# A novel method for tissue-specific RNAi rescue in *Drosophila*

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## ABSTRACT

**Targeted gene silencing by RNA interference allows the study of gene function in plants and animals. In cell culture and small animal models, genetic screens can be performed—even tissue-specifically in *Drosophila*—with genome-wide RNAi libraries. However, a major problem with the use of RNAi approaches is the unavoidable false-positive error caused by off-target effects. Until now, this is minimized by computational RNAi design, comparing RNAi to the mutant phenotype if known, and rescue with a presumed ortholog. The ultimate proof of specificity would be to restore expression of the same gene product *in vivo*. Here, we present a simple and efficient method to rescue the RNAi-mediated knockdown of two independent genes in *Drosophila*. By exploiting the degenerate genetic code, we generated *Drosophila* <u>R</u>NAi <u>E</u>scape <u>S</u>trategy <u>C</u>onstruct (RESC) rescue proteins containing frequent silent mismatches in the complete RNAi target sequence. RESC products were no longer efficiently silenced by RNAi in cell culture and *in vivo*. As a proof of principle, we rescue the RNAi-induced loss of function phenotype of the eye color gene *white* and tracheal defects caused by the knockdown of the heparan sulfate proteoglycan *syndecan*. Our data suggest that RESC is widely applicable to rescue and validate ubiquitous or tissue-specific RNAi and to perform protein structure–function analysis.**

## INTRODUCTION

In model organisms, such as yeast, worm, fly and mouse, significant progress has been made to create tools for the manipulation of individual genes, such as random mutagenesis and gene targeting altering the genomic sequence, or RNA interference (RNAi) targeting of mRNA for degradation. Lethality and pleiotropy compromise loss-of-function mutant studies for many genes, rendering identification of late or tissue-specific phenotypes difficult. Gene knockdown by RNAi, when combined with the transgenic *Gal4*/UAS system in *Drosophila* (1), is cell-autonomous and inducible in space and time. It is also nontransitive (2,3), allowing isoform-specific knockdown. *Drosophila* genome-wide RNAi libraries have been generated recently, making large-scale tissue-specific reverse genetic screens possible [(4) and http://www.shigen.nig.ac.jp/fly/nigfly].

A major problem of RNAi, however, is off-target effects (5–7). Even for a fully annotated genome, tolerance for mismatches makes the design of unique RNAi target sequences very difficult. This is particularly compounded in *Drosophila*, where long double-stranded RNA of several hundred base pairs is usually required for efficient knockdown (8), thus increasing the risk of hitting unrelated genes because of tandem trinucleotide repeats or sequences homologous to the RNAi target (5,6,9,10). Apart from that, activation of the innate immune system (11) and competition with the endogenous RNAi machinery (12) have been reported in mammals.

Clearly, improved RNAi reliability by experimental validation is highly desirable, ideally through rescue of the RNAi phenotype by the targeted gene itself. We set out to test if alteration of the cDNA at the nucleotide level while maintaining the encoding of the native protein would circumvent RNAi-mediated silencing and thus permit rescue. We then used this approach to unravel a novel function for the *Drosophila* proteoglycan *syndecan (sdc)* in tracheal development.

## MATERIALS AND METHODS

### RNAi design and synthesis

The *Sdc* RNAi target region was selected to be larger than 300 bp, to target all existing splice forms, and not to contain sequences longer than 17 bp that are identical in other loci. UAS::*Sdc*^dsRNA was designed as a genomic-cDNA hybrid construct targeting 357 bp of *sdc* exons 6 and 7.

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The forward genomic part consisting of *sdc* exon 6, intron 6 and exon 7 was cloned from gDNA into pUAST, followed by the 3-kb *sdc* intron 5 to improve genomic stability. Then, exons 6 and 7 cDNA were cloned in reverse orientation and transformed into Sure2 repair-deficient bacteria (Stratagene) to avoid hairpin repair (Figure 4) (13). The UAS::$w^{dsRNA}$ line was obtained from D. Smith (13).

## RESC design

The boundaries of the RESC sequence were defined by the RNAi target boundaries. In total, 235 silent mutations were introduced into the 687-bp *w* RNAi target region and 72, 33 and 17 mutations to obtain 6-mer, 12-mer or 24-mer changes into the 357-bp *Sdc* RNAi target region (Supplementary Figure 1). GC content, restriction sites, splice sites, repetitive nucleotide sequences and unavoidable gaps (Methionine, Tryptophan) were taken into account, leaving stretches of maximally five nucleotides unchanged. Preferably, amino acids with different codon usage were swapped. Finally, the RESC sequence was verified at amino acid level to be wild type (align p).

## $w^{RESC}$ synthesis

$w^{RESC}$ was synthesized with 16 desalted oligonucleotide primers (Invitrogen) in alternating directions, each 70 nucleotides in length ($+/-1$ or 2 bp, G or C at 3' end) and with 20-bp overlap each. To reach unique restriction sites for further cloning, the two flanking primers were extended over the target region to contain wild-type sequence at their 5' end. Primer pairs (first round) or purified PCR fragments together with the two outside primers (from round 2 on) were subjected to standard PCR elongation (Figure 1b). Finally, a 795-bp BspEI–SnaBI fragment was obtained from four rounds standard PCR and cloned to exchange the wild type *w* BspEI–SnaBI fragment in pUAST.

## $sdc^{RESC}$ synthesis

For $sdc^{RESC}$ every 6th, 12th or 24th nucleotide within the exon 6/7 RNAi target region was mutated (Supplementary Figure 1). $sdc^{RESC}$ was synthesized with eight primers in alternating directions, 72–76 bp in length ($+/-1$ or 2 bp, G or C at the 3' end) and with 22–25-bp overlap. To reach the unique KspI restriction site in exon 5 the RESC sequence was extended by wild-type sequence. The RESC fragment was obtained from three rounds standard PCR (Figure 1b) and cloned to exchange the *sdc* wild-type sequence in pUAST-*sdc*. All inserts were verified by sequencing.

## Cell culture

Two Mio *Drosophila* S2 cells per 1 ml Drosophila serum-free medium (Invitrogen) were transiently transfected with 5 µl Cellfectin (Invitrogen) and 3 µg total DNA (pUAST-*sdc* with or without RESC modification, pUAST-$Sdc^{dsRNA}$ or empty vector, and pMT-*Gal4*, 1:1:1 ratio), induced with 700 µM $CuSO_4$ 5 h after transfection, and harvested 48 h later.
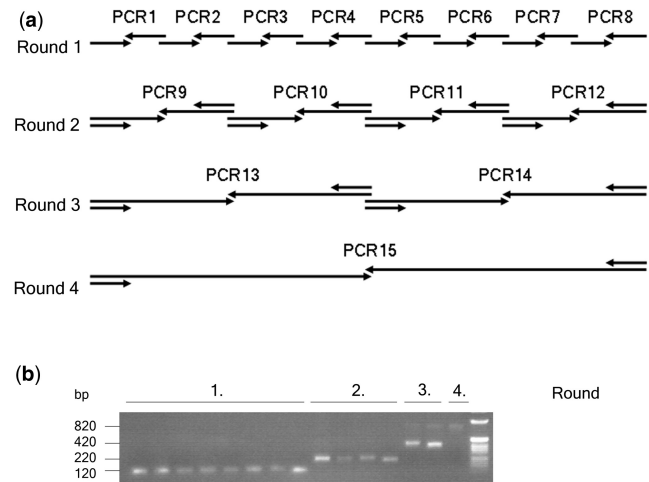


**Figure 1.** Generation of RESC DNA from oligonucleotides. (**a**) Illustration of primer positions and PCR reactions required to generate a RESC DNA fragment in four rounds of PCR starting from 16 oligonucleotides. In each round, the two corresponding PCR products and outside primers from the previous round are used for amplification. Arrows indicate direction of elongation. (**b**) Agarose gel showing $w^{RESC}$ PCR products and their size obtained in four PCR rounds as illustrated in (a).

## Western blot

S2 cells or whole anesthetized flies were homogenized in lysis buffer (1% Triton X-100, 140 mM NaCl, 10 mM Tris) with complete protease inhibitor cocktail (Roche), boiled in reducing SDS sample buffer and loaded on 4–15% Biorad Ready Tris–HCl gels, and transferred to positively charged Zeta-probe blotting membrane (Biorad). The membrane was blocked with 0.5% Casein (Merck) in 0.6 M NaCl, incubated with α-Sdc (14) or α-tubulin antibody, and detected with alkaline phosphatase-conjugated secondary antibody (Promega) and CSPG (Tropix) on Amersham Hyperfilm.

## Fly lines

Fly stocks were kept on standard fly food. Transgenic flies ($sdc^{RESC}$, $w^{RESC}$) were generated by standard P-element-mediated germline transformation. Transgenic lines were generated from $sdc^{RESC}$ with 6-mer changes. UAS::$w^{dsRNA}$ flies were obtained from D. Smith, btl-*Gal4* from the Japan National Institute of Genetics, all other lines from the Bloomington Stock Center. *sdc* cDNA was obtained from J. Lincecum, $sdc^{23}$ stock from G. Vorbrüggen.

## Phenotype analysis

The tracheal morphology of third instar larvae was analyzed in a filet preparation by stereomicroscopy. Larvae were cold anesthetized in 4°C PBS and dissected with iris spring scissors (Fine Science Tools) from the ventral side. Although the lumen of the tracheae is easily visible with bright field microscopy, this does not reliably reflect morphology because air filling is not always complete. Hence, reporter lines were generated that express membrane-bound CD8-GFP fusion protein in the trachea with btl::*Gal4*. Eye colors of 5–7-day-old adult flies were analyzed under white light with a stereomicroscope (Leica).

## Primers

*sdc RESC, 6-mer changes.*
For 1
Atcgatggtccgcgcgatcggtggcaacgatggagatattacagagcgcggacc
gggtgctggtggtagcaatgtg

For 2
Tcaatagtcagccgtccgatacaaaaggcattgatcataggccgaacggaaac
gaggtggtgataatgagtgag

For 3
Tcccaaccgggaatactggcagctgtgattggaggtgcggtcgtcggcctcctgt
gcgcgattctcgtcgtgatg

For 4
Gacgaaggatcgtatgccctggatgagcctaagcgttcgccagccaataattcc
tatgccaagaacgcgaacaac

Rev 1
TTTGTATCGGACGGCTGACTATTGACGTTGGTG
TTCGGGTCTAGTTCATGCACATTGCTACCACCA
GCACCCGG

Rev 2
CAGCTGCCAGTATTCCCGGTTGGGGAGAAGAAA
CTGGACGTCCGATCATCCTCACTCATTATCACC
ACCTCGTTTC

Rev 3
CATCCAGGGCATACGATCCTTCGTCTTTCTTTCG
CATTCGGTAGACGATAAACATCACGACGAGAA
TCGCGCACAG

Rev 4
GAGGTACCCTCGAGCCGGAGCTCGCATATTCTC
ACGCATAGAATTCTCTGTTGTTCGCGTTCTTGG
CATAGG

*sdc RESC, 12-mer changes.*
For 1
Atcgatggtccgcgcgatcggtggcaacgatggagatattacagagcgcggacc
gggtgctggtggcagcaatgtg

For 2
Tgaatagtcagccctccgatacaaagggcattgatcacaggccgaacggcaac
gaggtggtcataatgagcgag

For 3
Tcccagcccggaatactggctgctgtgattggcggtgcggtcgttggcctcctctg
cgccattctcgtggtcatg

For 4
Gacgagggatcgtatgcgctggatgagccaaagcgttcgccggccaataattc
gtatgccaaaaatgcgaacaac

Rev 1
TTTGTATCGGAGGGCTGACTATTCACGTTGGTG
TTGGGGTCTAATTCGTGCACATTGCTGCCACCA
GCACCCGG

Rev 2
CAGCAGCCAGTATTCCGGGCTGGGAGAAGAAG
CTGGACGTGCGATCATCCTCGCTCATTATGACC
ACCTCGTTGC

Rev 3
CATCCAGCGCATACGATCCCTCGTCTTTCTTCCT
CATTCGGTACACGATAAACATGACCACGAGAAT
GGCGCAGAG

Rev 4
AGAGGTACCCTCGAGCGCGAGTCGCATTATCTC
ACGCGTAGAATTCGCGGTTGTTCGCATTTTTGG
CATACG

*sdc RESC, 24-mer changes.*
For 1
Atcgatggtccgcggatcggtggcaacgatggagatattacagagcgcggacc
gggtgctggtggcagcaacgtg

For 2
Tgaatagtcagccctccgacacaaagggcattgatcacaggcccaacggcaac
gaggtggtcatcatgagcgag

For 3
Tcccagcccggaattctggctgctgtgattggcggtgccgtcgttggcctcctctg
cgccatactcgtggtcatg

For 4
Gacgagggatcctatgcgctggatgagccaaagagatcgccggccaataattc
gtatgcgaaaaatgcgaacaac

Rev 1
TTTGTGTCGGAGGGCTGACTATTCACGTTCGTG
TTGGGGTCTAATTCGTGCACGTTGCTGCCACCA
GCACCCGG

Rev 2
CAGCAGCCAGAATTCCGGGCTGGGAGAAGAAG
CTCGACGTGCGATCATCCTCGCTCATGATGACC
ACCTCGTTGC

Rev 3
CATCCAGCGCATAGGATCCCTCGTCTTTCTTCCT
CATGCGGTACACGATAAACATGACCACGAGTAT
GGCGCAGAG

Rev 4
AGAGGTACCCTCGAGCGCGAGTGCCATTATCTC
ACGCGTAGAACTCGCGGTTGTTCGCATTTTTCG
CATACG

*w RESC primers.*
For 1
Ggctccggatggcggcagctggtcaaccggacacgcggactattctgcaacga
gcgacacatcccagctc

For 2
Gagtcgcgtaccctggagagttgctggcagtcatgggtagctcaggcgcgggc
aagaccacgctcctgaac

For 3
Ccaggtatccccgtcgggcatgaggctcctgaacgggcagccagtcgatgcaa
aagaaatgcaagcgcgg

For 4
Attgggtcgctcaccgcgcgagagcatctcatctttcaagcgatggtccgaatgc
cgcggcacctcacttac

For 5
Tcatacaagaactcagcttgtcgaagtgccaacataccattataggcgtcccgg
gacgggtcaagggactaag

For 6

Tgcatcggaagcgctgacggaccctccactgcttatatgtgacgaacctacgag
tggcctagatagtttc

For 7

Aaactcagccaaaagggaaagacggtgattctcactatccaccaacctagctct
gaactattcgaactgttc

For 8

Gagtcgcctttctaggaaccccttcggaggcagtagatttcttctcatacgtgggt
gcccagtgtcctac

Rev 1

CTCTCCAGGGTACGCGACTCCACAAACATTCTT
CAGTAAGTGCTTTCTAGGAGCTGGGATGTGTCG
CTCG

Rev 2

TGCCCGACGGGGATACCTGGATCCCTTGTGGG
GACCGGAAAGCCAGCGCGTTCAGGAGCGTGGT
CTTGCC

Rev 3

CGCGCGGTGAGCGACCCAATGAATAGATCGTC
TTGTTGCACGTACGCACACCGCGCTTGCATTTC
TTTTG

Rev 4

CAAGCTGAGTTCTTGTATGACTTGGTCGACTCG
TGCGACGCGTTGGCGGTAAGTGAGGTGCCGCG
GCATTC

Rev 5

CCGTCAGCGCTTCCGATGCAAAGGCTAGCCTTT
TCCGCTCGCCTCCGCTTAGTCCCTTGACCCGTCC
CGG

Rev 6

TTTCCCTTTTGGCTGAGTTTCTTAAGGACTTGCA
CCACGGAATGAGCAGTGAAACTATCTAGGCCA
CTCG

Rev 7

GGTTCCTAGAAAGGCGACTCGCCCTTCAGCCAT
AAGCAGAATCTTATCGAACAGTTCGAATAGTTC
AGAG

Rev 8

CCTACGTAAAAGTCCGCCGGATTGTAGTTGGTA
GGACACTGGGCACCCAC.

## RESULTS

### Rescue of RNAi-mediated *w* loss-of-function

RNAi specificity is ideally demonstrated by re-expression of the knocked-down gene. However, a transgene containing the original RNAi target sequence is subject to RNAi as well, and deletion of the target sequence while maintaining gene function is possible only if noncoding sequence is targeted. A possible way to circumvent this problem may be to introduce silent mismatches into the rescue construct such that the mRNA sequence is altered while maintaining the protein coding sequence. The degenerate nature of the genetic code permits sequence alterations for 18 out of the

20 amino acids and the stop codon (exceptions are Methionine and Tryptophan), allowing approximately one change every third nucleotide of a cDNA. We reasoned that even when considering the tolerance of the RNAi for some mismatches, this degree of alteration would be sufficient to escape the RNAi machinery.

To test this idea *in vivo* and *in vitro*, we chose to rescue two genes in *Drosophila*, one with a known and one with a novel phenotype. The *white (w)* gene is required for red eye color in *Drosophila* and was previously shown to be effectively knocked down by RNAi *in vivo* (13,15) (Figure 2a). We decided to rescue *w* with a rescue construct containing 235 silent mutations in the 687-bp target sequence and named it *w*RESC (RNAi Escape Strategy Construct). Since RNAi in *Drosophila* is not transitive, only the *w* RNAi target sequence but not the flanking sequences were mismatched. To introduce this high number of mutations, site-directed mutagenesis by which only a few new mutations can be generated per round of PCR is not an efficient approach. In addition, a natural RESC template that could be used for amplification does not exist. Hence, we decided to create the new sequence completely from custom-synthesized oligonucleotides. We divided the target fragment including overlapping wild-type sequence on both ends for further cloning into 16 oligonucleotides, 70–75-nt long and with an overlap of 20–25 nt. By successive elongation of the oligonucleotides and purification of intermediate fragments the final *w*RESC fragment was obtained after four rounds of PCR (Figure 1a and b). The *w*RESC fragment was cloned to replace the corresponding part of the wild-type sequence of *w* in a standard vector used for P-element-mediated germline transformation, where *w* routinely serves as a marker gene expressed under its own promoter. The original vector in which the *w* sequence was unaltered served as a control. Several lines of both *w* and *w*RESC transgenic flies were obtained which restore eye color to *w* null mutant flies, showing that the *w*RESC transgene is functional (Figure 2b). However, whereas *w* RNAi efficiently targeted combinations of up to three *w* transgenes, resulting in complete loss of eye color (Figure 2a and c), *w*RESC was not targeted, as judged by the fact that *w*RESC and *w* RNAi + *w*RESC flies show identical eye color (Figure 2b).

### Sdc RESC in vitro

Available mutants of the single fly homolog of the vertebrate transmembrane heparan sulfate proteoglycan *syndecan (sdc)* are all derived from imprecise excisions causing deletions that potentially affect a nearby gene on the complementary strand called *Smad anchor for receptor activation (sara)*. *sara* shares the enhancer region with *sdc*, making *sdc* an ideal candidate to confirm the validity of the RESC strategy.

While a maximum number of nucleotides were mismatched in *w*RESC, we now first tested to what extent the nucleotides in *sdc*RESC had to be exchanged in order to escape RNAi. We generated three UAS::*sdc*RESC vectors for rescue experiments, with mismatches every 6th, 12th or 24th bp in exons 6 and 7, giving rise to 72, 33 and 17 mutations in the 357-bp target region, respectively
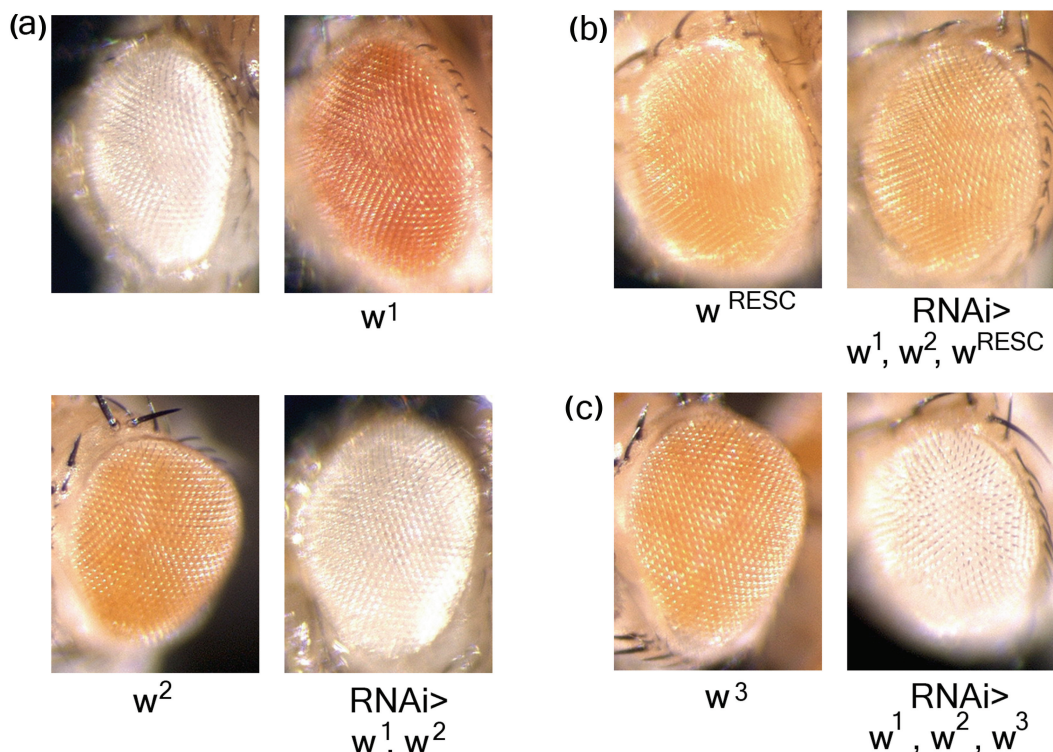
**Figure 2.** Photographs (100×) from eyes of age-matched adult flies demonstrating eye color rescue with $w^{RESC}$. $w$ RNAi causes total loss of eye color, which is restored with $w^{RESC}$ but not $w^3$. Genotypes are (**a**) $w^{1118}$ (upper left); $w^{1118}$;tub::$Gal4,w^{+(1)}/+$ (upper right); $w^{1118}$;;UAS::$w^{dsRNA},w^{+(2)}/+$ (lower left); $w^{1118}$;;tub::$Gal4,w^{+(1)}/UAS::w^{dsRNA},w^{+(2)}$ (lower right). (**b**) $w^{1118},w^{+(RESC)}/w^{1118}$ (left); $w^{1118},w^{+(RESC)}/w^{1118}$;; tub::$Gal4,w^{+(1)}/UAS::w^{dsRNA},w^{+(2)}$ (right) (**c**) $w^{1118},w^{+(3)}/w^{1118}$ (left); $w^{1118},w^{+(3)}/w^{1118}$;; tub::$Gal4,w^{+(1)}/UAS::w^{dsRNA},w^{+(2)}$ (right).

(Supplementary Figure 1). In *Drosophila* S2 cells in culture, all *sdc* constructs were expressed at similar levels in the absence of RNAi, but under *sdc* RNAi conditions the expression levels decreased with increasing similarity between hairpin sequence and target region in the rescue construct. Expression of *sdc*$^{RESC}$ with 24-mer-mismatch spacing was not different from wild-type *sdc*, suggesting that a mutation every 24th base pair is insufficient to escape silencing by RNAi. The 12-mer changes resulted in some level of escape; however, the strongest effect was observed with the 6-mer construct (Figure 3a).
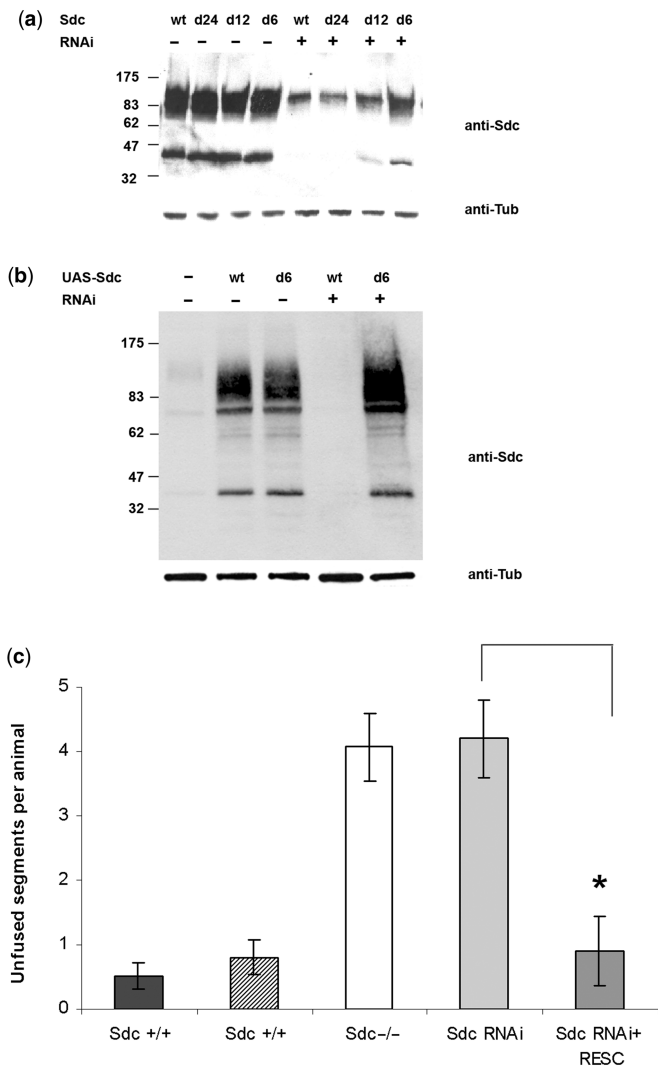
### *Sdc* RESC *in vivo*

To confirm the RESC effect *in vivo*, transgenic flies were generated that inducibly express *sdc*$^{RESC}$ with 6-mer mismatches. In the absence of RNAi, both *sdc* and *sdc*$^{RESC}$ were efficiently translated, while under RNAi conditions most wild-type *sdc* but very little, if any, *sdc*$^{RESC}$ was degraded (Figure 3b).

Both, *sdc* mutants and RNAi animals, show an almost identical nonfusion phenotype in the dorsal branches of the tracheal system (Schulz *et al.*, submitted for publication). When *sdc*$^{RESC}$ was expressed in the tracheae of *sdc* RNAi animals, the dorsal branch phenotype was completely reversed (Figure 3c), confirming that Sdc is necessary for tracheal development and that the RESC technique permits tissue-specific rescue of RNAi-mediated phenotypes.
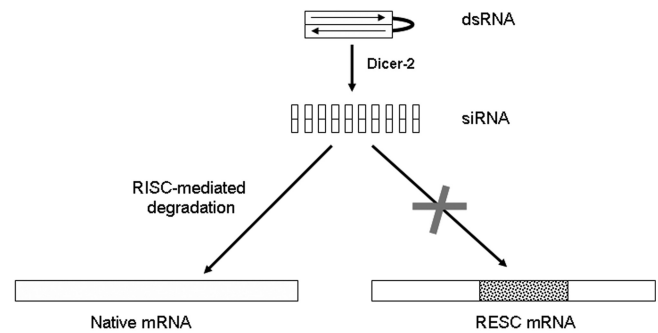
## DISCUSSION

The discovery of RNAi and its exploitation for the versatile, rapid and efficient study of gene function has the potential to revolutionize genetics and genetic screens in small model organisms such as *Drosophila* and *Caenorhabditis elegans*. However, a major drawback of RNAi-mediated gene targeting is off-target effects, especially in *Drosophila* where long dsRNA give rise to many short dsRNA species. Thus far, off-target effects were addressed by *in silico* analysis of the RNAi sequence, use of more than one nonoverlapping RNAi per targeted gene, rescue by a putative ortholog or paralog, or comparison to known mutant phenotypes (16,17). The latter is of particular importance for conclusions about biological function but limited by the fact that null alleles are only available for a minority of genes, and that even among those not all show easily discernable phenotypes. The most powerful proof of RNAi specificity would be rescue by the endogenous target gene itself. Here, we present a simple method that exploits the degenerate genetic code to introduce frequent silent mismatches in the entire target region in an otherwise wild-type rescue construct. On average, exchange of every third base in a coding sequence is possible. Our data indicate that exchanging every sixth nucleotide—equivalent to three to four mismatches in a ~21-bp dsRNA—only in the segment targeted by the RNAi is sufficient to rescue RNAi mediated knockdown. In contrast to the *in vivo* experiments, the

**Figure 3.** $sdc^{RESC}$ rescue *in vitro* and *in vivo*. (**a**) αSdc western blot from *Drosophila* S2 cells transiently transfected with p*MT-Gal4*, with (+) or without (–) UAS::$Sdc^{dsRNA}$, and UAS::*sdc* with wild-type (wt), or RESC sequence (Δ24, 24-mer changes; Δ12, 12-mer changes; Δ6, 6-mer changes). (**b**) αSdc western blot from adult flies ubiquitously expressing Sdc without (wt) or with (Δ6) RESC sequence, in the presence (+) or absence (–) of ubiquitous *Sdc* RNAi. (**c**) Quantification of unfused segments of tracheal dorsal branches per animal, in wild-type ($sdc^{+/+}$), $sdc^{23}$ homozygous ($sdc^{-/-}$) mutants, tracheal *Sdc* RNAi alone, or with tracheal rescue $sdc^{RESC}$. $n \geq 10$; error bars = CI; *$P < 0.01$.



**Figure 4.** Illustration of the RESC rescue principle. Small interfering nucleotide duplexes (siRNA) derived from cleavage of long, double-stranded RNAi by the RNase *Dcr-2* guides the RNA-induced silencing complex (RISC) complex to complementary mRNA that is subsequently cleaved and degraded. RESC constructs are mismatched in the RNAi target region and therefore escape RISC-mediated degradation.

number of mutations required to modify the complete target sequence of the rescue construct. Instead, we suggest introducing mutations by using long, overlapping oligonucleotides as templates for amplification and synthesis of the entire RESC fragment. Assuming oligonucleotides that are 80-nt long and overlap by 20 bp, synthesis of a target region of 500 bp or less requires three PCR rounds or seven reactions with 8 primers, for target regions between 500 and 1000 bp four PCR rounds with 16 primers suffice. Due to the transitive nature of *Drosophila* RNAi, it is sufficient to mismatch only the direct RNAi target sequence, thus the size of the RESC fragment is independent from the total gene product size.

To simplify future RESC design, we created a simple and user-friendly web-based tool to generate RESC and the necessary oligonucleotides sequences for any gene at: https://med.kuleuven.be/cme-mg/lng/RESC/.

To demonstrate the feasibility and functionality of RESC, we rescued phenotypes associated with two independent genes *in vitro* and *in vivo*. The eye color phenotype of the *w* gene was restored by $w^{RESC}$ containing mismatches in 212 of 229 codons of the target region, but not in the nontargeted sequence. The targeting of *sdc* represents a common case where despite the availability of putative null alleles, their specificity cannot be completely established. In this case the combination of RNAi and RESC reveals novel tissue-autonomous and gene-specific functions. Furthermore, once a RESC construct is generated it can be exploited further in rescue-based structure–function analysis.

The combination of the transgenic RNAi and RESC rescue with the *Gal4*/UAS system and its variations offers several advantages. First, wild-type protein can be knocked down and exchanged for by wild-type or mutant protein using the same promoter at any time during development, and in any chosen tissue. Second, tissue-specific expression avoids 'anatomical off-target effects', in contrast to systemic RNA injection. Third, without *Gal4* both UAS::dsRNA and UAS::RESC can be kept in a combined stock in off-modus, avoiding modifier accumulation over several generations of stock keeping. Fourth, the effects are dominant, making screens more feasible.

rescue appears to be incomplete *in vitro*. Yet, as opposed to the stable expression under *in vivo* conditions, the expression of the transgenes in cell culture was achieved by triple transient transfections, and it is unlikely that all cells with knockdown also express the rescue construct. Constructs with mismatch of every 12th nucleotide or less, yielding ∼21-bp fragments with only two peripheral or a single central mismatch, were still largely degraded. This confirms that RNAi target recognition is extremely sequence specific and several mismatches per short dsRNA still allows for some translation (15,18,19).

When RNAi is performed with long hairpins of up to 1000 bp such as in *Drosophila* (8), site-directed mutagenesis would be too time consuming to introduce the high

Our technique has implications beyond *Drosophila*. In mammals, RNAi is already successfully used to knock down dominant mutant human disease genes such as Huntingtin (20,21), but in human gene therapy a problem may arise from targeting of the endogenous wild-type copy (20). A RESC-based approach rescue might be a valuable strategy to prevent this.

The main limitations of the *Gal4*-driven RNAi/RESC combination are intrinsic to the *Gal4*/UAS system. First, RESC expression levels are not endogenous and, due to position effects, subject to variation between the transgenic lines. The latter may be overcome by using a P-element replacement strategy or φC31 integrase to target a common locus. Overexpression is more difficult to avoid when using the *Gal4*/UAS system alone, since lowering expression by decreasing the temperature will also affect RNAi performance. To uncouple RNAi and rescue, two independent systems with different induction modes, such as the *Gal4* and *lexA* systems may be used simultaneously (22). Second, the use of tissue-specific driver lines can only cause knockdown in cells where the target mRNA is present, but it induces expression in all cells expressing the driver, which is not necessarily identical. Here, the endogenous promoter of the gene of interest would be ideal. Another limitation is that synonymous codons are not necessarily expressed with the same accuracy (23) and at the same level (24), but this is probably of minor importance given the efficiency of the rescue we observe. In our web-based tool we suggest the most frequently used alternative codon.

An alternative to the RESC approach might be the use of cDNA from other *Drosophila* species. However, finding a related species with the right balance between sufficient change at the nucleotide level to avoid knockdown and no critical change at the amino acid level in order to fully restore function is not an easy task.

In summary, the combination of RESC rescue with large-scale RNAi libraries are a powerful approach to study gene function *in vitro* and tissue-specifically *in vivo* with high reliability because off-target effects can be excluded experimentally.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

## ACKNOWLEDGEMENTS

## FUNDING

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