Reflections on the inhibition of RNAi by cell death signaling

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Mutations and most transgenes that induce ectopic cell death in *Drosophila* will produce an inhibitory effect on RNA interference (RNAi) in adjacent cells. When extensive cell death is sporadically induced using a heat shock promoted-head involution defective (*hs-hid*) transgene, molecular attributes of this inhibition can be studied. For a Green Fluorescent Protein (GFP) RNAi construct, cell death causes a greater accumulation of the mature mRNA and the double stranded RNA with an accompanying reduction in the homologous siRNAs. Endogenous transposable element expression is increased and there is an overall reduction in their corresponding siRNAs. The implications of this finding for the conduct of RNAi and potential reasons for its existence are discussed.

In a recent paper, we described the phenomenon that mutations or transgene constructs that cause ectopic cell death will inhibit RNA interference (RNAi).¹ This situation was first realized by combining an RNAi construct for the *white* eye color gene with the *Bar* eye mutation. A strip of red color was present in the eye behind the anterior region of the *Bar* eye. The *Bar* gene is known to involve a defect in the movement of the morphogenetic furrow in the eye disc during differentiation and thus the anterior portion of the eye is missing due to the cell death that ensues.² Other mutations that have a regional cell death produced a similar phenotype. Mutations that change the eye morphology by means other than cell death failed to exhibit such effects. Mosaic analysis using FLP-FRT somatic recombination^{3,4} illustrated that reversal of RNAi can occur

in normal cells adjacent to those sectors with a history of cell death. These results suggested that the cell death produced a signal to neighboring cells that was inhibitory directly or indirectly to RNAi.

By using transgene constructs that induce ectopic cell death in the eye,⁵ a similar reversal of RNAi was observed. Reversing the action of the cell death transgenes also reversed the inhibition of RNAi.¹ The effects could be extended to RNAi for Green Fluorescent Protein (GFP) illustrating the generality of the effects. Nevertheless, in the GFP RNAi strain we found no reason to suspect that programmed cell death as part of normal development had any impact on RNAi.

One construct that is regularly used in fly genetics has a heat shock promoter driving the *head involution defective* gene (*hs-hid*),⁶ was found to cause cell death sporadically at normal temperature due to the low level constitutive expression of the heat shock promoter.¹ This construct would cause specks of red in the *white* eye color RNAi flies and a generalized return of the expression of GFP in larvae and flies that otherwise had RNAi operating on GFP. This stock permitted an analysis on the molecular level. As expected, this introduction of *hs-hid* into the GFP RNAi strain caused an increase in GFP mRNA and there was also a greater accumulation of the double stranded GFP RNA. The amount of GFP siRNAs was diminished. These results suggest that the block occurs at the step of conversion of double stranded RNA to siRNA although no change in the amount of DICER-2 protein was detected suggesting that either a modification of the double stranded RNA or of DICER-2 is the target for the inhibition.

Keywords: RNAi, cell death, signaling, transposons, siRNA

Submitted: 06/22/11

Accepted: 07/18/11

<http://dx.doi.org/10.4161/fly.5.4.17370>

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Extra View to: Xie W, Liang C, Birchler JA. Inhibition of RNA interference and modulation of transposable element expression by cell death in *Drosophila*. *Genetics* 2011; 188:823-34; PMID:21596898; <http://dx.doi.org/10.1534/genetics.111.128470>

The presumed generality of the effect was confirmed by examining the expression of representative retrotransposons that are controlled in part by the endogenous RNAi machinery. The families of retrotransposons affected in the *hs-hid* stock were quite similar to those that change in tissue culture cells in which the RNAi machinery has been compromised.⁷⁻¹⁰ In addition, an endogenous gene, *mus308*, which is controlled in part by the endogenous RNAi machinery,^{7,11} was also modulated in its expression. Deep sequencing of the small RNA population found a reduction in the total population of siRNAs and potentially a slight impact on the piRNAs.

Why?

The immediate question that comes to mind with regard to this phenomenon, is why does it exist? At this point, the answer remains unknown. However, as with most experimental results, it is likely a reflection of a natural process. One rationale for the existence of RNAi is as a defense against viruses and transposons that contain a double stranded RNA intermediate in their life cycle.¹² Certainly, many viruses have evolved a virulence protein that counteracts the effects of RNAi, so the viruses are in an arms race with the host with regard to RNAi. One might suggest that RNAi is the first line of defense against viruses but that this line of defense regularly fails (i.e., viral infection). With viral infection and cell death, the dying cell might send a signal to neighboring cells to induce another mechanism that attacks double stranded RNA. Such a mechanism would need the first line of defense to fail regularly in order to evolve but the fact of viral infection provides this evidence. In mammals, the introduction of double stranded RNA into a cell induces the interferon response¹³ that includes the induction of ADAR (Adenosine deaminase acting on RNA) that acts as a double stranded RNA editing enzyme that converts A residues to inosine. This edit makes the dsRNA unavailable as a substrate for RNAi.^{14,15} When the two forms of this mammalian enzyme were transformed into flies, one of them can compete with RNAi and cause the interference reaction to be reduced.

There is a single ADAR gene in flies and knockdowns of it were combined with RNAi transgenes to test whether it could be responsible for the cell death induced inhibition of RNAi. Neither was there any evidence for an effect on the inhibition of RNAi nor for an increased expression of ADAR in the *hs-hid* strain.¹ Thus, these tests proved negative for an involvement of ADAR in the RNAi inhibition. If there were a modification of the dsRNA that prevents it from entering into the RNAi pathway, the basis of such modification would need to be further investigated. There are no data available on whether cell death signaling modifies DICER-2.

Recently, Saleh and colleagues¹⁶ have found that defects in the system for double stranded RNA (dsRNA) uptake were exceptionally susceptible to virus infection. The results imply that dsRNA can serve as a signal that is spread throughout the fly to confer immunity. It will be interesting to determine whether there is any relationship or not with the cell death inhibition of RNAi.

What is the Signal?

Cells induced to undergo death will emit proliferation signals to their neighboring cells.¹⁷⁻¹⁹ Even under circumstances in which the cell death is triggered but prevented by the expression of the anti-apoptotic protein, p35, there will still be proliferation signals emitted that will cause an overgrowth of the tissue. When this circumstance was combined with the RNAi constructs, the prevention of cell death also prevented the reversal of RNAi. This evidence indicates that the proliferation signals sent to neighboring cells are unlikely to be the same as those that trigger the inhibition of RNAi. It should also be noted that in some cases in which cell death is rapid and extensive, such as the heat shock induction of the *hs-hid* transgene that kills the larvae or flies quickly, there is no evidence of RNAi reversal. Why this is the case remains unknown as well as whether there is a signal generated under these conditions. The nature of the signal in those cases of cell death in which RNAi is reversed is an interesting direction for future research on this topic.

Implications for Somatic Mutations

The finding that chronic ectopic cell death causes an increase in the expression of multiple retrotransposons raises an interesting issue with regard to somatic mutation. The whole process of RNAi is involved with silencing transposons apparently to prevent deleterious mutations. Thus, processes that inhibit interference and allow an increase of new insertions of the transposons²⁰ would likely have an increased mutation rate. Indeed, as cases of hybrid dysgenesis illustrate,²¹ increased expression of retrotransposons is correlated with an increased mutation frequency so it is possible that any condition that regularly causes sporadic cell death would also be capable of fostering an increase in somatic mutation rate in adjacent cells that contribute to the adult fly.

An interesting issue is whether such a phenomenon occurs in mammals. If so, then any chronic irritation that causes sporadic cell death would potentially increase somatic mutation frequency and be an agent for cancer development. However, the interferon response noted above might be a more prominent response that will shut down the expression of retrotransposons and viruses to prevent such from happening.

Does the Reversal of RNAi by Cell Death Signaling Occur in the Germline?

At present, there are no data that provide any clue as to whether the germline is susceptible to reversal of RNAi processes. If this does occur, then the possibility arises that there would be an increase in germinal mutations in the presence of sporadic ectopic cell death. If this indeed occurs, then this phenomenon would become a factor in discussions of "spontaneous" mutation frequencies.

The PIWI interacting RNAs (piRNAs) are prevalent in the germline^{21,22} and the question arises as to whether they are affected by sporadic ectopic cell death. We did find a slight but repeatable reduction of total piRNAs in the *hs-hid* larvae but not to the extent as found for siRNAs. If the piRNA system were also affected by cell

death, then germinal mutation frequencies would likely be increased. Such a test for an impact on piRNAs would also be informative about the mechanism involved in the inhibition process more generally.

Basic and Practical Applications of RNAi

RNAi has become a powerful reverse genetic tool that is used routinely in basic studies to knockdown specific genes to learn about their function. There have also been a number of practical uses of RNAi that have been proposed and in some species placed into practice.²³ Depending on the generality of the cell death induced RNAi inhibition phenomenon across species and depending on the types and conditions of cell death that trigger a reversal of RNAi, this phenomenon could impact the use of RNAi and be a necessary condition to understand before appropriate conclusions can be drawn from RNAi experiments.

Acknowledgments

Work on this topic in our laboratory has been supported by NSF grant MCB 0923607.

References

1. Xie W, Liang C, Birchler JA. Inhibition of RNA interference and modulation of transposable element expression by cell death in *Drosophila*. *Genetics* 2011; DOI:101534/genetics.111.128470.
2. Fristrom D. Cellular degeneration in the production of some mutant phenotypes in *Drosophila melanogaster*. *Mol Gen Genet* 1969; 103:363-79.
3. Golic KG. Site-specific recombination between homologous chromosomes in *Drosophila*. *Science* 1991; 252:958-61.
4. Xu T, Rubin GM. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* 1993; 117:1223-37.
5. Xu P, Vernooy SY, Guo M, Hay BA. The *Drosophila* microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. *Curr Biol* 2003; 13:790-5.
6. Grether ME, Abrams JM, Agapite J, White K, Steller H. The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev* 1995; 9:1694-708.
7. Czech B, Malone CD, Zhou R, Stark A, Schlingeheyde C, et al. An endogenous small interfering RNA pathway in *Drosophila*. *Nature* 2008; 453:798-802.
8. Ghildiyal M, Seitz H, Horwich MD, Li C, Du T, et al. Endogenous siRNAs derived from transposons and mRNAs in *Drosophila* somatic cells. *Science* 2008; 320:1077-81.
9. Kawamura Y, Saito K, Kin T, Ono Y, Asai K, et al. *Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. *Nature* 2008; 453:793-7.
10. Chung WJ, Okamura K, Martin R, Lai EC. Endogenous RNA interference provides a somatic defense against *Drosophila* transposons. *Curr Biol* 2008; 18:795-802.
11. Okamura K, Chung WJ, Ruby JG, Guo H, Bartel DP, Lai EC. The *Drosophila* hairpin RNA pathway generates endogenous short interfering RNAs. *Nature* 2008; 453:803-6.
12. Li HW, Ding SW. Antiviral silencing in animals. *FEBS Lett* 2005; 579:5965-73.
13. Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signaling, antiviral responses and virus countermeasures. *J Gen Virol* 2008; 89:1-47.
14. Scadden AD, Smith CW. RNAi is antagonized by A→I hyper-editing. *EMBO Rep* 2001; 2:1107-11.
15. Heale BSE, Keegan LP, McGurk L, Michlewski G, Brindle J, et al. Editing independent effects of ADARs on the miRNA/siRNA pathways. *EMBO J* 2009; 28:3145-56.
16. Saleh MC, Tassetto M, van Rij RP, Goic B, Gausson V, et al. Antiviral immunity in *Drosophila* requires systemic RNA interference spread. *Nature* 2009; 458:346-50.
17. Ryoo HD, Gorenc T, Steller H. Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. *Dev Cell* 2004; 7:491-501.
18. Huh JR, Guo M, Hay BA. Compensatory proliferation induced by cell death in the *Drosophila* wing disc requires activity of the apical cell death caspase Dronc in a nonapoptotic role. *Curr Biol* 2004; 14:1262-6.
19. Fan Y, Bergmann A. Distinct mechanisms of apoptosis-induced compensatory proliferation in proliferating and differentiating tissues in the *Drosophila* eye. *Dev Cell* 2008; 14:399-410.
20. Kalmykova AI, Klenov MS, Gvozdev VA. Argonaute protein PIWI controls mobilization of retrotransposons in the *Drosophila* male germline. *Nucleic Acids Research* 2005; 33:2052-9.
21. Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, et al. Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 2007; 128:1089-103.
22. Aravin AA, Hannon GJ, Brennecke J. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* 2007; 318:761-4.
23. Aigner A. Applications of RNA interference: current state and prospects for siRNA-based strategies in vivo. *Appl Microbiol Biotechnol* 2007; 76:9-21.