Validating RNAi Phenotypes in Drosophila Using a Synthetic RNAi-Resistant Transgene

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Abstract

RNA interference (RNAi) is a powerful and widely used approach to investigate gene function, but a major limitation of the approach is the high incidence of non-specific phenotypes that arise due to off-target effects. We previously showed that RNAi-mediated knock-down of pico, which encodes the only member of the MRL family of adapter proteins in Drosophila, resulted in reduction in cell number and size leading to reduced tissue growth. In contrast, a recent study reported that pico knockdown leads to tissue dysmorphology, pointing to an indirect role for pico in the control of wing size. To understand the cause of this disparity we have utilised a synthetic RNAi-resistant transgene, which bears minimal sequence homology to the predicted dsRNA but encodes wild type Pico protein, to reanalyse the RNAi lines used in the two studies. We find that the RNAi lines from different sources exhibit different effects, with one set of lines uniquely resulting in a tissue dysmorphology phenotype when expressed in the developing wing. Importantly, the loss of tissue morphology fails to be complemented by co-overexpression of RNAi-resistant pico suggesting that this phenotype is the result of an off-target effect. This highlights the importance of careful validation of RNAi-induced phenotypes, and shows the potential of synthetic transgenes for their experimental validation.

Introduction

For more than 10 years, silencing of gene expression by RNA interference (RNAi) in Drosophila melanogaster has provided a powerful approach to complement classic mutant studies for assessing loss of gene function in vitro and in vivo. The recent advent of transgenic libraries of inverted repeat constructs capable of expressing dsRNA for virtually any gene of interest in vivo [1], has led to the wide uptake of heritable RNAi technology. The use of the UAS-GAL4 expression system to target dsRNA expression to different cell types or stages of development has facilitated targeted genetic screens and allowed manipulation of gene function in different cellular contexts [2]. However, a major limitation of the approach is the high incidence of non-specific phenotypes that arise due to off-target effects [3,4]. One way to mitigate the risk of misinterpreting RNAi-induced phenotypes is to use independent dsRNA constructs that target non-overlapping sequences of the same gene: if two or more independent lines produce the same effect one can have more confidence that the resulting phenotype is due to knockdown of the gene of interest. However, conflicting results for multiple dsRNAs targeting the same gene are difficult to interpret in the absence of other information. In silico predictions of the potential for off-targets (e.g. [5]) can be indicative in this regard, but a genetic complementation test is the best way to validate specificity of the RNAi-induced phenotype. Complementation experiments with wild type transgens do not control for specificity because the ectopic miRNA may act by titrating RNAi knockdown of endogenous genes, whether they be on- or off-targets. In mammalian systems, where short siRNA molecules are used to induce RNAi, an effective solution to this problem is to test for rescue of the RNAi-induced phenotype using RNAi-resistant transgenes containing silent mutations in the region targeted by the siRNA [6,7]. Here we have applied this principle to assess complementation of longer dsRNA molecules (typically around 0.5 kb in length) in Drosophila to distinguish between on and off-target effects.

The MRL (Mig-10, RIAM, Lamellipodin) family of proteins has been demonstrated to modulate the actin cytoskeleton in response to extracellular signals to effect changes in cell morphology, adhesion and migration [8,9]. In addition to these roles, we previously showed that pico knockdown leads to a reduction of aspect of this growth effect of pico and a reduced cell doubling time compared to wild-type control cells, without any change in cell cycle phase and cell density. One aspect of this growth effect of pico was re-examined in a recent paper [11]. In contradiction with our previous results [10], it was...
reported that RNAi targeting of pico lines leads to severe tissue
dysmorphology rather than a strict growth phenotype in the adult
wing [11]. Here we have analysed the source of the apparent
disparity and find that RNAi lines from different sources exhibit
different phenotypic effects. Unlike the construct we previously
reported, the commercially available NIG-Fly RNAi lines
MS1096-GAL4 alone, in agreement with dsCheck predictions.

Wing dysmorphology phenotypes of picoRNAiIR4/R3 are
not rescued by over-expression of an RNAi-resistant pico
transgene
To test if the wing dysmorphology phenotype resulting from
overexpression of picoRNAiIR4/R3 was due to off-target effects
of the inverted repeat construct, we developed an RNAi-resistant
form of pico (picoIR) that could be used in genetic complementation
tests. This was done by incorporating numerous silent polymor-
phic mutations into a synthetic gene construct encoding the short
isoform of Pico (picoIR). Changes in the codon usage that we
introduced consequently meant that, in the regions targeted by the
RNAi, homology with the inverted repeat sequences was limited to
no more than 8 contiguous base pairs (Fig. 2). To assess the
resistance of ectopic pico to dsRNA for pico, we generated
transgenic flies capable of expressing Venus-tagged pico under
UAS-GAL4 control and analysed the levels of the Venus-tagged
ectopic protein in the presence or absence of the inverted repeat
constructs. Venus, a variant of enhanced Yellow Fluorescent
Protein, was readily detectible when the tagged protein was
ectopically expressed in the wing pouch under the control of
MS1096-GAL4. Notably, ectopic expression of Venus-Pico was
not modified by co-expression of picoRNAiIR4 or picoRNAiR3,
demonstrating that ectopic Pico is resistant to RNAi-mediated
 knockdown (Fig. 3A).

Next we tested whether the phenotypic effect of MS1096>G-picoRNAiIR4 or picoRNAiR3 could be rescued by overexpression of
Pico’ (Fig. 3B). Previously we reported that overexpression of wild
type pico short with MS1096-GAL4 resulted in modest tissue
overgrowth. Similarly, ectopic-tagged Venus-pico’ resulted in a
107% increase in adult wing size, indicating that the Venus tag
does not interfere with its ability to promote growth. When we
coexpressed pico’ together with picoRNAiR3 the adult wings
resembled those of ectopic pico alone, indicating that the reduced
wing size resulting from MS1096>G-picoRNAiIR3 was rescued by
expression of pico’. Notably, the effect of pico’ and picoRNAiR3 was
not simply additive, indicating that the level of Pico’ is likely to be
much more than two fold that of endogenous levels. In contrast,
ectopic pico’ failed to rescue the cramped wing resulting from
picoRNAiR3 overexpression (Fig. 3B). Taken together, these data
confirm that specific RNAi-mediated knockdown of pico results in
a tissue growth phenotype, and indicate that the tissue dysmor-
phology phenotype associated with picoRNAiR3 is attributable to an
off-target effect.

Discussion
Comprehensive validation of an RNAi-induced phenotype
requires demonstration that: i) the expression level of the intended
target is reduced by the RNAi; ii) the expression of any potential
off-targets is not affected; and, iii) the RNAi-induced phenotype
can be reversed by expression of the wild type protein; genetic
complementation should be with an RNAi-resistant form of the
gene, and not rely on overexpression of wild type mRNA that could sequester siRNA molecules from both off- and on-targets alike. Applying these principles to the validation of pico RNAi lines, we set about to determine whether the discrepancy in reported phenotypes for pico knockdown could be explained by potential off-target effects. Firstly, we assessed the levels of pico mRNA in wing discs expressing pico inverted repeat constructs. qRT-PCR revealed that both of the RNAi constructs tested (picoRNAiR4 and picoRNAiR2/R3) were effective in knocking down pico mRNA levels. The efficacy of picoRNAiR4 is consistent with our previous observations that levels of ectopically expressed epitope-tagged Pico were reduced in the presence of picoRNAiIR4 [10]. Next, we analysed potential off-targets in silico and in extracts. Unlike picoRNAiR4, picoRNAiR2/R3 was predicted to affect the expression of numerous off-target genes. qRT-PCR analysis confirmed that the expression level of 4 out of 5 of the predicted off-targets tested were indeed reduced by picoRNAiR2/R3. Notably, although the number of siRNA oligomers that matched the off-target genes was low (e.g. 5 predicted off-target 19 mers as compared to 482 on-target 19 mers, Table 1), they nevertheless drastically affected...
Transgenic is widely applicable to the validation of any RNAi general, the strategy that we have employed using synthetic correctly attributed to knockdown of the gene of interest. In specificity of RNAi-induced phenotypes to ensure they are targets. To do this, we tested the ability of a synthetic transgene to rescue importance of testing the specificity of RNAi lines experimentally. This highlights the importance of testing the specificity of RNAi lines experimentally. Off-targets were determined using dscheck (http://dscheck.rnai.jp/), which generates all possible 19 mers that can be theoretically generated from a longer dsRNA sequence and determines their homology to Drosophila genes identified by their Flybase ID and Celera Genomics (CG) number. The number of 19 mers matching each gene found by the sequence comparison are tabulated, where Mis = 0, Mis = 1, Mis = 2 correspond to the number of 19 mers with 0, 1 and 2 mismatches for each gene, respectively.

Table 1. List of potential off-targets for pico inverted repeat constructs.

| mis = 0 | mis = 1 | mis = 2 | FlyBase ID | CG number |
|---------|---------|---------|------------|-----------|
| 459     | 0       | 0       | FBgn0261811 | CG11940-PB |
| 459     | 0       | 0       | FBgn0261811 | CG11940-PB |
| 0       | 2       | 7       | FBgn0022787 | CG4261-PA  |
| 0       | 2       | 3       | FBgn0031988 | CG8668-PA  |
| 0       | 2       | 2       | FBgn0027866 | CG9776-PA  |
| 0       | 2       | 2       | FBgn0021760 | CG2435-PA  |
| 0       | 2       | 2       | FBgn0033460 | CG1472-PA  |
| 0       | 2       | 2       | FBgn0021760 | CG2435-PB  |
| 0       | 2       | 2       | FBgn0021760 | CG2435-PC  |
| 0       | 2       | 2       | FBgn0027866 | CG9776-PA  |
| 0       | 1       | 7       | FBgn0052251 | CG2251-PA  |
| 0       | 1       | 5       | FBgn0031116 | CG1695-PA  |
| 0       | 1       | 5       | FBgn0031116 | CG1695-PA  |
| 0       | 1       | 5       | FBgn00263289 | CG5462-PA |
| 0       | 1       | 5       | FBgn00263289 | CG5462-PA |
| 0       | 1       | 5       | FBgn00263289 | CG5462-PD |
| 0       | 1       | 5       | FBgn00263289 | CG5462-PD |
| 0       | 1       | 5       | FBgn00263289 | CG5462-PC |
| 0       | 1       | 3       | FBgn0033558 | CG12344-PA |
| 0       | 1       | 3       | FBgn0039554 | CG5003-PA  |
| 0       | 1       | 3       | FBgn00261388 | CG15720-PA |
| 0       | 1       | 3       | FBgn0053519 | CG30175-PA |
| 0       | 1       | 3       | FBgn0031299 | CG4629-PC  |
| 0       | 1       | 3       | FBgn0028474 | CG4119-PA  |
| 0       | 1       | 3       | FBgn0024329 | CG7717-PA  |
| 0       | 1       | 3       | FBgn0024329 | CG7717-PB  |
| 0       | 1       | 3       | FBgn0031299 | CG4629-PA  |
| 0       | 1       | 2       | FBgn0051301 | CG13101-PA |
| 0       | 1       | 2       | FBgn0028647 | CG1902-PA  |
| 0       | 1       | 2       | FBgn0037391 | CG2017-PB  |

Off-targets were determined using dscheck (http://dscheck.rnai.jp/), which generates all possible 19 mers that can be theoretically generated from a longer dsRNA sequence and determines their homology to Drosophila genes identified by their Flybase ID and Celera Genomics (CG) number. The number of 19 mers matching each gene found by the sequence comparison are tabulated, where Mis = 0, Mis = 1, Mis = 2 correspond to the number of 19 mers with 0, 1 and 2 mismatches for each gene, respectively.

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the gene expression of these off-target genes. This highlights the importance of testing the specificity of RNAi lines experimentally. To do this, we tested the ability of a synthetic transgene to rescue the RNAi-induced phenotypes. Phenotypic analysis of picoRNAiIR4 and picoRNAiR2,R3 revealed two distinct effects: picoRNAiIR2,R3 promoted loss of normal wing morphology, whereas picoIR4 induced a reduction in wing size. Importantly, we found that the effect of picoRNAiR4 was not rescued by overexpression of the picoRNAiR2,R3 refractory to this approach. Untranslated regions targeted by an RNAi construct can simply be omitted from any rescue construct or refractory to this approach. Untranslated regions targeted by an RNAi construct can simply be omitted from any rescue construct. Efficient gene synthesis of long stretches of DNA has become increasingly affordable in the last few years and rivals the cost of conventional cloning approaches. Almost any coding region of a gene can be rendered resistant because, on average, every third base can be substituted due to the degeneracy of the genetic code. However, regions containing multiple codons for either Methionine or Tryptophan, which do not allow sequence substitutions if the amino acid sequence is to be maintained, are refractory to this approach. Untranslated regions targeted by an RNAi construct can simply be omitted from any rescue construct. By extension, this approach could be utilised to test the role of critical domains or single amino-acids by engineering the synthetic transgene to harbour additional genetic changes that affect the coding sequence of the ectopic protein. The use of the GAL4-UAS system means that knockdown and complementation experiments can be performed in a tissue-specific or stage specific.
manner. Furthermore, a range of UAS-expression vectors are publicly available with fluorescent and epitope tags that can be used to readily monitor the ability of the transgene to elude RNAi-mediated knockdown. However, the use of GAL4-UAS system for genetic complementation tests is not without its limitations. Firstly, the use of a GAL4 driver (and to a lesser extent changes to codon usage) means that levels of the synthetic transgene are not endogenous. Secondly, whereas an on-target RNAi should only have an effect in cells where there is endogenous expression of the target gene, the protein produced from a synthetic rescue construct may be ectopically expressed in cells where there is no endogenous expression. This may be mitigated by the use of an enhancer trap GAL4 line driving GAL4 under the control of the target gene promoter, but enhancer trap lines that faithfully replicate the endogenous expression pattern only exist for a minority of genes.

Another strategy that has recently been developed to test genetic complementation in *Drosophila melanogaster* is based on the use of cross-species transgenic constructs [14,15]. This method uses the genomic DNA of different *Drosophila* species such as *Drosophila pseudoobscura* that are divergent enough from the host sequence to make the genomic constructs RNAi-resistant. However, this approach is also not without its limitations. Cross-species constructs may also show differences in their pattern of expression relative to the endogenous gene in *Drosophila melanogaster*, not least because of position effects associated with the insertion site of the transgene. In addition, the genomes of the donor species often carry non-synonymous substitutions affecting the amino-acid sequences of the gene products. Very minor changes in amino acid sequences can abolish functional rescue in interspecies crosses for example by drastically affecting the ability of proteins from divergent species to interact correctly with one another [16]. In addition, because the constructs are not tagged it is hard to readily assess whether protein levels are refractory to the effects of the RNAi.

In summary, we report here the results of experiments to test the specificity of two RNAi constructs for the *pico* gene that display different phenotypic effects. Notably one of the RNAi constructs affects the expression of various off-target genes despite the prediction that only a limited number of siRNA oligomers generated from the full-length dsRNA target these loci. We find the use of synthetic gene fragments resistant to knockdown by RNAi to be an effective approach to distinguish between specific and non-specific effects.

**Materials and Methods**

Fly husbandry and genetics

Flies were reared at 25°C under standard conditions. *picoIR2-3* RNAi lines came from NIG-Fly (National institute of Genetics: 11940R); *picoIR4* was described in a previous paper [10]. Phenotypic analysis of adult wings and effect of *pico* RNAi lines on *pico* mRNA expression was determined using the wing imaginal disc driver MS1096-GAL4. Genotypes were as follows:

1B, D  MS1096-GAL4
1B, D  MS1096-GAL4; UAS-picoRNAIR2/+; UAS-picoRNAIR3/+  1B  MS1096-GAL4; UAS-picoRNAIR4/+  1C  UAS-picoRNAIR4/+  1C  MS1096-GAL4/Y; UAS-picoRNAIR4/+  1C  UAS-picoRNAIR4/+; UAS-picoRNAIR3/+  1C  MS1096-GAL4/Y; UAS-picoRNAIR4/+; UAS-picoRNAIR3/+  2A,B  MS1096-GAL4/Y; UAS-Venus-pico/+  2A,B  MS1096-GAL4/Y; UAS-Venus-pico/+; UAS-picoRNAIR3/+  2A, B  MS1096-GAL4/Y; UAS-Venus-pico/+; UAS-picoRNAIR3/+  2A, B  MS1096-GAL4/Y; UAS-picoRNAIR3/+.

**Wing Area Analysis**

Male adult flies wings were dissected and fixed in 75% ethanol, and mounted on glass slides in Canadian Balsam mounting medium (Gary’s Magic Mountant) and examined by light microscopy. The wing area, exclusive of the alula and the costal...
cell, was measured using NIH ImageJ (http://rsb.info.nih.gov/ij/), n = 35 per genotype. To avoid observer bias in the measurements of wing areas, experimenters were blinded to the genotype of flies. A t-test was applied to test for a significant effect of MS1096-GAL4, UAS-picoRNAi on wing size by comparison with UAS-picoRNAi alone.

RNA isolation and qRT-PCR
3rd instar larval imaginal tissues were dissected in cold Phosphate Buffered Saline buffer, put in RNAlater (Invitrogen), quickly frozen in liquid nitrogen and stored at −80°C until isolation of RNA. 3 pools of imaginal discs were made for each condition tested (MS1096-GAL4; MS1096>picoIR4; MS1096>picoIR2-3) corresponding to at least 24 imaginal discs/pool. RNA extractions were performed using the Ambion RNAqueous-Micro...
Kit (Invitrogen). RNA concentrations were measured at 260 nm and RNA integrity was evaluated on a 2% agarose gel. 1 μg of total RNA samples were subjected to reverse-transcription using High capacity RNA-to-cDNA kit (Applied biosystems/Invitrogen). Primer design was performed using Primer3 online software, http://frodo.wi.mit.edu/ [17]. The specificity of primers was assessed by sequencing of PCR products (GATC Biotech), and alignment of the resulting sequences by performing BLASTN against the Drosophila melanogaster transcriptome. cDNA were amplified in real time using the qPCR Master mix plus for power SYBR Green I assay (Invitrogen) and analysed with the StepOnePlus Real-Time PCR System (Applied Biosystems). Each run included triplicates of control cDNA corresponding to a pool of imaginal discs from MS1096-GAL4 and MS1096->picoRNAi lines, no-template controls and samples. The threshold cycle (Ct) was determined for each sample and control cDNA. A calibration curve was calculated using the Ct values of the control cDNA samples and the relative amounts of unknown samples were deduced from this curve. The level of expression for genes tested with different RNAi lines was compared to wild-type expression (in deduced from this curve). The level of expression for genes tested with different RNAi lines was compared to wild-type expression (in an MS1096-GAL4 strain) and expressed as a percentage of the latter.

RNAi-resistant construct design

Codon usage in the transcript picoRB was modified with the introduction of silent mutations to render it resistant to RNAi-mediated knockdown, whilst minimising the use of rare codons that are recognised by low abundance tRNA species. 187/493 bp were changed in the region targeted by picoRNAiIR4 and picoRNAiIR5 as shown in Fig. 2. pico was synthesised and subcloned into pDONR221 Gateway cloning vector (Invitrogen). Gateway LR reactions were performed to shuttle picoR from the pDONR221 entry vector into pTVW (UAST promoter with an N-terminal Venus tag) vector (Drosophila Genomics Resource Center) for expression in flies with an N-terminal Venus Tag. Transgenic flies were generated using P element mediated germline transformation of a w*118 strain.

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Author Contributions

Conceived and designed the experiments: DB VJ. Performed the experiments: VJ. Analyzed the data: DB VJ. Wrote the paper: DB VJ.

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