Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays

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To evaluate the specificity of long dsRNAs used in high-throughput RNA interference (RNAi) screens performed at the Drosophila RNAi Screening Center (DRSC), we performed a global analysis of their activity in 30 genome-wide screens completed at our facility. Notably, our analysis predicts that dsRNAs containing ≥19-nucleotide perfect matches identified *in silico* to unintended targets may contribute to a significant false positive error rate arising from off-target effects. We confirmed experimentally that such sequences in dsRNAs lead to false positives and to efficient knockdown of a cross-hybridizing transcript, raising a cautionary note about interpreting results based on the use of a single dsRNA per gene. Although a full appreciation of all causes of false positive errors remains to be determined, we suggest simple guidelines to help ensure high-quality information from RNAi high-throughput screens.

The use of RNAi-mediated gene knockdown in cell-based experiments provides a direct link from sequence to the target gene's function[1]. The usefulness of this approach depends critically on the assumption that gene silencing is highly specific, that is, limited to the knockdown of the targeted gene without interfering with the expression or function of other genes or proteins. Studies in mammalian cells, however, have shown that RNAi can lead to the degradation of nontargeted mRNAs that happen to contain a cross-hybridizing region to the small interfering RNA (siRNA) trigger[2,3] or to the translational silencing of unrelated transcripts by siRNAs acting as microRNAs[4,5]. Additionally, some siRNAs can induce common, nonspecific changes in gene expression (such as the interferon response) in a sequence-independent manner[6]. These unintended effects have been collectively referred to as off-target effects, which if not controlled for can seriously limit the utility of RNAi.

Although RNAi has been widely used in *Caenorhabditis elegans* and *D. melanogaster* research[6], off-target effects were not thought to be a major problem in these organisms. First, long dsRNAs (instead of siRNAs) are used for RNAi in *D. melanogaster* and *C. elegans* because these organisms, in contrast to mammals, have no interferon response to dsRNAs. Second, it is widely presumed that the large excess of 'good' or 'specific' siRNAs generated from a long dsRNA by the Dicer enzyme[7] outcompetes the potential sequence-specific off-target effect associated with the odd 'bad' or 'nonspecific' siRNA. Surprisingly, this argument has remained experimentally untested, although biocomputational predictions have started to call this into question[8,9].

The need for a more definite answer takes on renewed urgency in light of the proliferation of high-throughput RNAi screens performed in *C. elegans* and *D. melanogaster* to systematically interrogate gene function at near- or full-genome scale. The quality of information derived from such screens relies heavily on the fact that false positive and false negative error rates can be evaluated and ideally kept low, and in particular that long dsRNAs do not cause off-target effects. The large collection of dsRNAs available at the DRSC (http://flyrnai.org)[10,11], coupled with the completion of over 30 genome-wide screens in *D. melanogaster* cultured cells, provided an excellent experimental opportunity to systematically analyze the aggregate results of the screens and to estimate the false positive and false negative error rates inherent to the methodologies and reagents. Overall, we observed good reproducibility within screens confirming that the screening platform and high-throughput screening methodologies were robust. Similarly, based on screens where the major players in a pathway had been previously identified, we found that the rate of false negatives was not a major issue. Although these findings underscored the reproducibility and effectiveness of the experimental technique and the various cell-based assays used, they did not address the issue of false positives owing to off-target effects. As a careful *in vivo* validation of the 100–300 genes typically identified in a screen is not practical, we performed a computational meta-analysis based on the results from the large number of different screens performed in the DRSC. Notably, our analysis showed that off-target sequences that are identified *in silico* in long dsRNAs can lead to off-target effects and contribute to a significant false positive error rate. Further, we present evidence that the presence of an off-target sequence is sufficient to knock down the cross-hybridizing transcript, raising a cautionary note in the interpretation of results based on the use of a single dsRNA.

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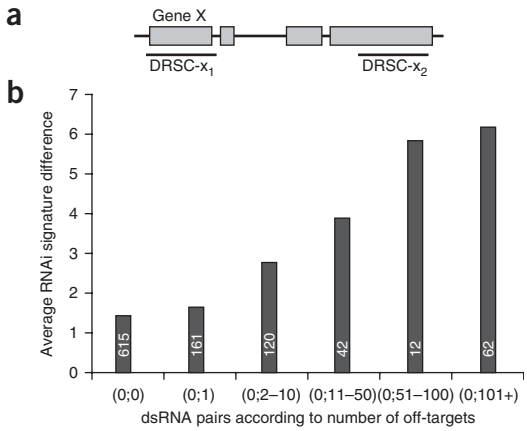

Figure 1 | Signature difference across 30 screens shown by pairs of dsRNAs that differ in their number of predicted off-target sequences but target the same gene. (**a**) Schematic representation of a gene targeted by two distinct dsRNAs (DRSC-X_1 and DRSC-X_2) in the DRSC collection. (**b**) We developed a simple string search algorithm (http://flyrnai.org/RNAi_find_frag_free.html), to determine whether each quality-controlled dsRNA contained any 21-nt perfect match (no gaps allowed) to one or more untargeted *D. melanogaster* genes (annotated in Berkeley Drosophila Genome Project (BDGP), release 3.2). Pairs of distinct dsRNAs targeting the same gene were placed in six categories, according to the number of off-target sequences predicted for each dsRNA. In each category, one of the dsRNAs has zero off-targets and the other has either zero (0;0), one (0;1) or more (0;2–10), (0;11–50) and so on off-targets. Within each category, we examined whether dsRNAs in a given pair caused the same outcome (30 genome-wide screens analyzed) or differed. Each dsRNA in the pair scored as a hit in a subset of screens. The signature difference for each pair is the number of screens found in the symmetric difference of these two subsets. The average signature difference in each category corresponds to the sum of the signature differences of pairs divided by the number of pairs within each category. The number of pairs analyzed in each category is indicated.

RESULTS

Evidence of false positives in genome-wide RNAi screens

The initial dsRNA library available at the DRSC was based on early annotations[12,13] and targeted all predicted *D. melanogaster* open reading frames (ORFs). In several cases, two adjacent ORFs originally thought to be distinct were later re-annotated as a single gene (**Fig. 1a**). These revisions resulted in a limited redundancy in our library, such that 1,372 genes were targeted by two or more dsRNAs. This fortuitous event allowed us to monitor how pairs of dsRNAs targeting the same gene behaved across 30 genome-wide screens. To minimize differences in knockdown efficiency, we selected only dsRNAs that passed high quality control for concentration and integrity. Surprisingly, in a substantial number of cases, only one of two dsRNAs targeting the same gene led to an observable phenotype in a given screen, resulting in a signature difference between the dsRNAs (**Fig. 1b**). Assay noise alone is unlikely to be responsible for the difference observed, as the overall results from primary screens showed good reproducibility. Instead, it suggested that the signature difference might be linked to the sequence of dsRNAs in such pairs, perhaps through a difference in predicted off-target sequences.

Using a simple algorithm[11], we determined whether any of the possible 21-mers present in a long dsRNA might be complementary to sequences found in other *D. melanogaster* transcripts, and found that 17.6% of the 21,306 dsRNAs in our collection showed one or more off-targets (data not shown). We then specifically examined whether these computationally predicted off-targets were the source of the discrepancy noted for some dsRNA pairs targeting the same gene. We selected pairs in which one dsRNA always lacked predicted off-targets, and the other contained either no or increasing numbers of off-targets. We binned the pairs into six groups according to the number of off-targets predicted in the second dsRNA and calculated the signature difference within each group. This analysis indicated a clear increase in the average signature difference from bin to bin as differences in the number of predicted off-targets increased between each pair (**Fig. 1b**). This was the first indication that sequence-specific off-target effects might be associated with a dsRNA and contribute to false positives.

Analysis of off-target effects based on 19-nt homology

To investigate the functional consequence of having multiple predicted off-targets in dsRNAs, we analyzed how their presence might skew the likelihood of a dsRNA to score in any of the screens performed at the DRSC. As sequences of homology shorter than 21 nt can lead to off-target effects in mammalian cells[2,14], we varied the length of the perfect homology from 23 to 16 nt and recomputed the number of predicted off-target(s) in the DRSC dsRNA collection. For each set length, we calculated the predicted number of off-targets for the dsRNAs and assigned dsRNAs to four different groups (bins) according to their off-targets (0, 1, 2 or ≥ 3). Within each bin, we calculated a 'hit rate' by dividing the number of dsRNAs associated with a phenotype in at least one screen by the total number of dsRNAs contained in that bin. Glancing at the hit rate alone, the trend is clear: the rate increases with the number of predicted off-targets. A χ^2 analysis confirmed that the trend was significant and demonstrated that dsRNAs with perfect matches as short as 19 nt to genes other than their targets scored at a higher rate than predicted by chance alone. Sequence matches of 18 nt and even 17 nt appeared to be statistically relevant, but we found no significant correlation for homologies of 16 nt in length (**Table 1**). In light of the sharp drop-off in the χ^2 value for sequence matches below 19 nt, we set this length as the critical threshold for the rest of our experiments. At this threshold, about 61% of the dsRNAs in our library had no predicted off-targets, 32% had between 1 and 10, and 7% had 10 or more predicted off-targets (**Fig. 2a**). We next examined how the hit rate of dsRNAs varied with increasing numbers of off-targets. To make the analysis more stringent, we restricted it to dsRNAs that caused a phenotype in five or more primary screens. When compared to dsRNAs with no off-targets, there was a dramatic increase in the percentage of dsRNAs that scored as hits in five or more screens once the predicted number of off-targets reached 20 or greater, but the increase was already apparent for dsRNAs with only one off-target (**Fig. 2b**).

To rule out the contribution of confounding factors other than the presence of off-target sequences, we first analyzed whether differences in concentration among the dsRNAs in our collection might affect their reported activity. Although higher concentrations of siRNAs are more likely to cause off-target effects[2], our statistical analysis did not show a correlation between dsRNA concentration and hit rate, a trend that may have been obscured by the high working concentrations of dsRNAs used in the screens (data not shown). Next we asked whether dsRNAs targeting genes with

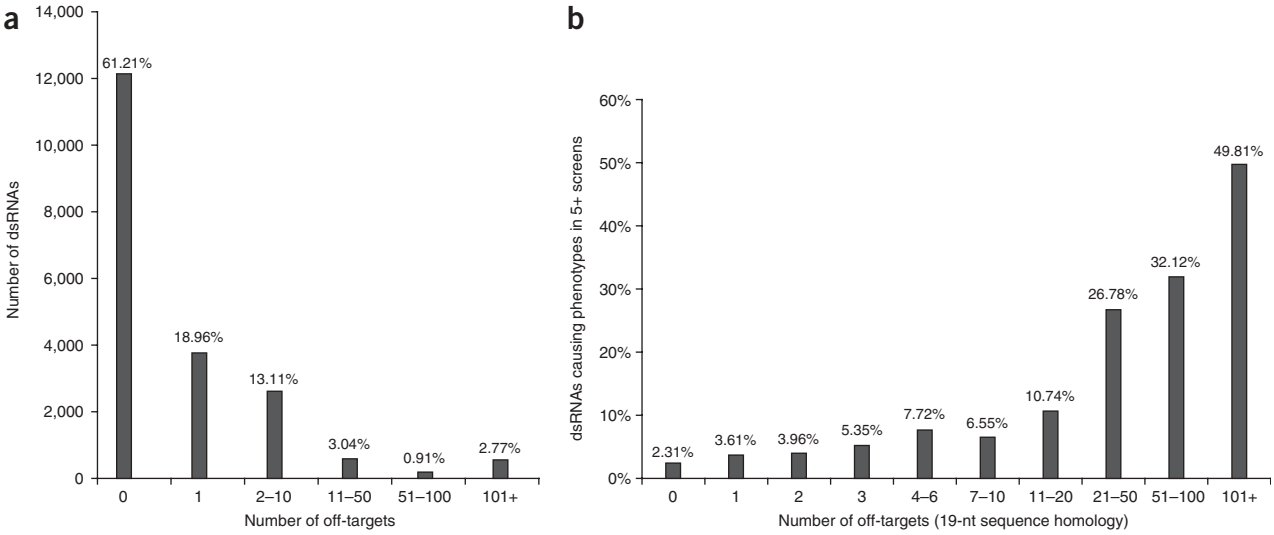

Figure 2 | Off-target sequences in the DRSC dsRNAs collection and their effect on hit rate frequency. (**a**) dsRNAs with predicted off-targets in the DRSC collection. Using the same algorithm as in **Figure 1**, we determined for each dsRNA the number of 19-nt perfect matches (no gaps allowed) to one or more *D. melanogaster* genes (annotated in BDGP, release 3.2) other than the intended target. Percentages of dsRNAs with 0, 1, 2–10, 11–50, 51–100 and 101 or more off-targets are indicated. (**b**) Results reported in 30 genome-wide screens performed at the DRSC were used to plot the percentage of dsRNAs that score in five or more screens in relation to their number of predicted off-targets (based on the 19-nt threshold). Increase in the rate at which dsRNAs cause a phenotype in five or more screens correlates with number of predicted off-targets.

general metabolic functions and commonly found as hits in our screens were associated with a higher number of predicted off-targets. First, we found a high enrichment in Gene Ontology (GO) terms dealing with ribosomal function, transcription, mRNA metabolism and splicing, translation, cellular biosynthesis and ubiquitination in dsRNAs scoring in five or more screens, independently of their number of predicted off-targets (**Supplementary Table 1** online). A χ^2 analysis then demonstrated that for the most part there was no obvious bias for dsRNAs in these GO categories to have a high number of off-target sequences with the exception of dsRNAs targeting the RNA polymerase II transcription factor and related categories (**Supplementary Table 2** online). These sequences, however, accounted for only 6.6% of the off-target–rich dsRNAs associated with a phenotype. Further, the mild enrichment in this group may reflect the fairly common occurrence of a polyglutamine stretch (a characteristic of the OPA repeats[15]) in the activation domain of transcription factors. Our analyses show that although certain GO categories of genes with basic metabolic

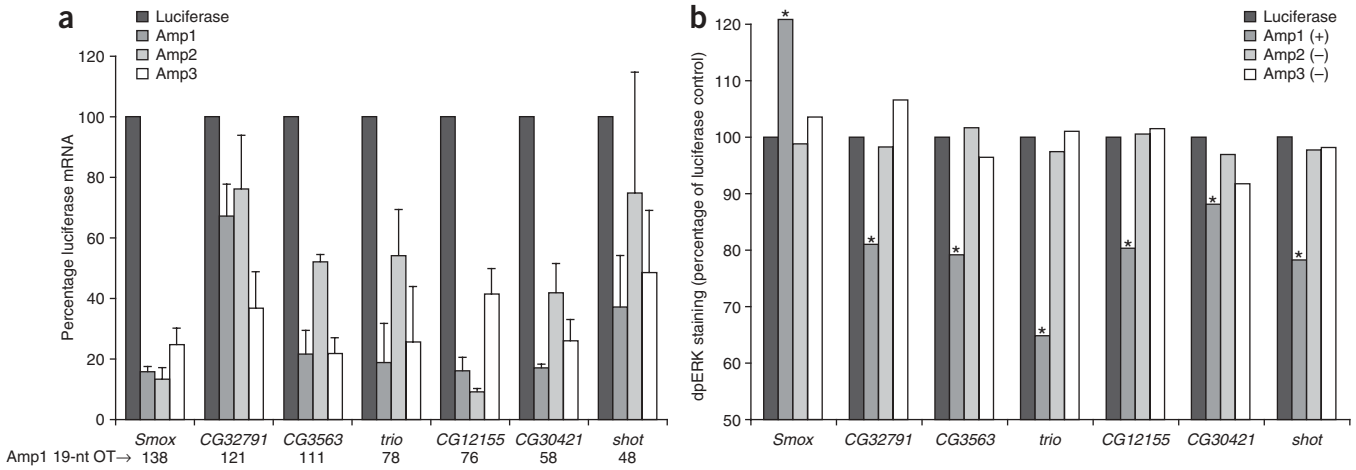

Figure 3 | Predicted off-target sequences in dsRNAs lead to false positives in an ERK activation RNAi screen. (**a**) For each gene targeted by a dsRNA (Amp1) with the indicated number of computationally predicted 19-nt off-targets (indicated below the gene name), two additional dsRNAs (Amp2 and Amp3) were synthesized, and all three dsRNAs were tested for extent of mRNA knockdown by qRT-PCR, relative to a luciferase dsRNA negative control. Error bars, s.d.; $n = 4$ except for *trio* and *shot*, where $n = 3$. (**b**) The phenotype of each dsRNA (Amp1, Amp2 and Amp3) in the ERK activation assay, relative to a luciferase dsRNA negative control, and the *P* value for each comparison. Only the Amp1 original dsRNA for each gene (with predicted 19-nt off-targets; see **Supplementary Table 4**) significantly affected ERK activation (denoted by + in the legend). Note there was no correlation between phenotype and extent of mRNA knockdown (**a**), indicating the phenotype of the first dsRNA (Amp1) is probably due to an off-target effect. $^*P < 0.006$. All other changes were statistically insignificant.

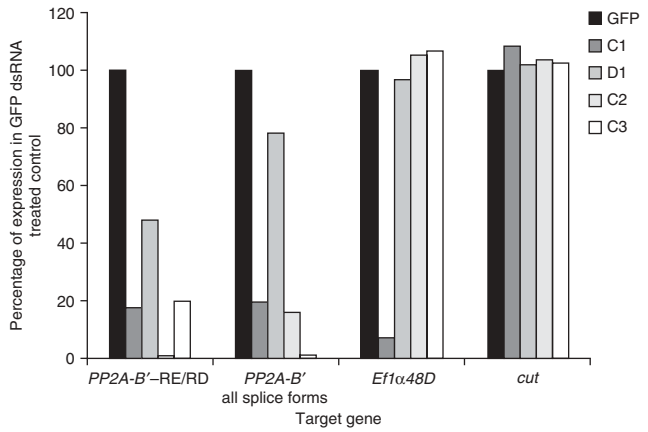

Figure 4 | Comparison of fold change expression differences and knockdown efficiency after treatment with pairs of dsRNAs directed against *PP2A-B′*. Total RNA from cells treated with control GFP, C1, D1, C2 and C3 dsRNA were subjected to real-time qRT-PCR with gene-specific primers to detect the mRNA levels of specific *PP2A-B′*–RD and *PP2A-B′*–RE splice forms, the mRNA levels of all splice forms of *PP2A-B′*, the mRNA level of the predicted off-targets *Ef1α48D* and *cut*. *PKA* was used as control. The C1 and D1 dsRNAs specifically target the *PP2A-B′*–RD and *PP2A-B′*–RE splice forms, whereas the C2 and C3 dsRNAs target all splice forms of *PP2A-B′* mRNA. The expression levels of different mRNAs in C1, D1, C2 and C3 dsRNA treated samples are reported as a percentage of expression in the GFP dsRNA–treated sample, which is set at 100%. The specific dsRNA treatment is indicated in the legend and the mRNA being detected is indicated along the *x* axis.

functions will be enriched in the hits of genome-wide RNAi screens, this enrichment alone cannot fully account for why dsRNAs with multiple off-targets score more frequently. Instead, the presence of off-target sequences in a dsRNA remains the strongest predictor for an off-target effect.

Predicted off-targets correlate with false positives

We evaluated the predictive value of off-targets by testing seven dsRNAs, which consistently scored as hits in the primary and subsequent secondary mitogen-activated protein kinase (MAPK) screens. The assay measured MAPK activation by quantifying extracellular regulated kinase (ERK) activity at baseline and under insulin stimulation using a fluorescently conjugated antibody that recognizes the di-phosphorylated (activated) form of *D. melanogaster* ERK (dpERK). The seven dsRNAs elicited strong-to-intermediate changes in the amount of dpERK in various cell lines but contained multiple off-target sequences ranging from 138 to 48. For each dsRNA, we designed two new, distinct dsRNAs that were devoid of any predicted off-targets (**Supplementary Table 3** online). For these new dsRNAs, we carefully examined the efficiency of knockdown and ability to affect dpERK status, and compared these to data achieved with the original dsRNAs. Despite the fact that one or two of the new dsRNAs led to equivalent target knockdown to that obtained with the original dsRNA (**Fig. 3a**), none of the new dsRNAs caused any significant change in dpERK staining, in stark contrast to the original set (**Fig. 3b** and **Supplementary Table 4** online). Further, each of the seven original dsRNAs included several off-targets to genes that were also identified as hits in the MAPK screen (data not shown). These results clearly demonstrate that the phenotype observed with the original dsRNAs is due to an off-target effect rather than knockdown of the primary target, and that use of dsRNAs predicted to have off-targets should be avoided.

Transcript knockdown by a 19-nt off-target sequence

To address whether a 19-nt off-target sequence can indeed knock down an unintended target, we focused on dsRNAs targeting the phosphatase regulatory subunit *PP2A-B′* gene, which had been identified as a hit in several genome-wide RNAi screens. We used custom cDNA microarrays (**Supplementary Methods** online) to compare the transcriptional signature elicited by the *PP2A-B′* dsRNA, DRSC16337 (D1), to those generated by three additional *PP2A-B′* dsRNAs (C1, C2 and C3) in SL2 culture cells. C1 targets the same splice form of *PP2A-B′* as D1 and is predicted to have two off-targets (*Ef1α48D* and *cut*) also represented in the 135 off-targets for D1. The C2 and C3 dsRNAs target all splice forms of the gene, and each is predicted to have one off-target. Analysis of the transcriptional profiles revealed that treatment with the relatively off-target–free and equally efficient C2 and C3 dsRNAs elicited responses that were similar to each other. In contrast, we observed a greater variation in the expression signatures of C1 and D1 dsRNAs. In this case, however, differences in knockdown efficiency of the *PP2A-B′* transcript by C1 and D1 (80% versus 50%, respectively) may partly account for the observed differences in gene expression (**Supplementary Fig. 1** online and **Fig. 4**).

We noticed that the expression level of *Ef1α48D*, a predicted off-target for both C1 and D1 dsRNAs, was significantly downregulated in the C1-treated sample as compared to the D1-treated sample. This downregulation was not due to a secondary effect of *PP2A-B′* knockdown as neither C2 nor C3 dsRNAs affected *Ef1α48D* expression. Furthermore, it is unlikely to be caused by a nonspecific effect of dsRNA treatment, as dsRNA against GFP did not affect its expression (**Supplementary Fig. 1** and **Fig. 4**). Thus, the C1 dsRNA not only downregulates its intended target (*PP2A-B′*) efficiently, but also causes the suppression of *Ef1α48D*. This indicates that 19-nt off-target sequences in long dsRNAs are not necessarily diluted out in the pool of diced siRNAs and can efficiently suppress the corresponding transcript of the off-target. But firm predictions for observing measurable knockdown of off-target mRNAs may not be straightforward as we did not observe an effect on the expression levels of 50 of the 135 predicted off-targets for D1 that were represented on the custom microarray (**Supplementary Fig. 1**). Further, the mRNA level of the gene *cut*, which is also a predicted off-target for both C1 and D1, was not affected in either C1 or D1 dsRNA–treated cells compared to control *GFP* dsRNA treated cells (**Fig. 4**).

DISCUSSION

As with any other large-scale methodology, it is important to identify and control for variables influencing rates of false negatives and false positives in genome-wide RNAi screens. The aggregate data from 30 genome-wide RNAi screens performed at the DRSC provided us with an opportunity to systematically address these issues. An early analysis of three published screens[16–18] had indicated that both reproducibility and rate of false negatives seemed to be manageable (a trend confirmed in the remaining screens). Eighty-five percent of the phenotypes identified in primary screens had been observed in duplicate and the effect(s) of 71–90% of selected dsRNAs that were associated with a phenotype in the primary screen had been confirmed when retested in secondary

Table 1 | Correlation between higher hit rate and predicted off-targets extends to perfect matches shorter than 21 nt

	Number of predicted off-targets				
	0	1	2	3+	
23 nt	16,780[a]	297	59	39	$\chi^2 = 43.548$
	7,236[b]	178	44	31	$P = 1.88 \times 10^{-9}$
	43.1%[c]	59.9%	74.6%	79.5%	
22 nt	16,339	355	47	39	$\chi^2 = 15.732$
	7,001	179	27	29	$P = 0.00129$
	42.8%	50.4%	57.4%	74.4%	
21 nt	15,574	632	94	39	$\chi^2 = 24.865$
	6,607	307	77	32	$P = 1.65 \times 10^{-5}$
	42.4%	48.6%	58.5%	82%	
20 nt	14,069	1,229	190	86	$\chi^2 = 25.343$
	5,860	589	106	52	$P = 1.31 \times 10^{-5}$
	41.7%	47.9%	55.8%	60.5%	
19 nt	11,365	2,159	372	173	$\chi^2 = 27.223$
	4,603	971	184	102	$P = 5.29 \times 10^{-6}$
	40.5%	45%	49.5%	59%	
18 nt	6,674	3,006	1,070	615	$\chi^2 = 6.795$
	2,629	1,241	456	277	$P = 0.0787$
	39.4%	41.3%	42.6%	45%	
17 nt	1,919	1,807	1,345	1,603	$\chi^2 = 3.859$
	714	714	541	660	$P = 0.277$
	37.2%	39.5%	40.2%	41.2%	
16 nt	205	285	337	1,092	$\chi^2 = 0.833$
	79	98	124	413	$P = 0.842$
	38.5%	34.4%	36.8%	37.8%	

Data from over 30 completed screens. For each set length, dsRNAs that have predicted off-targets of a greater length were excluded. For example, the 22-nt set includes no dsRNAs with 1 or more 23-nt off-targets.
[a]Total number of dsRNAs in the group. [b]Number of dsRNAs that led to a hit in at least one screen. [c]The observed hit rate (O) in each group was obtained by dividing the number of dsRNAs that led to a hit by the total number of dsRNAs in that group. For instance, there are 15,574 dsRNAs in our collection which show no predicted off-targets when their 21 nt fragments are analyzed. Of those, 6,607 cause a phenotype in one or more of our screens. The hit rate is therefore 6,607 / 15,574, or 42.4%. The χ^2 analysis was calculated as follows: $\chi^2 = \Sigma\ (O - E)^2 / E$, where E is the expected hit rate, which we defined for each row (corresponding to a homology length of 23 nt or 22 nt, etc.) to be equal to the sum of all hits divided by the sum of all dsRNAs corresponding to that row.

assays. Furthermore, the rate of false negatives was reasonable, as more than 80% of the genes known to be involved in these pathways had been identified (**Supplementary Table 5** online). In contrast, our analysis indicated that false positives could potentially be a concern. This became apparent when we compared how pairs of distinct dsRNAs targeting the same gene behaved across different screens. Contrary to our expectation, many dsRNA pairs scored differently in a substantial number of screens. Tellingly, the more two dsRNAs differed in their number of predicted off-targets, the more likely they were found to disagree. We extended our analysis to all dsRNAs in our collection and found that dsRNAs containing sequences with perfect homologies to mRNAs other than the intended target were statistically more likely to cause a phenotype than predicted by chance alone. Furthermore, sequence homologies as short as 19 nt were significant and needed to be considered as sources of false positives (**Table 1** and **Fig. 3**).

Based on the 19-nt threshold, 40% of dsRNAs in our collection contain one or more putative off-targets to other target(s), raising a note of caution when interpreting data obtained with these reagents and leaving open the possibility that their measured effect in any given screen could result from an off-target effect. At the same time, the *in silico* detection of off-target sequences only confers predictive value; it does not necessarily imply that any result obtained with a dsRNA containing such sequences should be viewed as artifactual. Rather, it should be considered suspect until confirmed by independent measures. Our analysis did not address whether there is, on average, a minimum number of predicted off-targets that can be tolerated. One vexing issue is that we cannot predict *a priori* which of the possible siRNAs will be generated with a given dsRNA by Dicer. Obviously, a large number of perfect, cross-hybridizing sequences in a dsRNA are likely to result in off-target effects (**Fig. 3**). Nevertheless, it is prudent to consider that even a single 19-nt off-target sequence might be capable of knocking down its unintended target, as was observed with the C1 *PP2A-B′* dsRNA. The predicted 19-nt match with a sequence in *EF1α48D* effectively knocked down its corresponding mRNA to less than 10% of its expression in control cells (**Fig. 4** and **Supplementary Fig. 1**). In contrast, the mere presence of off-target sequences may not universally correlate with drastic changes in expression of the mRNAs putatively targeted by these sequences as illustrated with the *cut* gene (**Fig. 4**). The expression level of all 50 mRNAs (out of 135), which could be assessed on our chip and be potentially affected by the off-target sequences in D1, remained unchanged. One possibility is that some or all were affected but at levels that could not be detected by the microarrays. Regardless, our results are consistent with the recent observation in mammalian cells[4] that perfect matches of 19–21 nt with expressed sequences other than the targeted gene are good predictors of siRNA-mediated off-target effects.

We validated dsRNAs identified as hits in a previous screen with multiple new dsRNAs lacking any 19 nt off-targets. We observed excellent validation rates for primary dsRNAs that lacked 19-nt off-targets (data not shown), with a rate of false positives undistinguishable from that of assay noise (typically around 15%). In contrast, dsRNAs with predicted 19 nt homologies were more variable in their validation rates. These results imply that, once dsRNAs with predicted 19-nt off-target sequences are removed, genome-wide screens appear to have a low false positive rate on par with assay noise, and that future screens should be carried out with improved dsRNA collections that directly address this issue.

Notwithstanding the contribution of off-target sequences to the rate of false positives, there are several additional factors that may contribute to this rate. Some are assay-specific (for example, experimental signal to noise ratio, normalization issues) whereas others are more procedure-specific (such as position in the screening plates). To minimize the rate of false positives, we propose a set of guidelines (**Supplementary Fig. 2** online). First, researchers using long dsRNAs should make full use of available algorithms that scan dsRNAs and make *in silico* predictions for the presence of homologies to all expressed sequences[8,11,19,20]. Second, researchers should avoid targeting gene regions that encode highly conserved

domains. These may have considerable similarity to coding regions in other genes but are unlikely to be picked-up as exact matches because of codon degeneracy. Simpler domains like the rather ubiquitous CAN trinucleotide repeats (OPA-like repeat), which encode polyglutamines and are often found in transcription factors and nucleic-acid binding proteins, are best avoided through rational dsRNA design. The low sequence complexity of these repeats makes them particularly prone to lead to off-target effects as they are iterated many times over a relatively compact region (50–150 nt). Third, researchers should use multiple dsRNAs to confirm data that cannot be easily validated by other means. Observing these simple guidelines should considerably help reduce the occurrence of false positives in genome-scale screens.

METHODS

dsRNA synthesis. We generated dsRNAs as previously described[21]. Briefly, we designed gene-specific primers to include T7 promoter sequences and used the resulting PCR products in *in vitro* transcription (T7 MEGAscript kit; Ambion) to generate both RNA strands in one reaction. We purified the dsRNAs using RNeasy columns (Qiagen) and checked their quality by gel electrophoresis.

Validation dsRNAs. We aliquoted 250 ng of each dsRNA 5–7 times, including 13–30 negative controls (luciferase dsRNA) into 384-well plates. After incubation, we either immediately fixed, or stimulated with insulin and fixed in formaldehyde S2R+ cells, and quantitatively assayed them for ERK activation[22]. We performed the assay in duplicate and calculated *P* values for each gene for 10–14 samples and 26–60 controls. For each gene, we represented the state of ERK activation, normalized to total ERK levels, as a percent of luciferase dsRNA (control) activity. We calculated the significance thresholds for determining knockdown effect on ERK activation using the false discovery rate (FDR) method[23]. For quantitative reverse-transcriptase PCR (qRT-PCR) analysis, we scaled the dsRNA knockdowns to 96-well plates and extracted total RNA using the Qiagen RNeasy 96 kit.

Real-time quantitative RT-PCR. We seeded 4×10^6 SL2 cells in each well of a 6-well cell culture dish (Falcon) in 1 ml of serum-free medium and treated the cells with 15 μg of dsRNA at room temperature (22–25 °C) for 45 min. After 45 min, we added 2 ml of serum-supplemented Schneider's medium and incubated the cells for an additional 72 h. We isolated total RNA with Trizol according to the manufacturer's specifications (GibcoBRL) and purified it using RNeasy columns. We prepared and distributed in a 96-well qPCR plate (ABI) a master mix containing the Quantitect SYBR Green PCR reagent (Qiagen), gene-specific primer pair and double distilled water. We used *Pka-C1* as a control. We added cDNA prepared with Quantitect Reverse transcription kit (Qiagen) from 1 μg of total RNA isolated from cells treated with control GFP, C1, D1, C2 and C3 dsRNA. The reaction was run on an ABI Prism 7900HT real-time PCR machine. For each experiment reactions were done in triplicate, resulting in a standard deviation and error of less than 0.5 in all cases.

Additional information. Details of custom microarray design and labeling and hybridization protocols are available in **Supplementary Methods**.

Accession codes. Gene Expression Omnibus (GEO): GSE5554.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

M.M.K., microarray design and analysis, RT-qPCR analysis of off-target effects; M.B., S.J.S. and P.H., data analysis and statistical evaluation; A.F., validation of dsRNAs.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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