Try to disarm the intruder or kill him!

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RNA interference and transgene-mediated cosuppression are trans-generational silencing mechanisms acting both at a post-transcriptional and epigenetic level. We have recently shown that both these procedures, which share several common factors and are commonly used to phenocopy gene deletions, also induce germ-line DNA damage and apoptosis. These observations shed new light on the cross-talk between different pathways devoted to the protection of genome stability in germ cells.

Complex genetic pathways have been selected by evolution in order to neutralize the effects of gene amplifications, viral infection, or transposons activation. Several proteins and small RNA molecules are active in these pathways. Silencing mechanisms involving dsRNA were discovered in the 90s in plants, fungi and nematodes.[1,2]

In *C. elegans,* gene expression can be abrogated at a given locus by introduction of dsRNA homologous to the gene of interest by feeding, soaking or microinjection. This phenomenon, discovered by Fire and Mello,[2] is known as RNA interference (RNAi) and proceeds by known steps: (1) dsRNA is processed by the Dicer protein into small interference RNAs (siRNA); (2) anti-sense siRNAs recognize and anneal to homologous mRNA transcripts and are amplified by an RNA-dependent RNA-polymerase (RdRp); (3) secondary siRNAs bind either to the bulk of the specific mRNA transcript leading to its degradation or migrate to the nucleus to the corresponding genomic sequence and induce silencing of the corresponding locus by means of histone methylation.[3]

Silencing of the homologous gene lasts for a few generations.

Introduction by microinjection of a transgene as dsDNA leads, in *C. elegans*, to the formation of long extrachromosomal arrays that are inherited through subsequent generations, although in a non-mendelian fashion.[4] When the coding sequence is under a somatic promoter, it will be expressed in the appropriate tissue, however, if the transgene carries a germ-line promoter, it will be silenced together with the endogenous locus in the gonad.[5,6] The extrachromosomal array can be maintained for several generations, however, once it is lost by random segregation, genomic silencing gradually disappears after two/three generations as in RNAi.[7] This phenomenon is known as transgene-mediated cosuppression. RNAi, cosuppression and transposon silencing share many steps and some, but not all, the main actors.[7-9]

Flock house virus (FHV) introduction in worms triggers a potent antiviral silencing that requires RDE-1, one of the Argonaute proteins essential for RNAi. This immunity system is capable of rapid virus clearance in the absence of FHV B2 protein, which acts as a broad-spectrum RNAi inhibitor.[10] Furthermore, it has also been shown that viral silencing is trans-generational.[11] Similarly, the Orsay virus (naturally infecting *C. elegans*) yields higher levels of viral RNA and infection symptoms in mutants defective in RNAi as compared with the wild-type N2 strain.[12] On the other hand, by introducing vaccinia virus in worms, it was shown that virus replication is significantly enhanced in *ced-3, ced-4, ced-9(gf)* and *egl-1(lf)* mutants, demonstrating that the

Figure 1. Co-suppression induces additional germ cell death events. *C. elegans* gonads labeled with SYTO12: the worms carrying an transgene extrachromosomal array suppressing GFP expression (on the right [*GFP*]) show an increment in the number of SYTO12 stained corpses.

core programmed cell death genes also antagonize virus replication in *C. elegans*.[13]

In our recent article, in collaboration with the group of Verena Jantsch, we demonstrated that both RNAi and transgene-mediated cosuppression induce parallel enhancement of germ-line apoptosis (**Fig. 1**).[14] Silencing-triggered germ-line apoptosis is a novel mechanism probably devoted in metazoa to the elimination of germ cells that have undergone endogenous sequence amplification, transposon activation or virus infection. But how do the two pathways, gene silencing and cell death, talk to each other?

Several observations exclude that apoptosis is enforced during cosuppression via the pairing checkpoint activated by the presence of the unpaired minichromosome: (1) extrachromosomal arrays carrying non meiotic transgenes, and therefore not inducing germ-line gene silencing, do not enforce germ-cell death; (2) the cosuppression effect and apoptosis enhancement are maintained when a long chromosomal array of a meiotic transgene is inserted into a chromosome and kept in homozygosis for over 50 generations, although no mini-chromosome is present and the intra-chromosomal array can properly pair; finally, (3) any time a cosuppressing extrachromosomal array is lost, the silencing effect decreases within a couple of generations and in parallel, apoptosis enhancement gradually also decreases.

That silencing dependent apoptosis is indeed due to the activation of the DNA damage checkpoint is demonstrated by its dependence on *cep-1/p53* and *sir-2.1* genes (both critical components of the DNA damage apoptosis pathway)[15,16] and by the associated enhancement of germ-line RAD-51 foci. These foci, indicative

of additional DSBs, do not depend on the action of the conserved meiotic protein SPO-11 inducing physiological DSBs at the onset of meiotic prophase through a topoisomerase-like transesterase mechanism. Furthermore, they also appear upon RNAi in a *ced-3* mutated background (where apoptosis does not take place),[17] suggesting that they are the cause of the checkpoint activation and not the consequence of apoptotic DNA degradation.

RNAi-induced apoptosis does not seem to be due to the great amount of RNA molecules artificially introduced in the gonad. In fact, if we inject dsRNA within a strain that does not carry the homologous locus or in a strain, as *rde-2*, that is defective in RNAi, no apoptosis enhancement is observed.

Alternative models to explain de novo formation of DSBs (revealed by the increase in RAD-51 foci), upon silencing, were taken into account, but none have withstood careful scrutiny. Initially, we thought that a possible inducer of silencing dependent DSBs might be the DCR-1 protein (*Dicer*) that had been shown to be processed by the CED-3 caspase from its RNase III form into a DNase.[18] This model would have been reasonable, since CED-3 is constitutively present into the germ line and Dicer could have been overproduced upon RNAi or cosuppression and therefore processed in part. However, this cannot be the case since DSB increase is, as stated before, also observed in a genetic background where the *ced-3* gene is defective. An alternative model is that since most factors active in RNAi and cosuppression are also necessary for transposon silencing, these factors might be diluted by engagement of the two silencing phenomena, leading to transposon activation and

DSB by excision. However, this model does not appear to be consistent with our data: (1) RNAi and cosuppression phenomena do not seem to be mutagenic, (2) inactivation of the apoptosis pathway does not confer a mutator phenotype,[19] and (3) in the *rde-2/mut-8* background, where transposons are active, apoptosis is not induced either before or after injection with dsRNA or dsDNA.[14] It seems, therefore, that transposon excision per se does not induces DNA-damage apoptosis in absence of the functional silencing program.

We have to imagine a more subtle tuning of the response against intruders in which on one side the overesspressed gene is silenced, both at the post-trancriptional and at the chromatin level, and, on the other, DSBs are induced and direct nuclei to apoptosis.

A potential model worth investigating envisions apoptosis triggering signals linked to sensors at the chromatin level. In the co-suppression experiments, we observed that when the extra-chromosomal array is lost during consecutive chromosome segregation cycles, the somatically-expressed selectable-marker phenotype disappears at the first generation, whereas the co-suppressed phenotype only gradually decreases in its effects over more than two generations[8] and levels of apoptosis decline in parallel.[14] The deacetylase sirtuin SIR-2.1, known to operate at the chromatin level and to participate in DNA damage induced apoptosis, is required for the rise of germ cell death during co-suppression. Interestingly, the yeast sir2 gene is known to alter DSBs genomic distribution.[20]

It is important to keep in mind that a large portion of the total RNA content of a metazoan cell is composed of noncoding RNA and that a large set of data assigned to these (long and small) non coding RNAs has fundamental roles in the regulation of gene expression during differentiation and development.[21-27] It is, therefore, also likely that an unbalanced RNA moiety could trigger mechanisms leading to cell elimination.

In *C. elegans*, damage-induced apoptosis is active in germ cells only, while activation of the DNA damage checkpoint in somatic cells only leads to repair and not

to cell death. However, in other organisms, accumulation of unrepaired DSBs in somatic tissues can lead to cell death. We cannot exclude that silencing-induced apoptosis is not a more widespread phenomenon and might be present in other organisms and cell types. Interestingly, when the first attempts were made to silence mammalian genes by RNAi, the cellular response to a large amount of dsRNA led to interferon-mediated apoptosis,[28,29] i.e., an apparently coevolved mechanism leads to a result similar to what we observe in *C. elegans.*

We were rather astonished that, after so many years in which RNAi and cosuppression have been used in genomic screening or to mimic gene depletion phenotypes in *C. elegans,* the accompanying increase in apoptosis had never been detected or described. However, it is likely that since mutations in most meiotic genes disrupt DNA repair and as such they result in a highly significant increase in apoptosis, a limited enhancement in cell death due to the silencing mechanism itself may have gone unnoticed when studying these genes. However, some colleagues have indeed stated in their papers that more apoptosis was seen after RNAi than in the corresponding mutant.[30] Also in the original paper where DNA damage checkpoint and apoptosis were first described,[15] it is noticeable that the apoptotic nuclei average in the *spo-11; rad-51*(RNAi) sample is about three times that observed in the *spo-11* single mutant, although much lower than that seen in the *rad-51*(RNAi) sample. We are now aware of the enormous impact that non coding RNAs have in the general regulation of various genes and pathways in metazoa[21-24] and, therefore, we would recommend caution in the interpretation of data obtained by gene depletion obtained by RNAi or transgene mediated cosuppression.

Future challenges will be to discover the crucial silencing molecules leading to induction of DSBs and triggering the DNA damage checkpoint and to investigate whether the silencing-induced apoptosis is conserved in evolution.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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