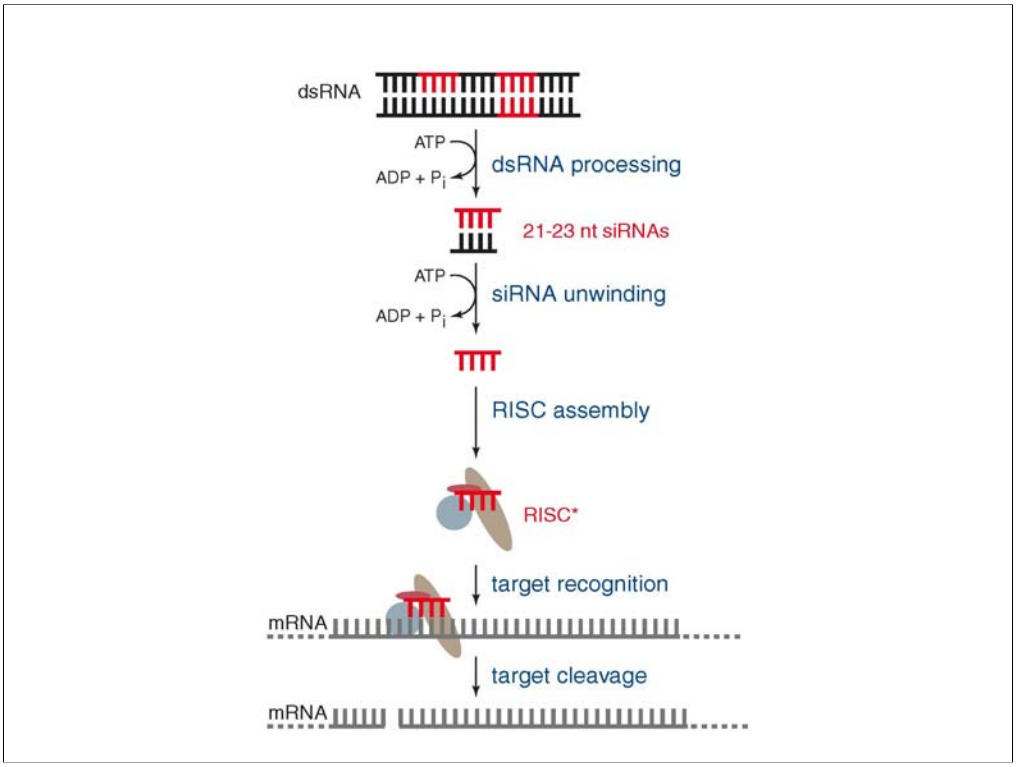
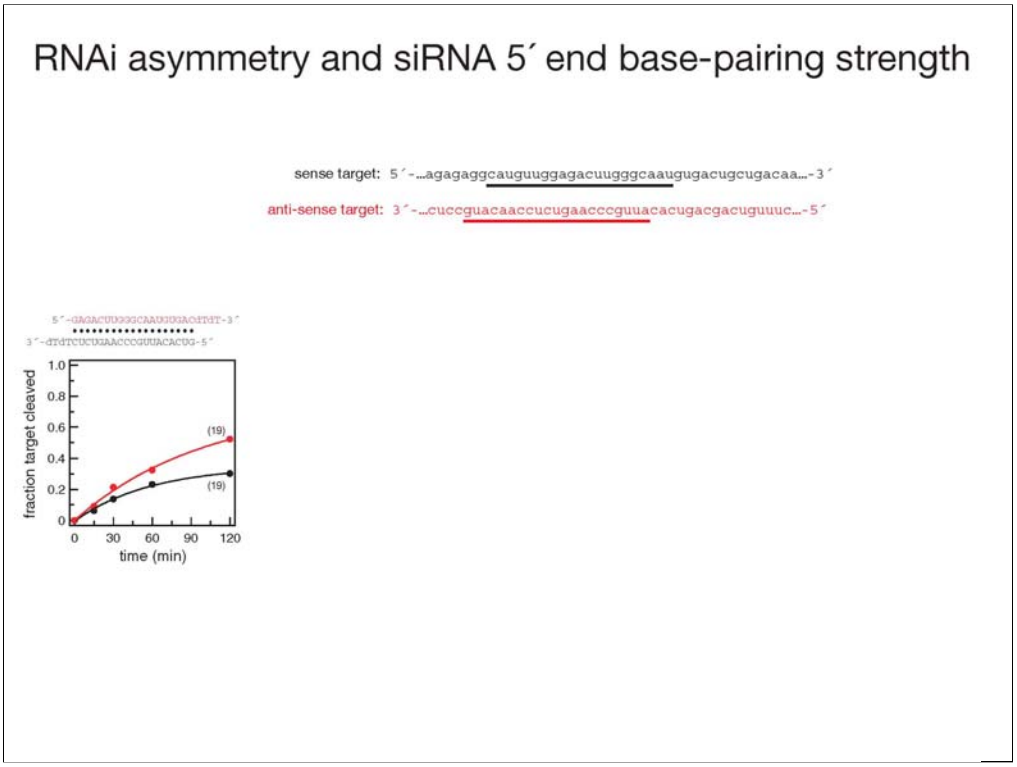
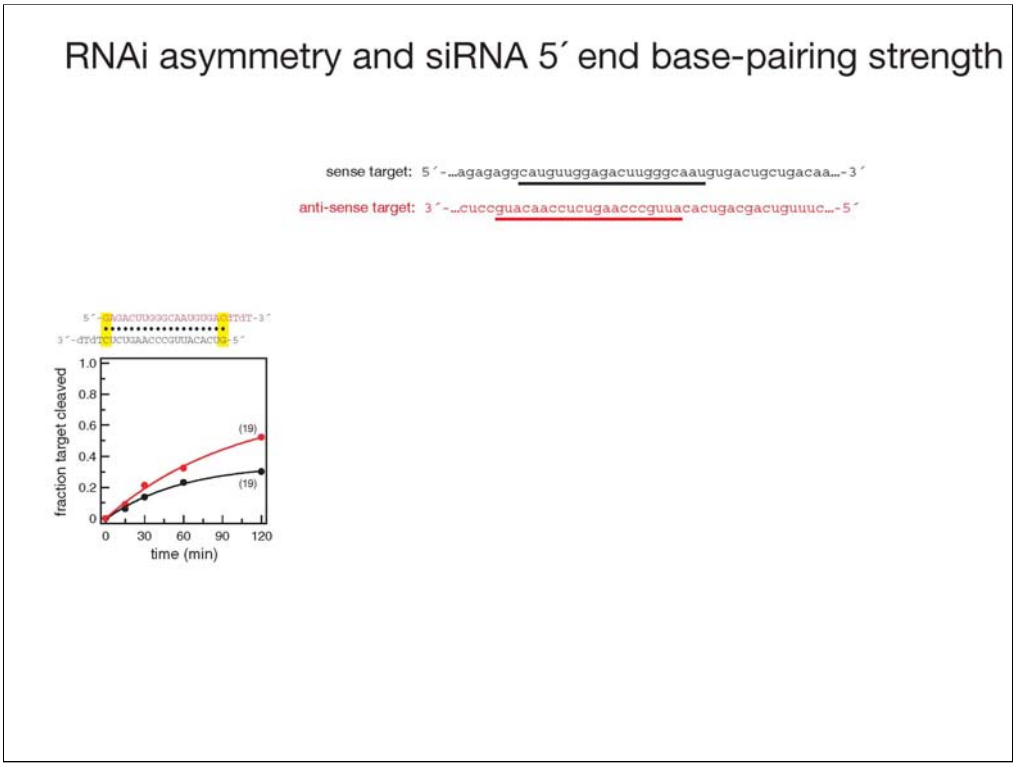


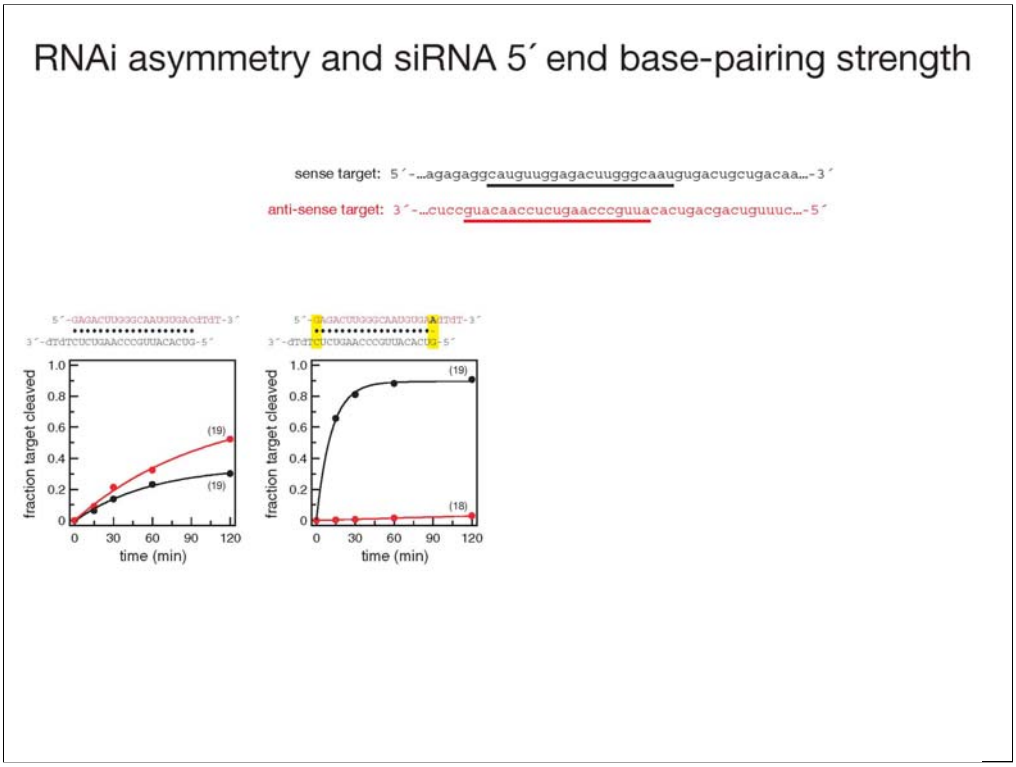
Slide 1. Biochemical experiments from several laboratories suggest a linear pathway for the RNAi pathway. The first step is the processing of long dsRNA into small interfering RNAs (siRNAs) by the ATP-dependent, RNase III enzyme Dicer. siRNAs must then be unwound in order for the strand which is to guide cleavage to produce the enzyme complex that mediates RNAi. This enzyme complex, the RNA-induced silencing complex (RISC), contains only one strand of the original double-stranded siRNA. The RISC can then use this strand as a guide, using RNA-RNA base pairing to bind a complementary target RNA, which it then cleaves. Cleavage involves breaking a single phosphodiester bond, and the cleaved pieces are subsequently degraded, reducing expression of the mRNA. A key question is, are both strands of an siRNA equally eligible for incorporation into the RISC?



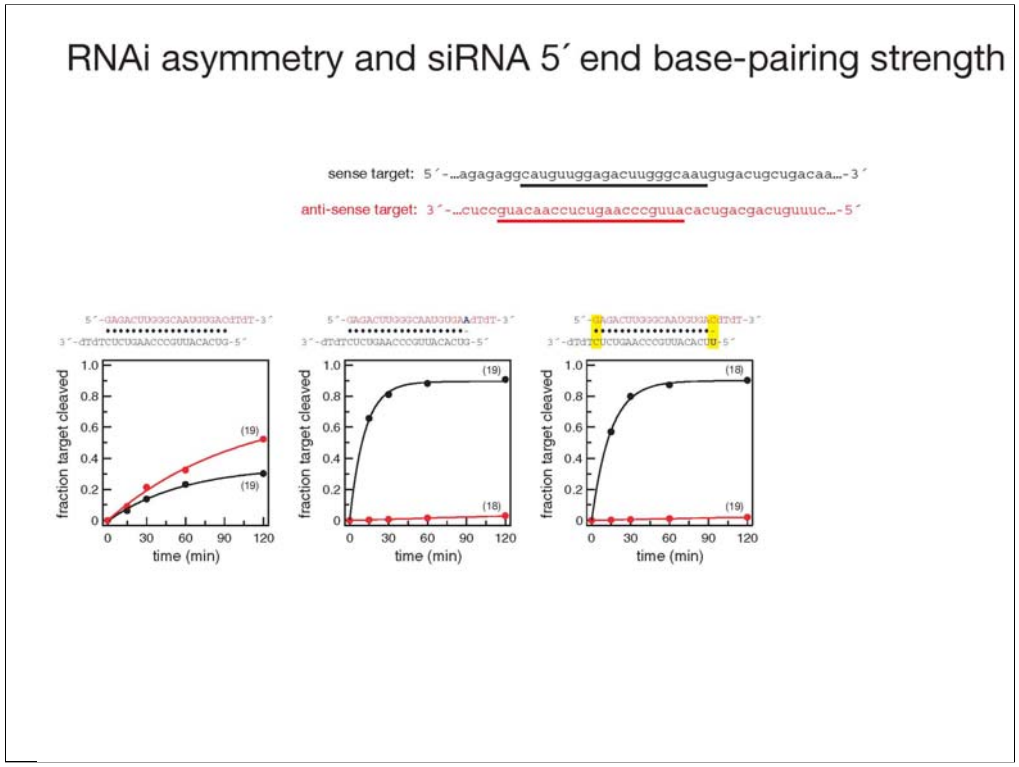
Slide 2. Here is an siRNA in which both strands can function in RNAi. The red strand can cleave the red target, and the black strand can cleave the black target. It's not a great siRNA (neither cleavage reaction proceeds with especial vigor in vitro or in vivo), but because both strands work to some extent, we'll call it a 'symmetric siRNA.' Are all siRNAs symmetric?



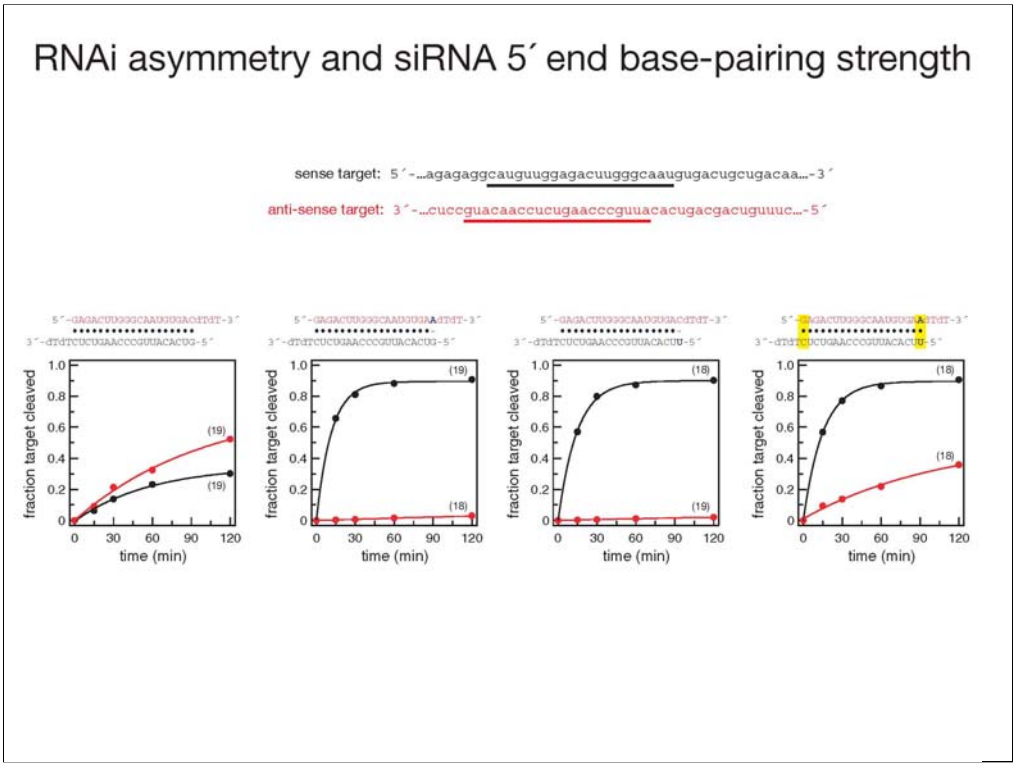
Slide 3. To answer this question, let's pose a hypothesis about siRNA symmetry. The ends of the siRNA, from the perspective of the 5´ ends of the two strands are symmetric structurally. That is, we can use a nearest neighbor analysis to estimate the local thermodynamic stability of the bases near each end. The 5´ end of the red strand is paired to the black strand with about the same strength as the 5´ end of the black strand is paired to the red.



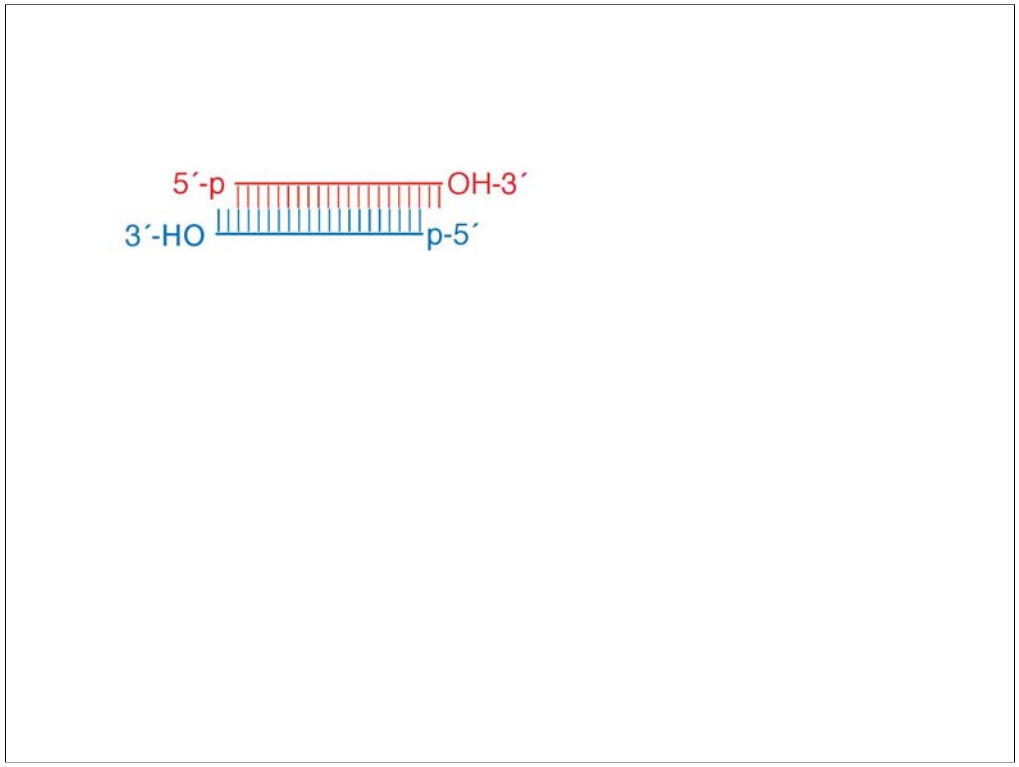
Slide 4. If we mutate one base on the red strand—base 19—to break a GC pair by creating a G:A mismatch, we dramatically alter the siRNA's function. Now it is functionally asymmetric. Only the black strand mediates RNAi.



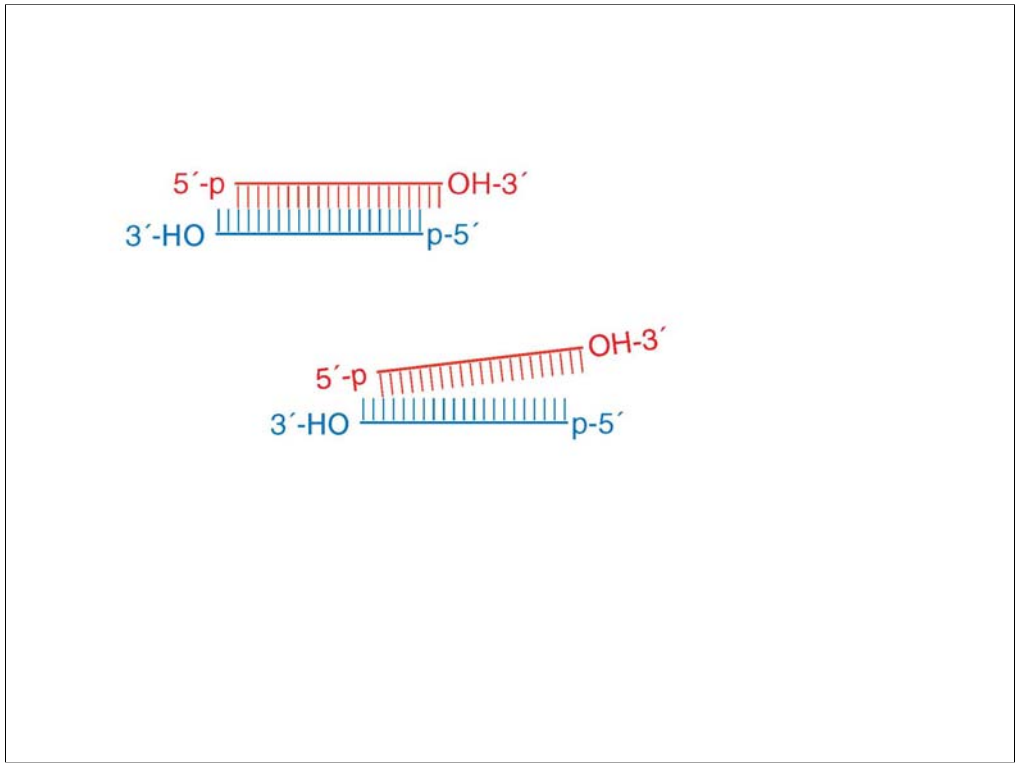
Slide 5. The precise nature of the mismatch is irrelevant. We can elicit the same effect by mutating the first base of the black strand. The mismatch *per se* is the cause of the functional asymmetry.



Slide 6. Pairing the mutant red strand with the mutant black creates an A:U base pair in place of the mismatch. The black strand is still highly functional, but now the red strand has recovered quite a bit of RNAi activity.



Slides 7-11. These and many more similar siRNA analyses led us to a simple model. The siRNA is unwound in a very special process in which the ends of the siRNA are interrogated for their thermodynamic stabilities. A comparison is made between the stabilities of the two ends, as well as an evaluation of the absolute strengths of pairing of each end. The end that is least stable, that is, the end that is easiest to unwind, is selected to be assembled into RISC. The process is remarkable in that the other strand seems to be destroyed. So we get the impression that for every cycle of RISC production, one strand is assembled into RISC and one strand is degraded. That is, the process is inherently asymmetric.



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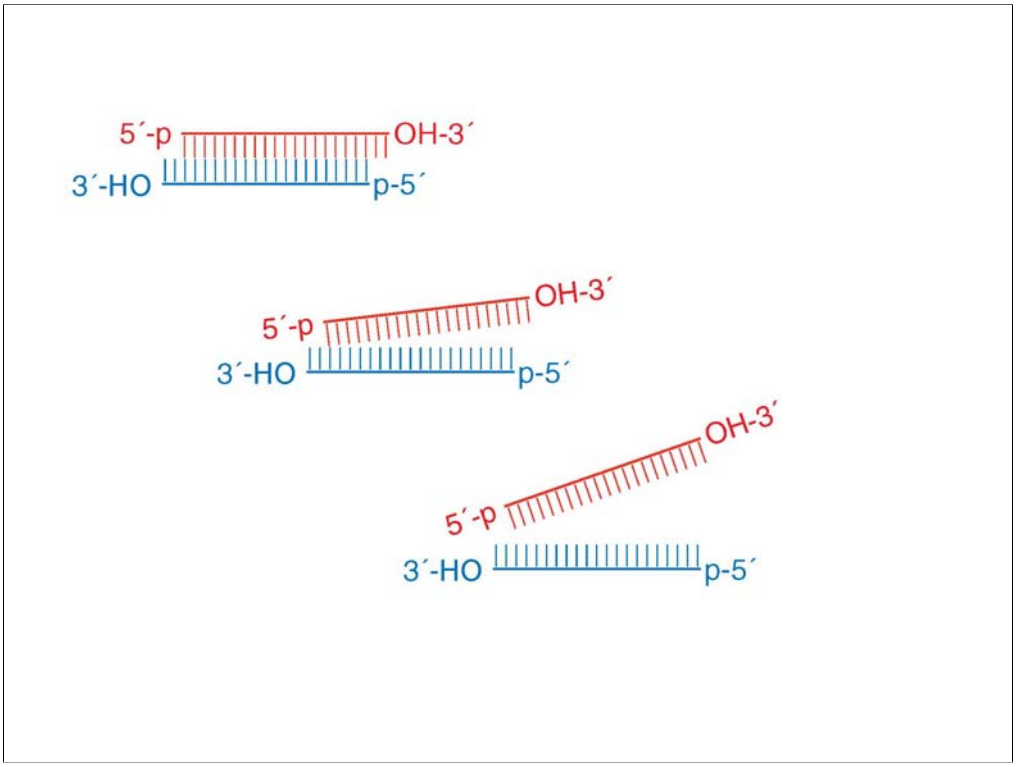

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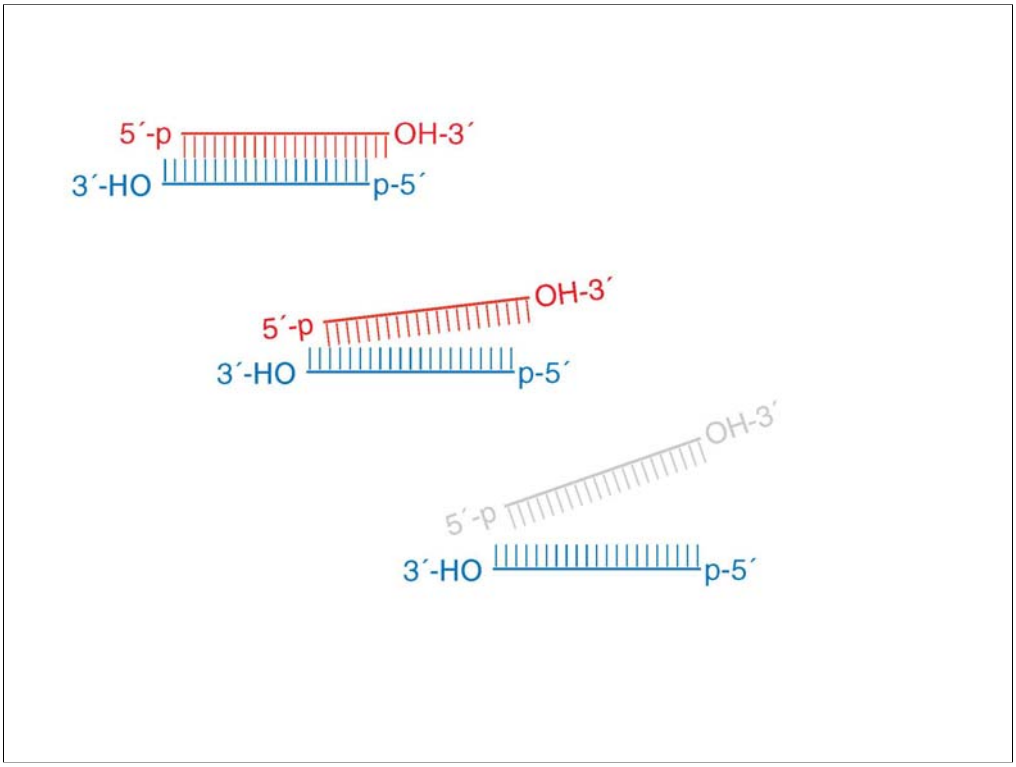

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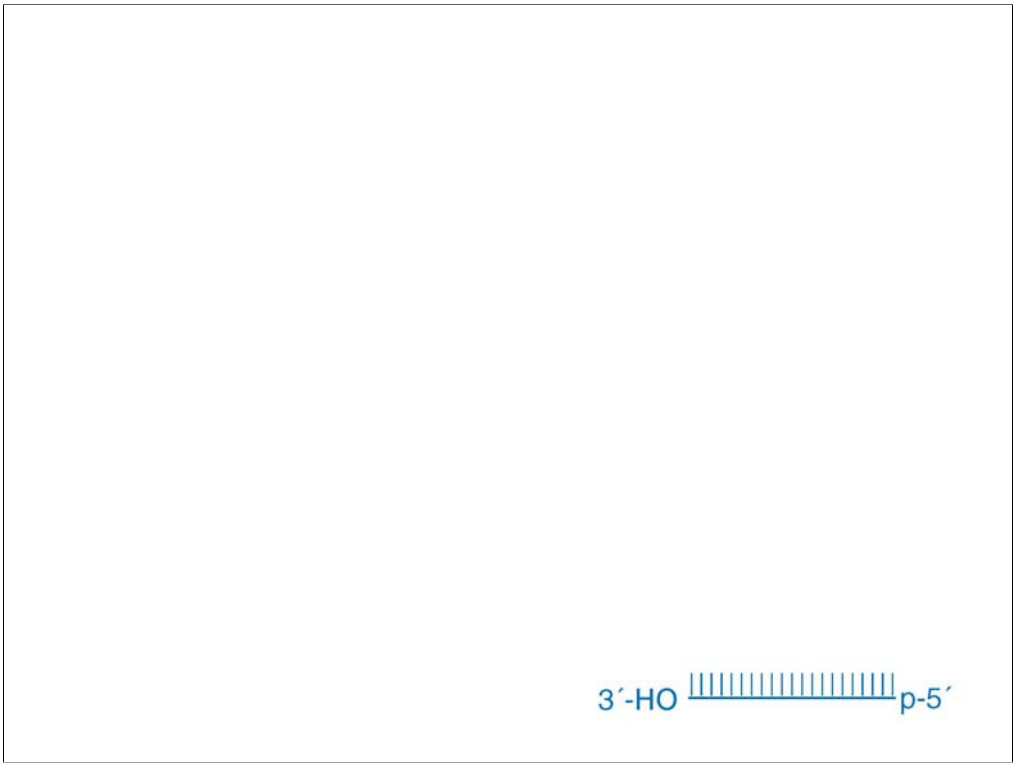

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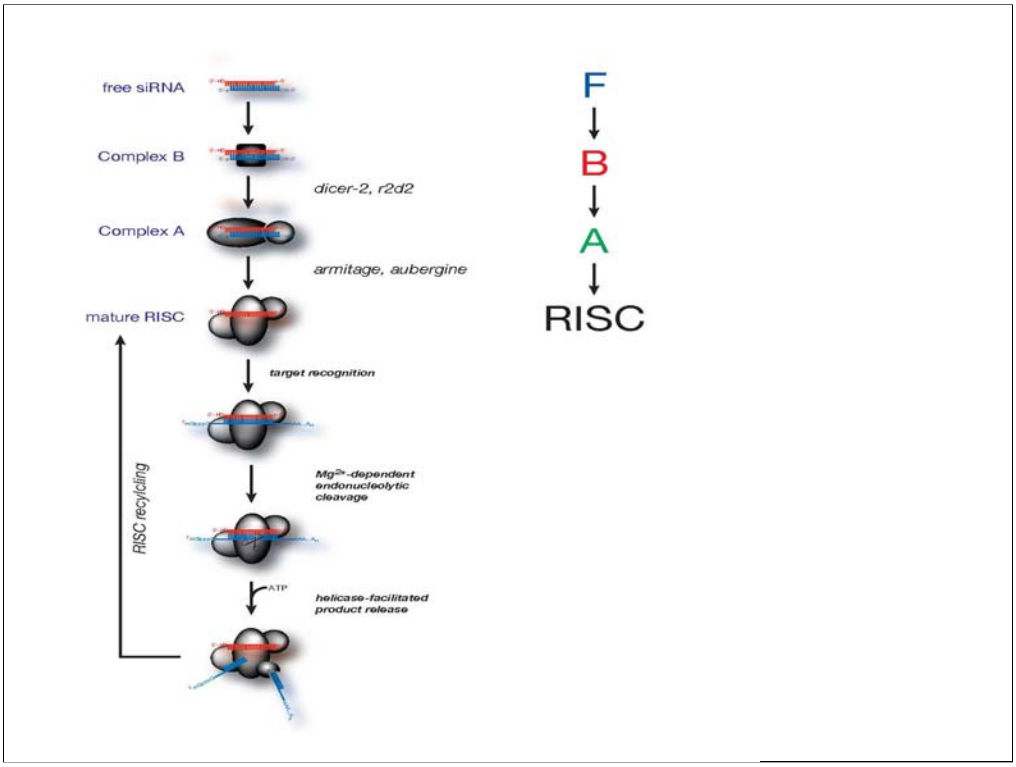
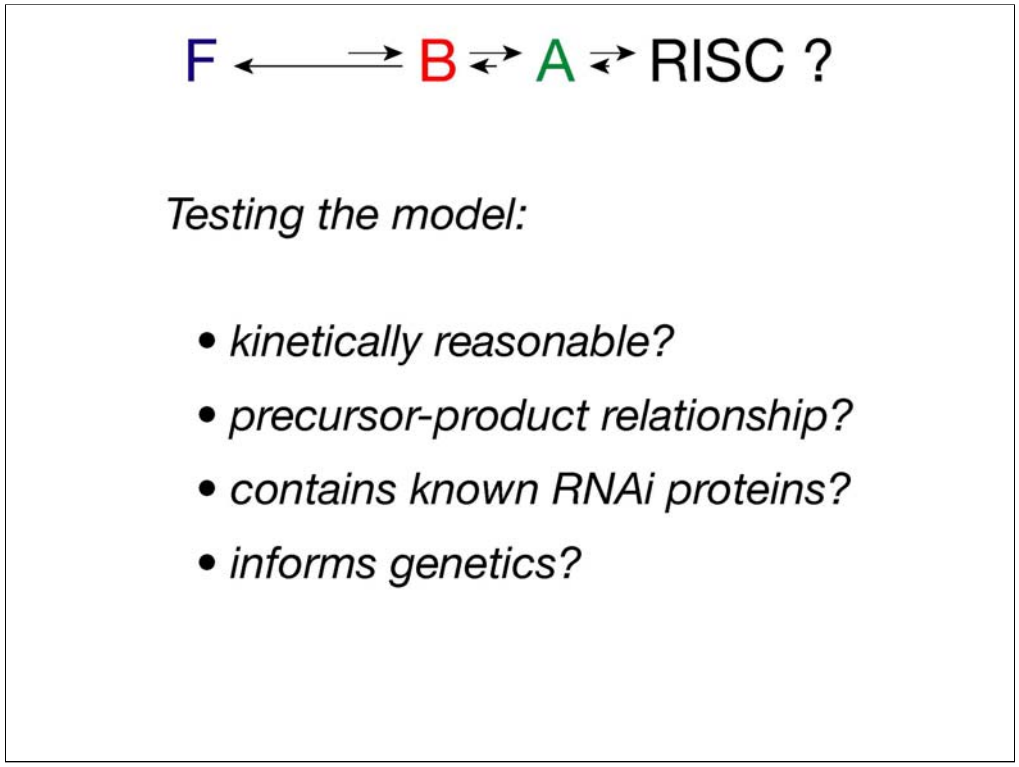
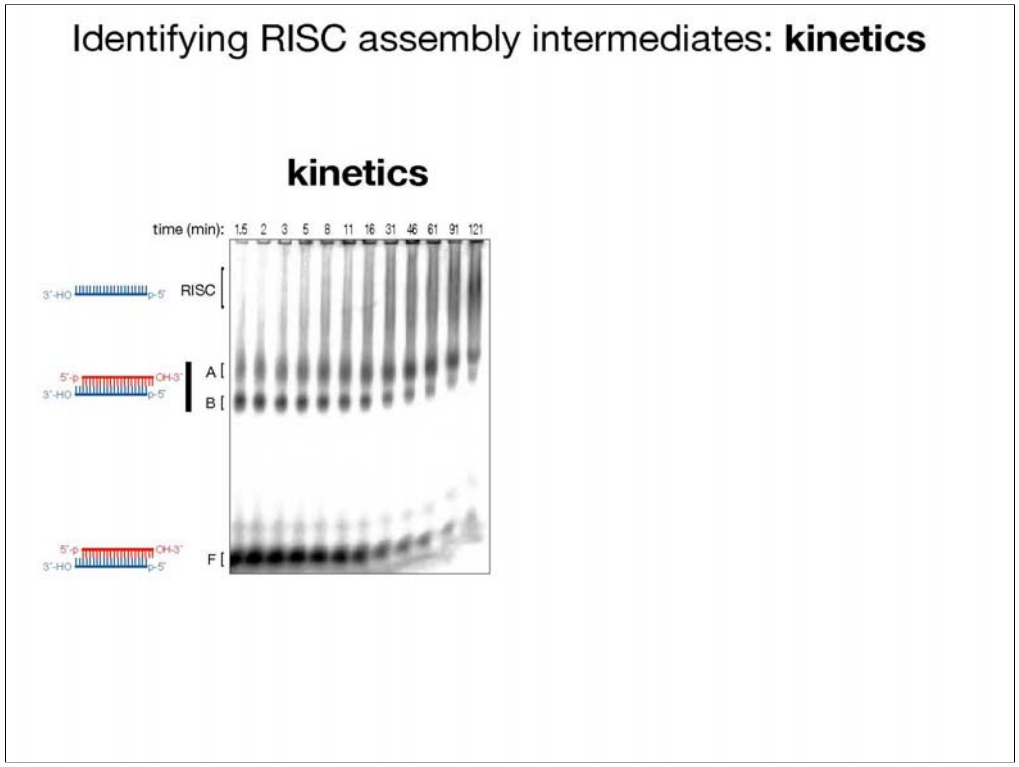


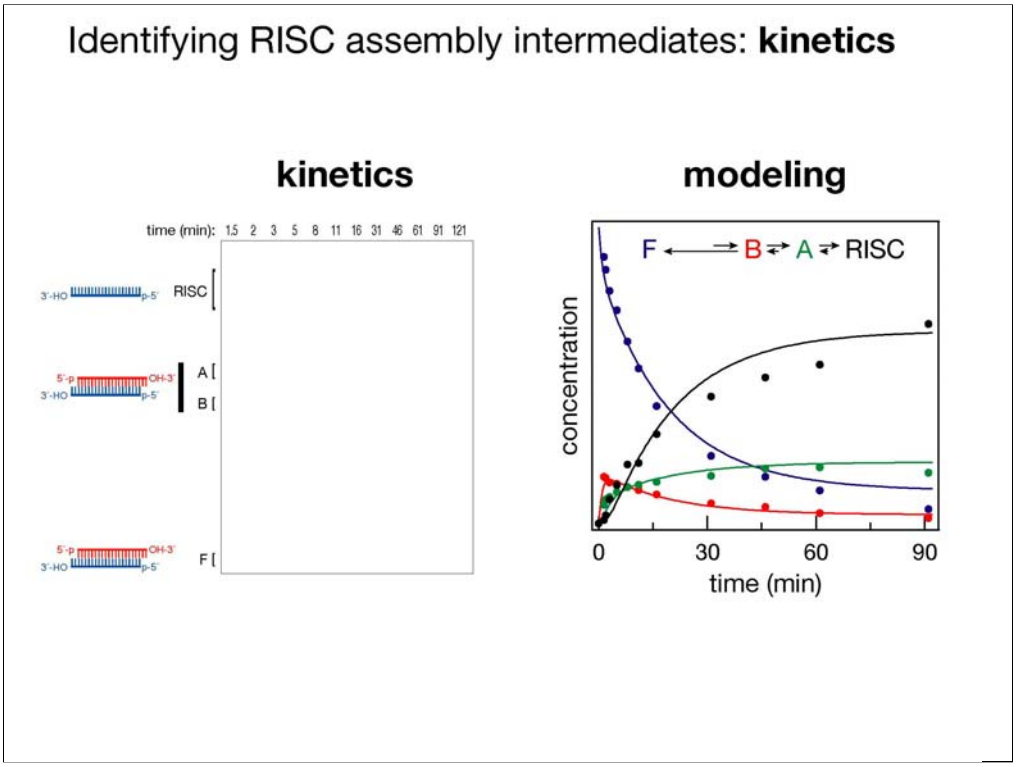
Slide 12. To begin to understand the basis of this, we have analyzed the pathway by which a RISC is built.



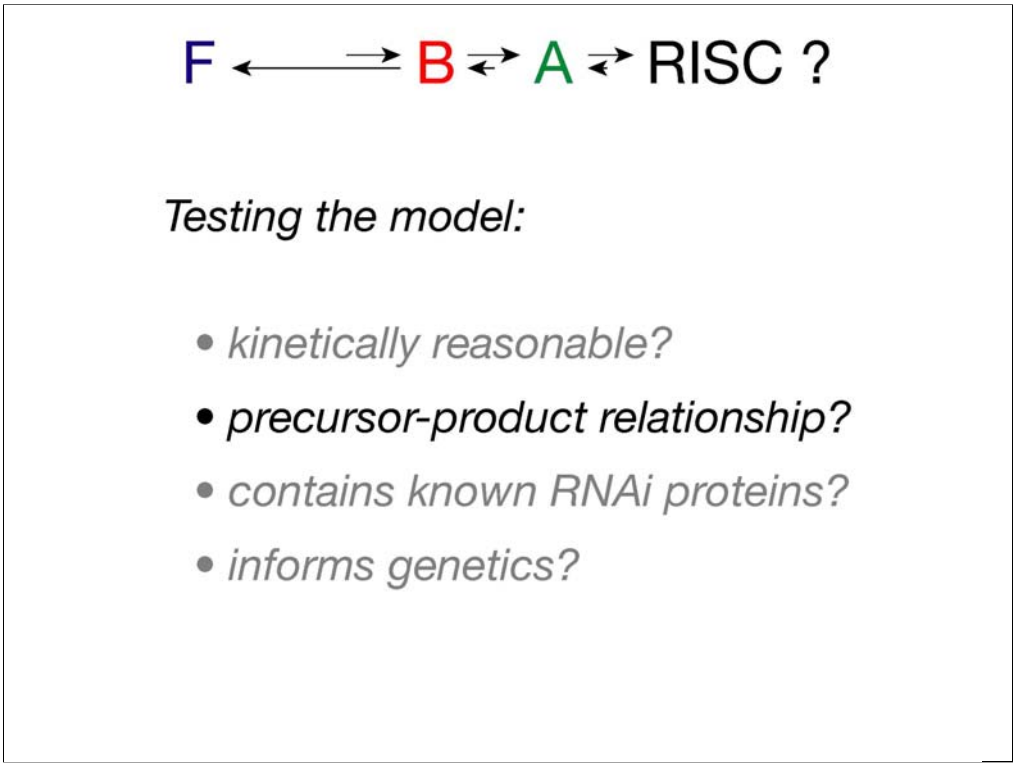
Slides 13. Analysis of RISC assembly in vitro in lysates from *Drosophila* embryos suggests that RISC assemble proceeds through two intermediate complexes, Complex B and Complex A. Free siRNA binds proteins to form B, then B is converted to A. Finally, A is converted to RISC. siRNA unwinding occurs between A and RISC. Is this idea right? We posed three tests. First, is the model kinetically reasonable? Second, is there a precursor-product relationship among the complexes? Third, do any of the intermediate complexes contain proteins known to function in loading siRNA into RISC? And finally, does this model help explain previous genetic observations?

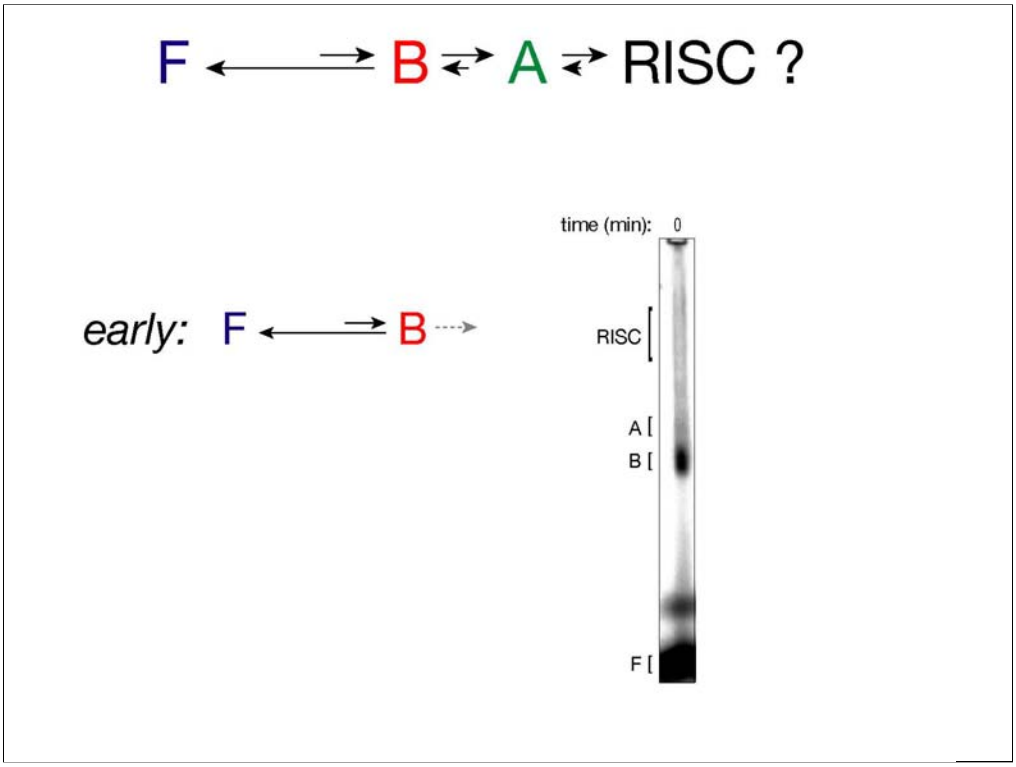


Slide 14. We performed a time course of complex assembly with 32P-siRNA, analyzing each time point by native gel electrophoresis…

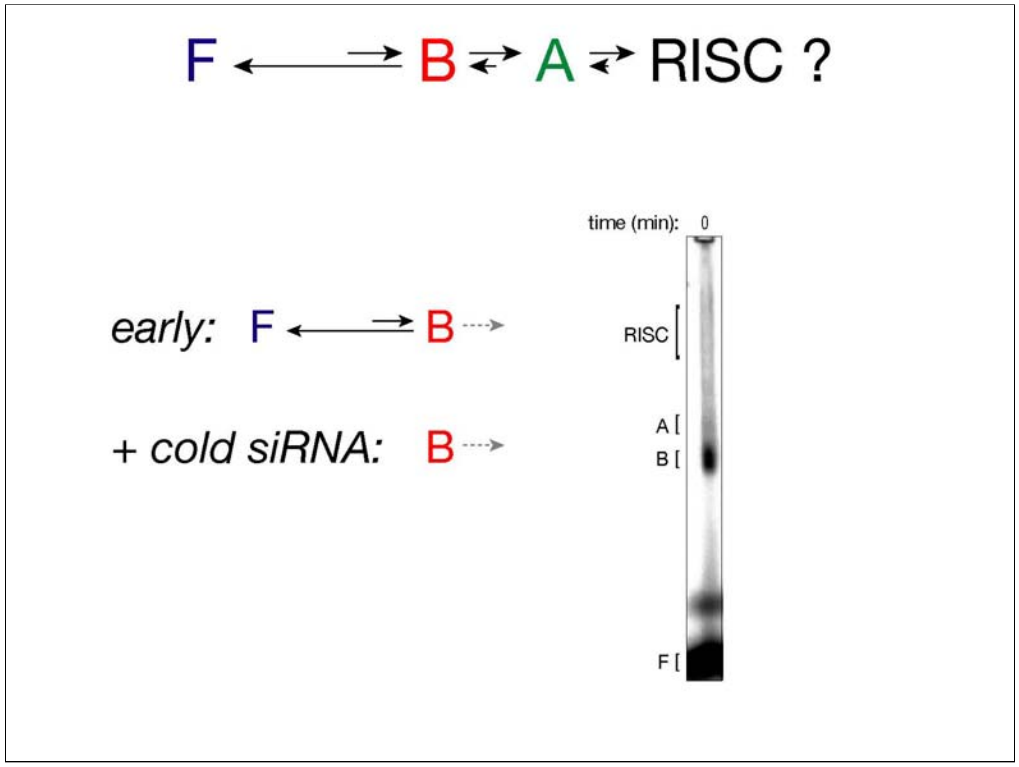


Slide 15. …then we modeled the data using a kinetic modeling computer program. The best fit to the data is the model shown at the top of the slide, in which the length of the arrows indicates the relative rate constants proposed by the kinetic model. It's a remarkably good fit! So the model is reasonable. But is it actually true?

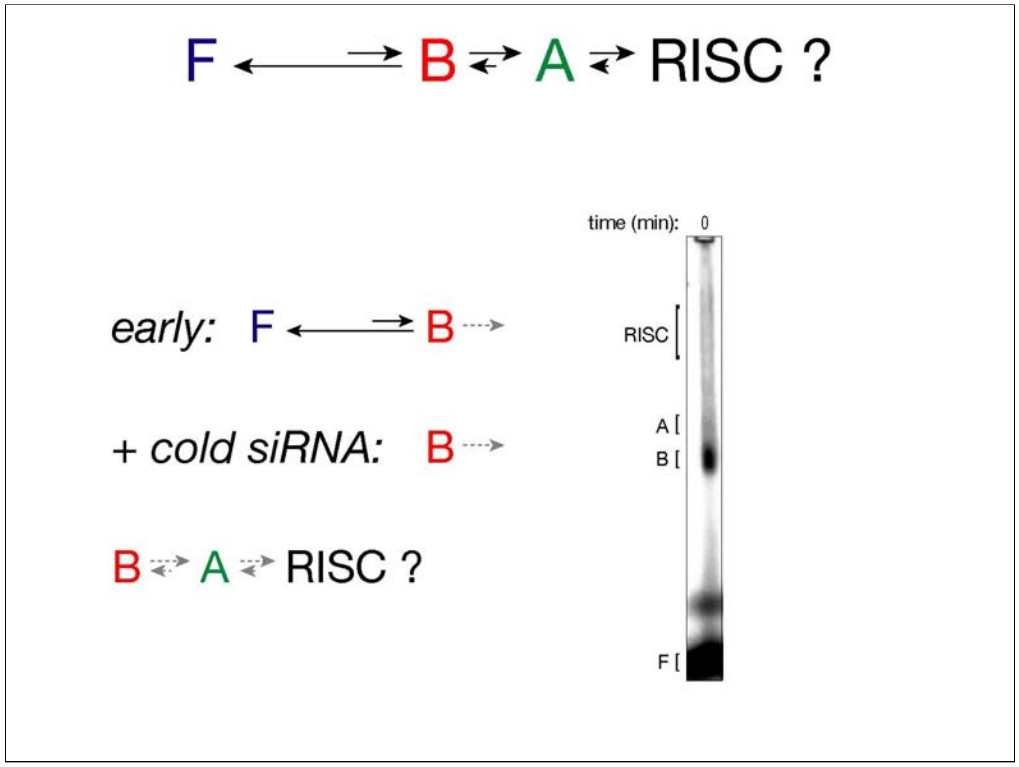

F ⟷ B ⇄ A ⇄ RISC ?
Testing the model:
• kinetically reasonable?
• precursor-product relationship?
• contains known RNAi proteins?
• informs genetics?



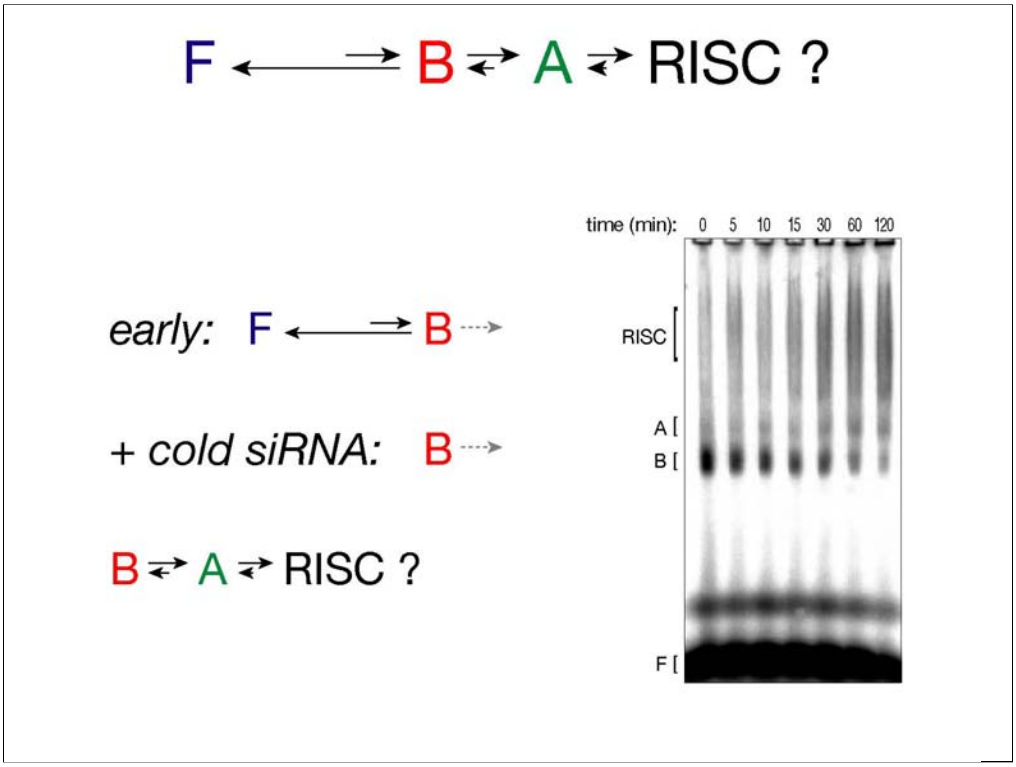
Slide 17. To test the precursor-product relationship, we assembled Complex B by incubating the siRNA for 5 min in the embryo lysate…



Slide 18. …then we added a large amount of unlabeled siRNA to prevent more Complex B from forming and to prevent any Complex B that fell apart from re-entering the pathway.



Slide 19-20. As I hope you can see, the Complex B disappears and is chased into Complexes A and RISC. So it looks like B is a precursor to the larger two complexes.



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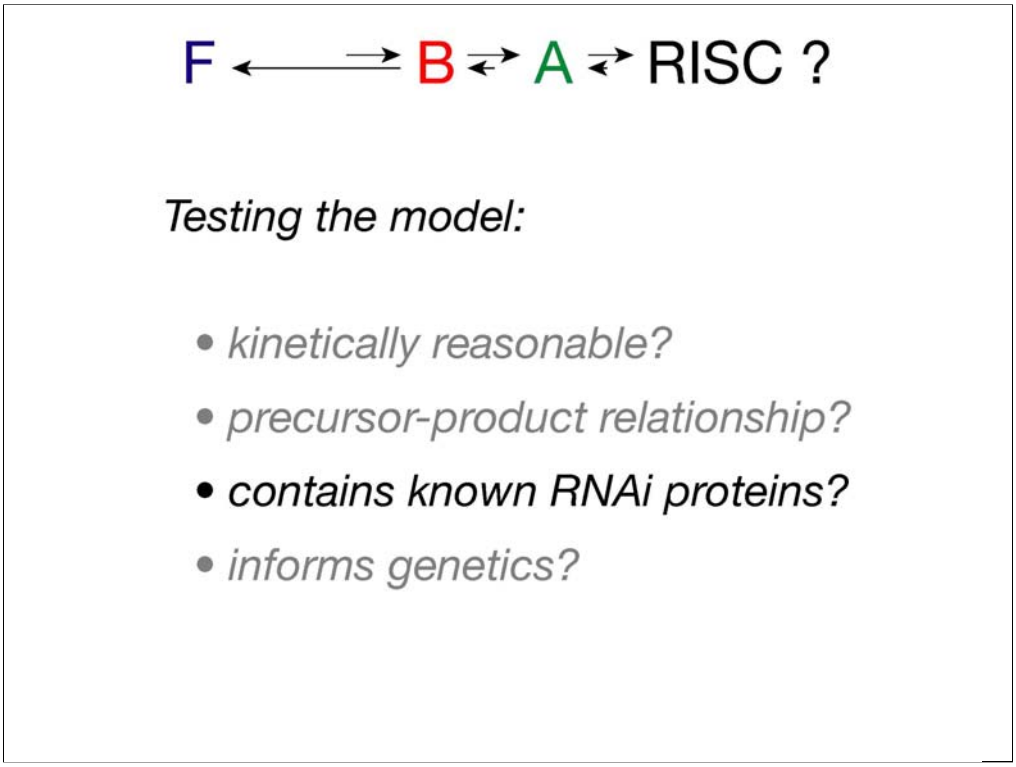
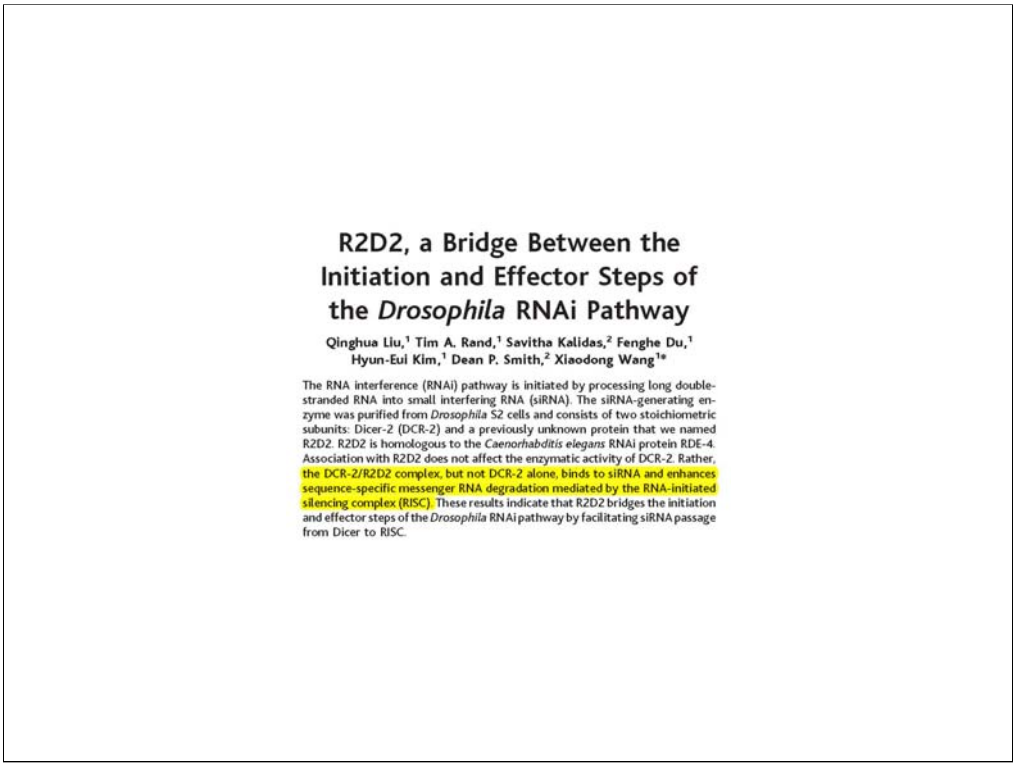


Slide 21. Do any of these complexes contain proteins known to be important for loading RISC with siRNA?

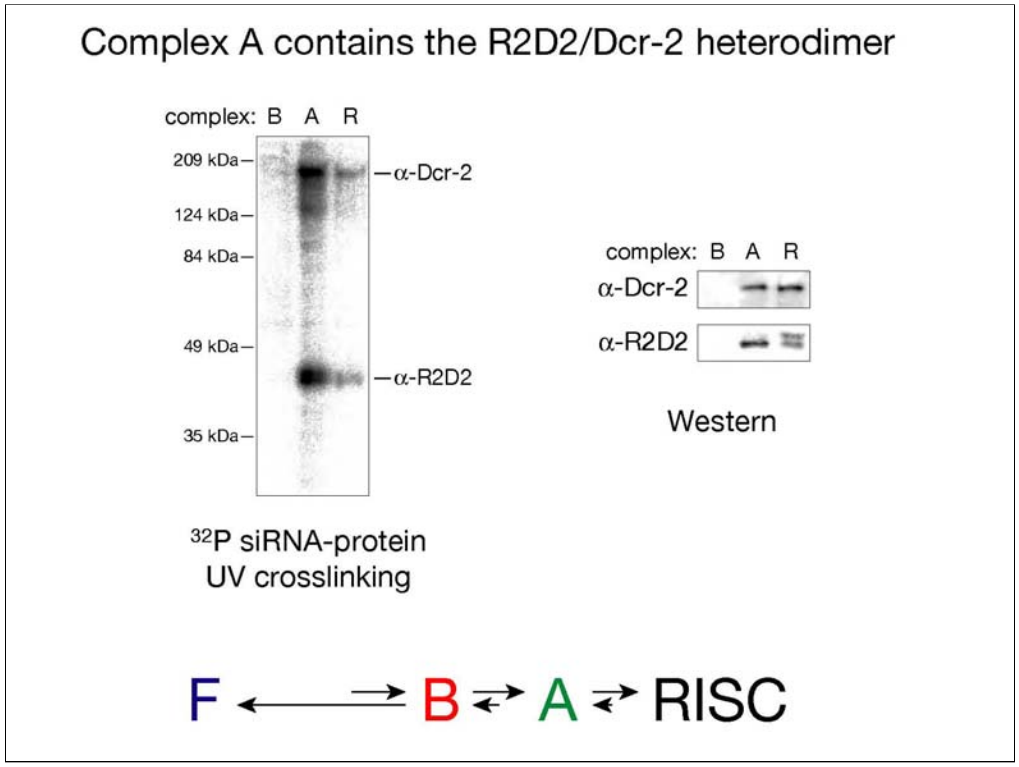


R2D2, a Bridge Between the Initiation and Effector Steps of the *Drosophila* RNAi Pathway

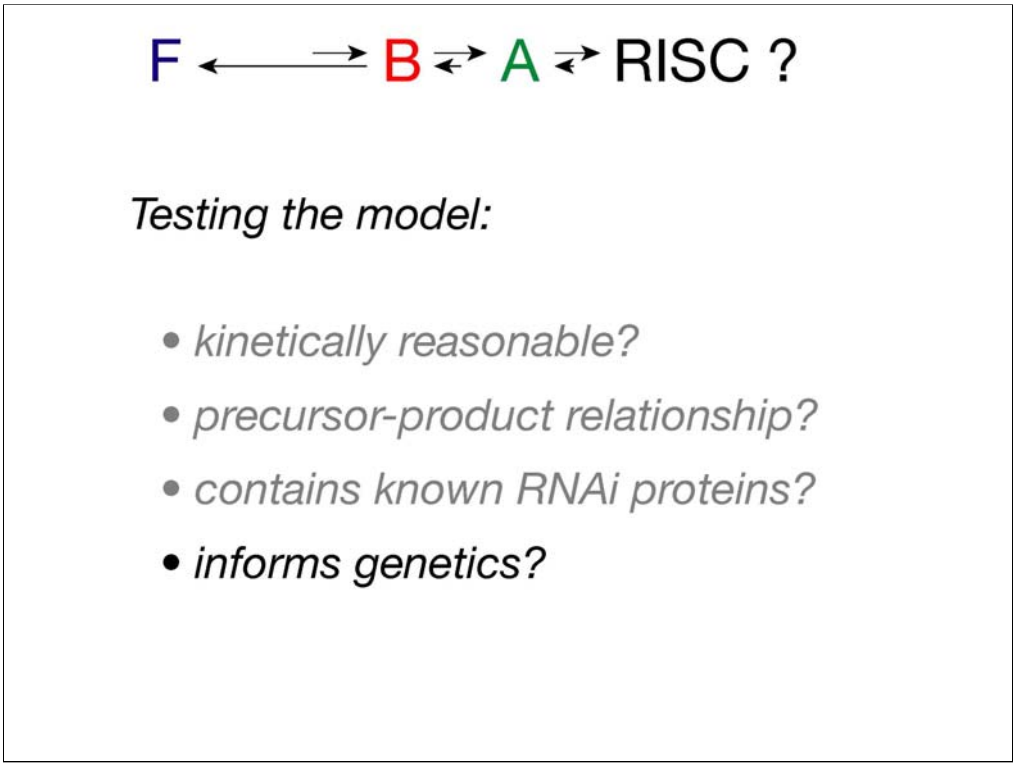
Qinghua Liu,[1] Tim A. Rand,[1] Savitha Kalidas,[2] Fenghe Du,[1] Hyun-Eui Kim,[1] Dean P. Smith,[2] Xiaodong Wang[1]*

The RNA interference (RNAi) pathway is initiated by processing long double-stranded RNA into small interfering RNA (siRNA). The siRNA-generating enzyme was purified from *Drosophila* S2 cells and consists of two stoichiometric subunits: Dicer-2 (DCR-2) and a previously unknown protein that we named R2D2. R2D2 is homologous to the *Caenorhabditis elegans* RNAi protein RDE-4. Association with R2D2 does not affect the enzymatic activity of DCR-2. Rather, the DCR-2/R2D2 complex, but not DCR-2 alone, binds to siRNA and enhances sequence-specific messenger RNA degradation mediated by the RNA-initiated silencing complex (RISC). These results indicate that R2D2 bridges the initiation and effector steps of the *Drosophila* RNAi pathway by facilitating siRNA passage from Dicer to RISC.

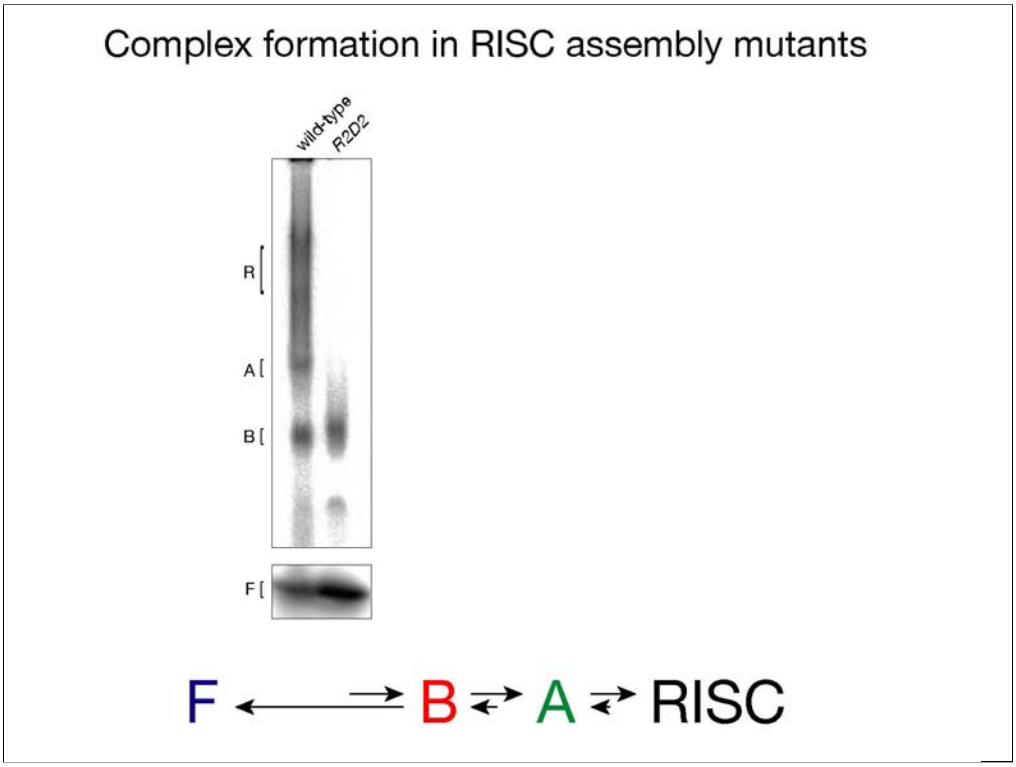
Slide 22. Well, we really only know two such proteins, R2D2 and Dicer-2, which Qinghua Liu and colleagues showed last year help transfer siRNA from the Dicing reaction mediated by Dicer to the RISC.



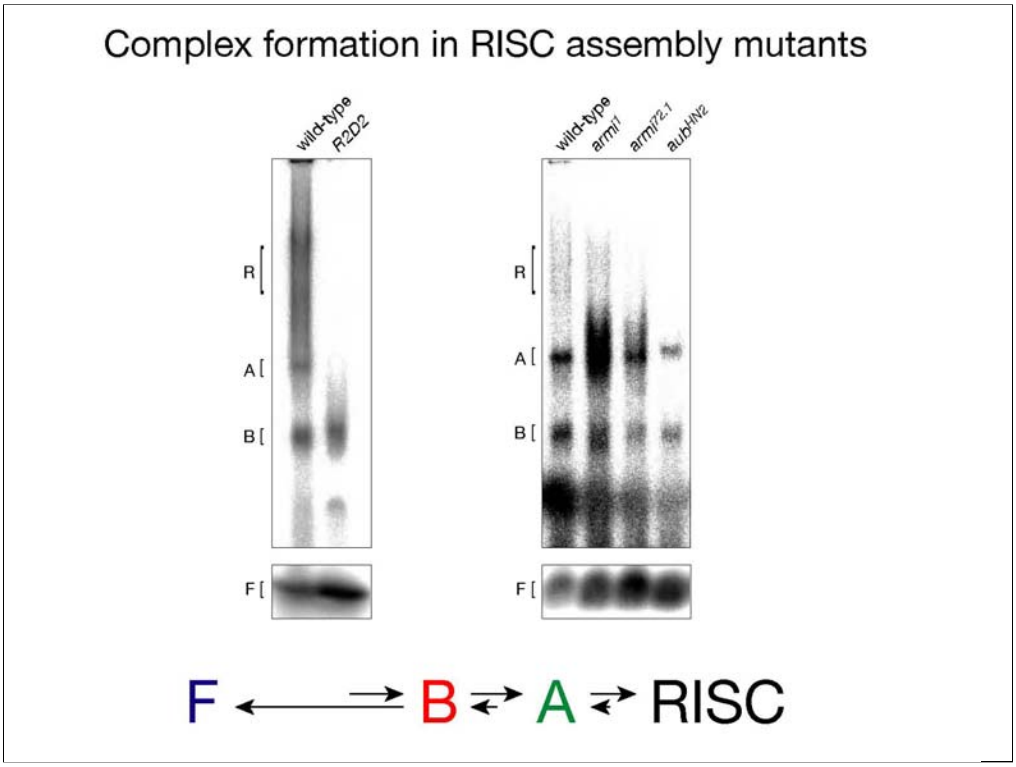
Slide 23. We cut each complex out the gel and looked at its contents by Western blotting (right panels). Complexes A and RISC both contain R2D2 and Dicer-2. If we use a radioactive, photocrosslinkable siRNA designed to crosslink to R2D2 and Dicer-2, and we crosslink the siRNA to the bound proteins using UV light, we also find R2D2 and Dicer-2 in Complex A and B. Similar results have been reported by Erik Sontheimer and colleagues.



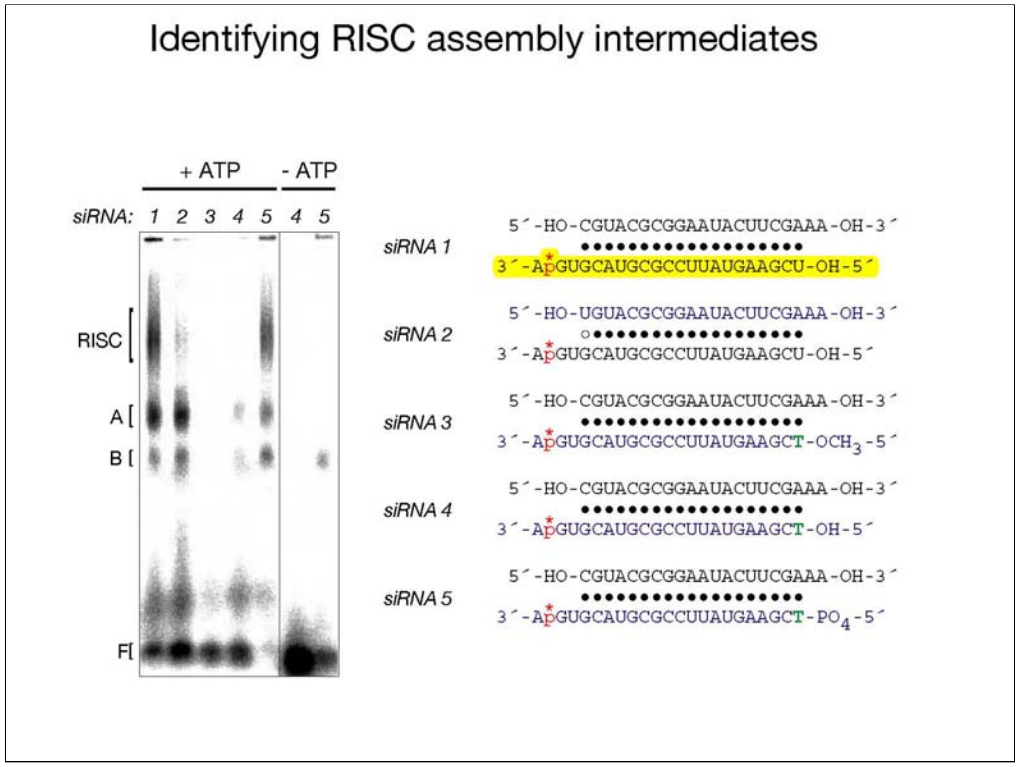
Slide 24. Finally, does the proposed pathway help us understand the genetics?



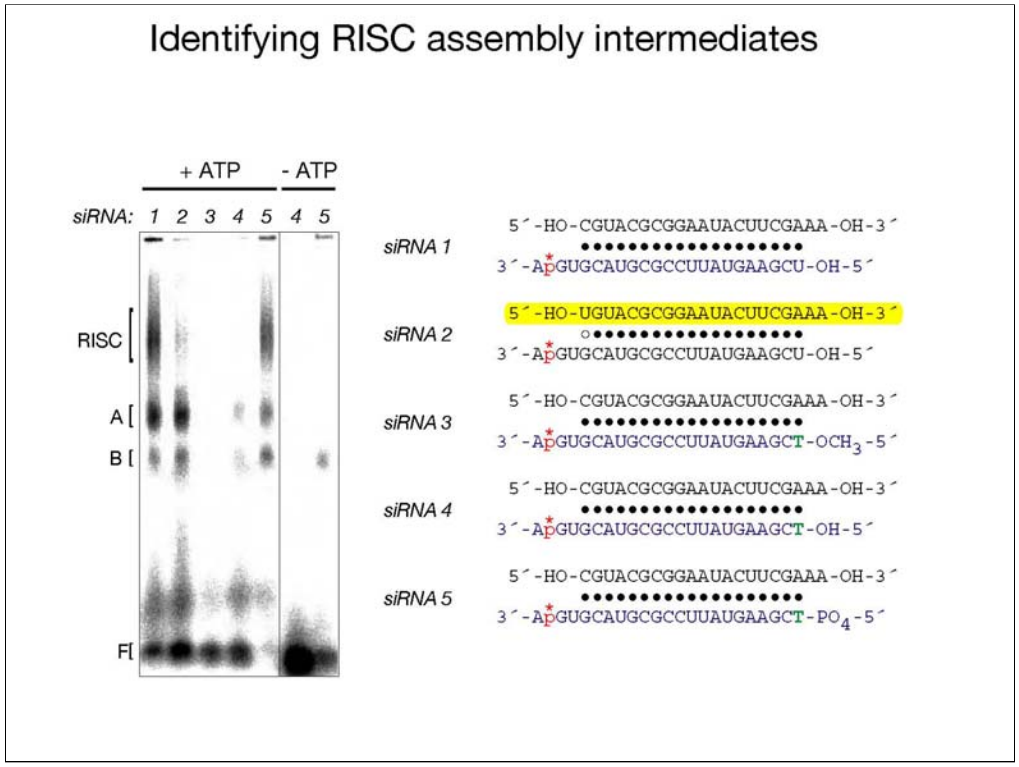
Slide 25. Lysates prepared from mutant flies lacking R2D2 don't form Complex A. They do make Complex B. R2D2 mutant flies can't do RNAi.



Slide 26. Neither can flies mutant for the genes *armitage* or *aubergine*. Lysates from these mutants make Complexes B and A, but they can't convert Complex A into RISC. So the model helps us understand the genetics indeed.



Slide 27. So let's return to the functionally asymmetric siRNAs. siRNA 1, shown here, loads the bottom, 32P-radiolabeled strand into RISC. All three complexes, B, A, and RISC, contain the radiolabeled strand.



Slide 28. If we radiolabel the bottom strand, but alter the thermodynamic stability of the 5´ ends of the two strands (siRNA 2), we invert the asymmetry. Now, the unlabeled strand functions and the labeled strand doesn't. Complexes B and A are just as 'hot' as before—so they must contain double-stranded siRNA. But little RISC is detected, because RISC contains largely the non-radioactive strand. So the model of the assembly of siRNA into RISC has begun to explain the molecular details of the process. But clearly much remains to be done.

Slide 29. Finally, I'd like to thank the wonderful students, post-docs and technicians who performed this work.