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Commentary

Matter Arising

Off Targets and Genome Scale RNAi Screens in *Drosophila*

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Commentary to:

Prevalence of Off-Target Effects in Drosophila RNA Interference Screens

Y. Ma, A. Creanga, L. Lum and P.A. Beachy

Nature 2006; 443:359-63

and

Evidence of Off-Target Effects Associated with Long dsRNAs in Drosophila Melanogaster Cell-Based Assays

M.M. Kulkarni, M. Booker, S.J. Silver, A. Friedman, P. Hong, N. Perrimon and B. Mathey-Prevot

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ABSTRACT

Recently, the issue of off-target effects (OTEs) associated with long double stranded RNAs (dsRNAs) used in RNAi screens, such as those performed at the *Drosophila* RNAi Screening Center and other laboratories, has become a focus of great interest and some concern. Although OTEs have been recognized as an important source of false positives in mammalian studies (where short siRNAs are used as triggers), they were generally thought to be inconsequential in *Drosophila* RNAi experiments because of the use of long dsRNAs. Two recent papers have disputed this contention and show that significant off-target effects can take place with the use of some long dsRNAs in *Drosophila* cells. Together, these studies provide evidence that OTEs mediated by short homology stretches of 19nt or greater within long dsRNAs can contribute to false positives in *Drosophila* RNAi screens. Here, we address how widespread the occurrence of OTE is in *Drosophila* screens, focusing on the DRSC dsRNA collections, and we discuss the implication for the interpretation of results reported in RNAi screens to-date. Lastly, we summarize steps taken by the DRSC to redress that situation and include a set of recommendations to observe in future RNAi screens.

ABBREVIATIONS

OTE, off target effect; dsRNA, double stranded RNA; siRNA, short interfering RNA; RNAi, RNA interference; DRSC, *Drosophila* RNAi Screening Center; nt, nucleotide

WHAT IS THE DRSC AND WHAT GOES ON THERE?

The *Drosophila* RNAi screening Center (DRSC) (http://flyrnai.org) was established at Harvard Medical School in 2003 to perform large-scale functional screens in *Drosophila* cells. It is open to all scientists and offers a unique genome-wide collection of dsRNAs that is prearrayed in 384-well plates, a state-of-the art infrastructure that includes robotics, plate readers, high content imaging capabilities, and a repository database.[3,4] Over the last three years, 65 genome-wide screens have been successfully completed at the Center, 14 of them resulting already in a publication (http://flyrnai.org/RNAi_screen_list.html).

WHAT IS AN OFF-TARGET EFFECT (OTE)?

Collectively, OTEs comprise all detectable phenotypic consequences arising from unintended interactions, whether dependent on nucleotide sequence between the silencing reagents and various nontargeted mRNAs in the cell, or independent of nucleotide sequence through the induction of an interferon response by dsRNAs in mammalian cells.[5-9]

WHAT IS THE NATURE OF THE PROBLEM?

Although RNAi has been widely used in *C. elegans* and *Drosophila* research, OTEs were not generally believed to be a significant issue in these organisms. First, long dsRNAs (instead of siRNAs), usually around 400 or more nt in length, are used for RNAi in *Drosophila* and *C. elegans*, as these organisms in contrast to mammals, have no interferon response to dsRNAs. Second, although a number of in silico analyses had clearly identified 21-23 nt long sequences in dsRNAs to have perfect homology to unintended target mRNAs.[4,10,11] the general consensus was that the occurrence of sequence-specific OTE would be minimal as the effect of the occasional "bad" or "non-specific" siRNAs

would be diluted by the large excess of "good" or "specific" siRNAs present in the pool of siRNAs generated after Dicer processing of the dsRNA. Surprisingly and until recently, this prediction had not been rigorously tested. Although recent experimental evidence has been presented to support the dilution effect provided by pooling siRNAs,[12] the extent to which it is protective is hard to predict. First, detection of OTEs and the protection from them observed with pools of siRNAs are highly concentration dependent.[12] Second, due to the 21-23 nt processivity of Dicer activity.[13,14] not all of the possible siRNAs will be represented in the pool and their precise identity cannot be easily predicted. Indeed, two independent studies clearly demonstrated that pools of siRNAs generated by Dicer in vivo would not always be free of OTEs.[1,2]

WHAT DO THE MA ET AL. AND KULKARNI ET AL. PAPERS SAY?

In both studies, the DRSC version 1.0 of dsRNAs (see below) was used in the screens analyzed. In the first study, Ma et al. demonstrate that the presence of 6 or more contiguous trinucleotides CA[AGCT] (or CAN repeats) found in some long dsRNAs used in *Drosophila* tissue culture RNAi screens were associated with OTEs, and led to false positives.[1] The study by Kulkarni et al. extends these findings and shows that dsRNAs containing ≥19 nt perfect matches (including but not exclusive to CAN repeats) corresponding to non-target specific transcripts are a likely source of OTEs.[2] As a result, both studies cautioned against interpreting phenotypes caused by dsRNAs containing any of the above problematic sequence(s) as they might result from either efficient knockdown of non-target specific transcripts or from other mechanisms leading to non-specific metabolic effects or cell toxicity.

Table 1 **The DRSC 1.0 and DRSC 2.0 dsRNAs collections**

	DRSC Version 1		DRSC Version 2	
	Total	**Percentage**	**Total**	**Percentage**
Total dsRNAs	21306		22490	
with no CAN[a]	20169		22022	
with CAR[b]	646	3.0%	117	0.5%
with CAN	1137	5.3%	424	1.9%
Total dsRNAs				
with (1+) OTs	8583	40.3%	6885	30.6%
with 0 OT	12723	59.7%	15605	69.4%
with 1 OT	4054	19.0%	3681	16.4%
with 2-10 OTs	2940	13.8%	2587	11.5%
with 11-50 OTs	715	3.4%	467	2.1%
with 51-100 OTs	214	1.0%	54	0.2%
with 101+ OTs	660	3.1%	96	0.4%
dsRNAs with 1+ OT and exactly 1gene target	N/A		6262	
Number and % of above dsRNAs with a 2nd OT free dsRNA to the same gene	N/A		5907	94.3%

The total number and breakdown of the various dsRNAs in the two collections are indicated. Predicted homologies are calculated based on a 19 nt threshold for perfect homology to other sequences than the intended target. [a]Six or more repeats of the trinucleotide CA[AGCU]. [b]Six or more repeats of the trinucleotide CA[AG]. The DRSC 2.0 collection has some dsRNAs with predicted OTs, but in 94.3 % of the cases, there is a second independent dsRNA in the collection that targets the same gene and is free of predicted OTs. The decision to keep DRSC 1.0 dsRNAs with ≤10 OTs is based on the observation that strict computational prediction is an overestimation of the possible OT sequences represented by the siRNAs generated by Dicer. Indeed, the number and type of siRNAs made from a long dsRNA is dictated by the processive cleavage of Dicer every 21-23 nt, starting at one or the other end of the dsRNA. Also, in 5.7% of the dsRNAs that have 1 or more OTs and are currently in version 2, it was not possible to design a second independent OT-free dsRNA due to various annotation issues (such as small gene size or the presence of closely related genes with similar sequence).

IS THE ISSUE WITH THE DRSC LIBRARY RESOLVED?

Yes. The DRSC dsRNA collection is the foundation on which all RNAi screens performed in our facility are based. We have therefore monitored closely the quality and performance of our dsRNAs in the screens, and have worked to address issues connected with their use as soon as we detected them.

The DRSC 1.0 and DRSC 2.0 collections. Our initial analysis (about two years ago) of the DRSC 1.0 library revealed that some dsRNAs caused a phenotype in many unrelated screens. This was readily apparent for dsRNAs that carried strings of CAR triplets (reminiscent of OPA-repeats[15]) as they tended to be more highly represented in the hits reported in our screens. This observation suggested that dsRNAs identified in multiple and unrelated screens corresponded to false positives, and that sequence homologies to multiple genes might lead to OTEs. To confirm this hypothesis, we developed a program to look for the presence of perfect sequence homologies between any of the possible siRNAs generated by the processing of a dsRNA to *Drosophila* transcripts other than the intended target (http://flyrnai.org/RNAi_find_frag_free.html).[4] Running the sequence of the dsRNAs through this program and looking at how the various dsRNAs had scored in a few DRSC screens confirmed that dsRNAs with ≥19 nt homology sequences to non-target genes tended to score as hits more often than predicted by chance alone. As a result, we set out to replace the questionable dsRNAs in our initial collection (DRSC 1.0) with new ones devoid of any predicted homology.

The important lesson from these studies is that the detection of a 17–19 bp perfect match remains currently the most reliable predictor for the minimal overlap required to detect an OTE (see Table 1, ref. 2) and that any result using a dsRNA containing even a single OT sequence should be viewed critically. Encouragingly, preliminary evidence obtained so far indicates that once the presence of 19 nt homology sequences has been eliminated from the design of new long dsRNAs, the risk of sequence specific OTE is considerably reduced. However, it should be stressed that we may not be aware of other rules governing OTEs, and additional work will be needed to resolve this issue more thoroughly. We are committed to continue this effort, as the full realization of the promises placed into RNAi to deliver unprecedented insights into the function of nearly all genes depends on it. It is equally important to mention that the mere presence of 17–19 nt homology detected in silico does not automatically translate into an OTE. The occurrence of an OTE can only be ruled in or ruled out if a second or third dsRNA is used to validate the initial finding. For this reason, we have started to build the DRSC validation collection.

The DRSC validation set. As mentioned earlier, the rules of what might constitute an offending sequence associated with OTEs are still unclear. Therefore, we highly recommend confirming the activity of a given dsRNA with a second independent dsRNA targeting the same gene. To facilitate this task, we are currently assembling a validation collection, which consists of new dsRNAs that are distinct from any dsRNA present in DRSC 2.0. In the Gene Lookup page and other places on the DRSC web page, we refer to these as

Table 2 ***Drosophila* libraries available for RNAi screens**

Collection and Coverage	Description	Comments
DRSC 1.0[19]: Genome-wide (http://flyrnai.com)	21,306 dsRNAs with average length of 400 bp	40% of dsRNAs have ≥ 1 predicted OT sequences (19 nt)
DRSC 2.0: Genome-wide (http://flyrnai.com)	22,490 dsRNAs with average length of 400 bp	94.3% of all annotated genes are targeted by at least 1 dsRNA free of OT.
Ambion: Best annotated *Drosophila* genes	13,071 genes (based on version 3.0 genome annotation) with average length of 300-800 bp	No data available with regard to OT sequences
Open Biosystems: Genome-wide Release 1.0 and 2.0	15,881 dsRNAs with average length of 200-800 bp	No data available with regard to OT sequences
BDGP[20,21]: Genes represented in cDNA set 1 collection	4,923 dsRNAs of variable size	No data available with regard to OT sequences
BDGP[22]: DGC1 and DGC2 70% annotated genes	~500 bp PCR fragments tailed with T7 promoter were amplified from these collections to serve as template for dsRNA synthesis	No data available with regard to OT sequences

Additional details for each of the nonDRSC collections are available from the respective web pages: Ambion library: (www.ambion.com/catalog/ProdGrp.html?fkApp=25&fkProdGrp=326); Open Systems library (www.openbiosystems.com/RNAi/Non%2DMammalian%20RNAi/Drosophila%20RNAi%20collections/); BDGP cDNA collections: (www.fruitfly.org/DGC/index.html).

"validation amplicons" or "validation dsRNAs." These dsRNAs are devoid of predicted perfect homologies to nontarget genes and correspond to every primary hit identified in a completed DRSC screen.

HOW DO I MAKE SURE THAT MY RNAi DATA ARE REAL?

Though challenging, controlling OTEs in genome-scale screens can be achieved through the applications of guidelines discussed in Echeverri and Perrimon[16] as well as in a more recent commentary endorsed by several leading RNAi laboratories.[17] Not accounting for the incidence of OTEs in a screen may lead to the unintended inclusion of false positives among lists of genes published after a large-scale screen. This risk will be minimal when the purpose of a screen is to identify and characterize only one or a handful of new components in the process of interest. In this case, any potential OTE would be quickly exposed in the follow-up validation experiments involving independent amplicons and corroborating data. However, it is not uncommon to identify over 300 genes in a screen, for which their corresponding dsRNAs yielded a phenotype of interest. Such a large number of candidate hits precludes a careful and thorough characterization, as it would make it unmanageable both in time and labor spent on this endeavor. In such cases, we recommend testing a second (and preferably a third) dsRNA targeting the same gene as the initial dsRNA associated with a phenotype, to ensure that the measured activity results from the specific knockdown of the intended target. In order to facilitate the implementation of these guidelines, the DRSC is committed to provide screeners with the means of confirming their primary results using a set of distinct dsRNAs (the DRSC validation set). In addition, as more screen results become publicly available, individual dsRNAs that have been associated with a phenotype in a screen can be checked against the list of dsRNAs reported as hits in published screens. Such knowledge can be used to gauge the specificity of the dsRNA and may help refine ultimately the choice of candidate genes to be actively pursued.

HOW MUCH OF A CONCERN ARE OTEs IN RNAi SCREENS?

Although it is best to assume that OTEs will occur in any given screen, the recent improvements in library designs will help minimize them. However, OTEs are not the only source of false positives. False positives can originate from a number of technical and statistical sources, and these have not received as much attention as OTEs have. In addition, we have noticed that OTEs, as well as the other sources of false positives, can be revealed more prominently in certain type of screens, depending of the assay, cell line and experimental conditions used. In this regard, one issue that deserves closer scrutiny when performing RNAi screens is the degree of specificity and distinctiveness of the phenotype scored in an assay. Indeed assays that are particularly sensitive to the overall "metabolic" health of the cells are at greater risk of being affected by nonspecific perturbations caused by experimental manipulations or reagents. This is a concern as OTEs effects often cause nonspecific toxicity. Therefore some assays will be more readily affected by OTEs as cell death can be triggered by many promiscuous cellular insults, including stress alone. For instance, it will be harder to sort out in a cell viability assay whether cell death results from OTEs associated with the use of dsRNAs or from the specific silencing of a candidate gene involved in this process. In addition, cell viability can affect the interpretation of results from assays that are not specifically designed to monitor this response. Healthy skepticism should be reserved for dsRNAs that score as hits in quantitative assays if they also cause cell death. Most quantitative assays involve a normalization step designed to control for cell number, and this normalization is often based on the ratio of the activity measured for the test reporter construct (the focus of the assay) versus that of a normalization reporter construct. At low cell numbers, many screeners have reported that issues of nonlinearity between the two reporters can complicate the interpretation of the results and, if not addressed, can lead to a high rate of false positives. Consequently, to minimize as much as possible the complications raised by OTEs in interpreting screen results, we recommend to design assays that are based on multiparametric measurements that rely on endogenous markers or capture elaborate cell biological responses. However no matter how distinct or specific the response assayed in a screen may be, the issue of OTE can only be unambiguously ruled out by showing that, at the minimum, two different dsRNAs (which have completely distinct sequences but target the same gene) will cause the same phenotype.[17]

WHAT ABOUT THE PREVIOUSLY PUBLISHED SCREENS?

Regarding the published screens conducted with the DRSC 1.0 version, information on the sequence for all dsRNAs has been openly available (http://flyrnai.org/), a policy that has enabled the recent detailed analyses by Ma et al. and Kulkarni et al.[1,2] Also, in addition to the published hit list, we have added for each screen a separate list that consists of only the candidate hits targeted by dsRNAs free of OT sequences (list available at http://flyrnai.org/). This is obviously a stringent measure and most likely overcautious because in silico detection of 19 nt perfect homology sequences in dsRNA is not necessarily sufficient to result in experimentally detectable OTEs. Obviously, the information content in the list of candidate genes available from the early screens will gain from being validated using additional dsRNAs. This process has been started for most of the screens, and our initial findings are very encouraging. We have observed excellent validation rates when new dsRNAs (DRSC validation set) were used to confirm the activity of the original dsRNAs that had been reported in the published screens and were predicted to lack the problematic 19nt homology. In fact, the validation rate was identical to the reproducibility observed when the original dsRNAs were retested in parallel to the new ones. In contrast, the validation rate for dsRNAs predicted to have OTs (using the 19nt perfect homology threshold) was not as high and declined according to the number of OTs found by our algorithm.

IS THIS AN ISSUE ONLY WITH THE DRSC LIBRARIES?

No. The two recent studies[1,2] have been done only with the DRSC 1.0 library, as the sequences are freely available and accessible. Similar analyses should be done with other *Drosophila* libraries (Table 2). A major issue with analysis of the libraries used in the other studies is that the sequence of the dsRNAs is not always made available, so the identity of the problematic dsRNAs and the extent to which they might cause OTEs are not known.

ARE OTEs AN ISSUE FOR IN VIVO RNAi?

It is not clear yet to what extent the issue of OTs is a concern for in vivo studies, either following the injection of dsRNAs into embryos, or the expression of transgenic hairpin constructs. There is no available rigorous analysis of this issue yet in the literature. However, a CAR repeat in a dsRNA targeting the m3 gene in the *E(spl)-C* locus has been reported to cause high mortality when injected in embryos, presumably through an OTE[18] suggesting that the problem is not restricted to cell-based assays only. Nevertheless, we expect to find out soon how much OTEs will prove to be a concern in vivo as the result of large-scale transgenic RNAi ongoing efforts should become available in the near future (www.imba.oeaw.ac.at/index.php?id=252; www.shigen.nig.ac.jp/fly/nigfly/). One interesting question is whether transgenic RNAi, unlike studies in tissue culture, might lead to fewer OTEs, especially if the level of expression of the hairpins is (or can be manipulated) to be below the threshold associated with many OTEs but sufficient enough to cause robust silencing of their mRNA targets. One aspect that has not been sufficiently addressed in RNAi screens in *Drosophila* is whether the range of dsRNA concentrations (as used in screens at the DRSC[3]) might perhaps be too high and could be decreased without affecting the overall knock-down efficiency. Providing dsRNAs at the minimal effective concentration would further reduce the chances of detectable OTEs (for additional discussion see refs. 8 and 12).

CONCLUSION

Like many new technologies, refining RNAi screening libraries depends on retrospective analysis of data and corrective action in the form of library updates. Our current understanding of OTs associated with long dsRNAs is likely not the end of the story as there might be other predictors (e.g., seed regions; ref. 9) that in certain context need to be avoided. Although the prediction of OTs and the design of better reagent is still evolving and will continue to be a focus of further efforts, the community experience with RNAi reagents have led to a better understanding of their specificities and provided recommendations for best usage of the technology. With this knowledge in hand, we expect to see many exciting applications of this powerful technology in the next few years.

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