Supplementary Discussion

Messages that respond to an ectopically introduced miRNA are enriched in evolutionarily conserved sites and tend to be messages that are depleted in tissues normally expressing the miRNA—two observations indicating that targeting by these miRNAs has some relevant relationship to that found *in vivo* and that such data can shed light on miRNA target recognition[9]. However, two key caveats limit interpretation of results from ectopically introduced miRNAs. Most importantly, the targets that were repressed in our HeLa experiments were not necessarily the biological targets of the introduced miRNAs because the miRNAs normally might not be expressed at consequential levels in the same cells that express the mRNA, or other factors, such as differentially expressed UTR-binding proteins, could prevent repression in the relevant cell type. A second caveat is that the amount of repression observed might not match that from the endogenous interactions. Transfection efficiency, either with respect to the fraction of cells transfected or the amount of miRNA delivered per cell, influenced the amount of repression that we observed. The short time frame inherent to transient delivery of the miRNA might also have imposed limits on the amount of repression observed. We measured protein effects 48 hours after miRNA delivery. This time frame was sufficient for cells to utilize the miRNA, but it only allowed for 1-2 cell divisions, depending upon the miRNA transfected (cells showed a delayed recovery after miR-124 transfection). In these circumstances, proteins with long half-lives might appear less downregulated than they actually are (e.g., a long-lived protein from a message totally repressed by the miRNA might appear to be repressed by only 50%). Moreover, miRNA effects are expected to diminish as the miRNA is diluted during cell division. The quantification caveat of transiently introduced miRNAs becomes especially acute when comparing effects on an mRNA to those of its protein because the kinetics of protein changes are not expected to parallel those of mRNA changes (even in a scenario involving mRNA destabilization without translational repression) because of delays in protein response imposed by the precursor-product relationship between the two and because the message and its protein can have very different intrinsic stabilities. To the extent that our miR-223 experiment measured steady-state changes in mature neutrophils, interpretation was not compromised by any of these target-identity and quantification caveats.

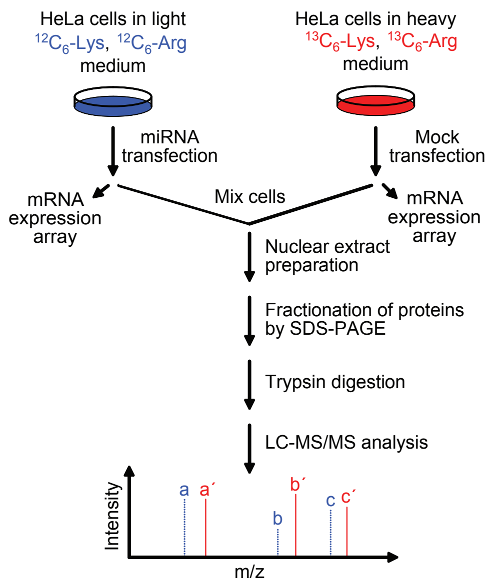
Supplementary Table 1. 3'-UTR motifs most confidently enriched in messages of proteins most upregulated in *mir-223*$^{-/Y}$ neutrophils (cutoff, 85th percentile).

Size	Rank	Motif	Canonical site (Fig. 1a)	Occurrence in upregulated 3' UTRs Observed	Expected	*P* value (Fisher's exact)
6 nt	1	..ACUGAC...	6mer	203	123	$< 10^{-12}$
	2	...CUGACA..		200	142	$< 10^{-4}$
	3	.AACUGA....		191	139	$< 10^{-3}$
7 nt	1	.AACUGAC...	7mer-m8	78	33	$< 10^{-9}$
	2	..ACUGACA..	7mer-A1	95	45	$< 10^{-9}$
	3	...CUGACAU.		84	44	$< 10^{-5}$
8 nt	1	.AACUGACA..	8mer	34	11	$< 10^{-4}$
	2	..ACUGACAA.		33	11	$< 10^{-4}$
	3	..ACUGACCA.		31	11	$< 10^{-2}$

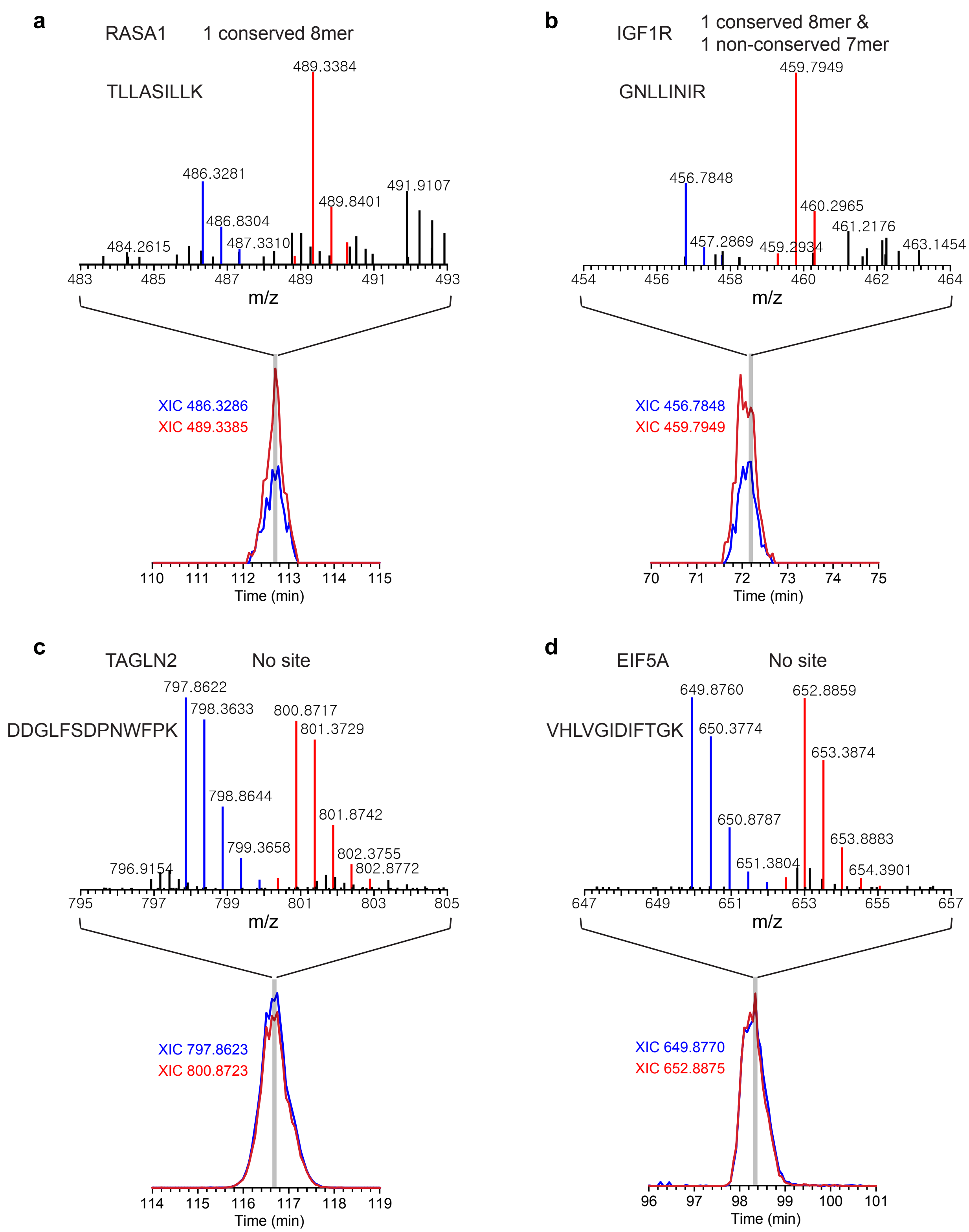
Supplementary Table 2. 20 miRNA families co-expressed with *mir-223* in wild-type neutrophils cultured *in vitro*, as detected by high-throughput sequencing.

Rank	Reads*	7mer-m8 site	miRNA Family
1	55,973	UGCUGCU	miR-15b/miR-195/miR-15a/miR-16/miR-16/miR-322/miR-497
2	41,848	AUAAGCU	miR-21/miR-590-5p
3	34,758	CUACCUC	let-7g/let-7i/let-7d/let-7a/let-7a/let-7b/let-7c/let-7c/let-7e/let-7f/let-7f/miR-98
4	23,871	UACUUGA	miR-26a/miR-26b/miR-26a
5	12,856	ACUUUAU	miR-142-5p
6	12,177	AACUGAC	miR-223
7	12,033	ACACUAC	miR-142-3p
8	11,579	UUCCGUU	miR-191
9	10,001	UGGUGCU	miR-29b/miR-29a/miR-29c/miR-29b
10	9253	GCACUUU	miR-106a/miR-106b/miR-20a/miR-93/miR-17/miR-20b
11	8464	CUGAGCC	miR-24/miR-24
12	7496	CUUUAUA	miR-340-5p
13	5962	GGCAGCU	miR-22
14	5363	ACUGUGA	miR-27b/miR-27a
15	4543	AUGCUGC	miR-103/miR-107
16	4019	AAUGUGA	miR-23b/miR-23a
17	3396	UGUUUAC	miR-30a/miR-30b/miR-30e/miR-30c/miR-30c/miR-30d/miR-384-5p
18	3126	UGCACUG	miR-152/miR-148a/miR-148b
19	2510	UUGCACU	miR-130a/miR-301a/miR-130b/miR-301b/miR-721
20	2344	CAAUGCA	miR-33
21	1659	UUUGCAC	miR-19b/miR-19a/miR-19b

*Reported are the numbers of sequencing reads that match the indicated miRNAs and also have a seed matching the indicted 7mer-m8 site, allowing for length heterogeneity at the 3' termini of the miRNAs.



Supplementary Figure 1. Schematic of quantitative proteomics experiment using SILAC. HeLa cells were grown in media containing either regular (light) Lys and Arg or $^{13}C_6$-labeled (heavy) Lys and Arg. Light cells were transfected with miRNA, and heavy cells were mock transfected. 48 hours after transfection, cells were harvested, and equal numbers from both populations were mixed and enriched for soluble nuclear proteins. Protein mixtures were separated by SDS-PAGE into 10 fractions and digested with trypsin. Peptides were analyzed by LC-MS/MS, which identified peptides and quantified the relative amounts of isotopic pairs of the same peptide separated by 6 mass units in the full MS scans (a-a', b-b' and c-c').



Supplementary Figure 2. Examples of protein quantifications by mass spectrometry. a, Extracted ion chromatogram (XIC) for the light (blue) and the heavy (red) versions of TLLASILLK, a representative peptide for Rasa1, which showed a 2-fold change upon miR-223 loss. Rasa1 derives from a message with one conserved 8mer in its 3' UTR. High mass accuracy allows for narrow window (<10 ppm) and thus highly selective extraction of the ions of interest. Above its chromatogram is the region of interest from the full MS scan that corresponds to the indicated region of the chromatogram (gray shading), displayed using the same color code for light and heavy isotopic envelops. **b,** Quantification for GNLLINIR, a representative peptide of Igf1r, which also displayed a 2-fold change upon miR-223 loss. **c** and **d,** Quantification of peptides from representative proteins that did not change with loss of miR-223.

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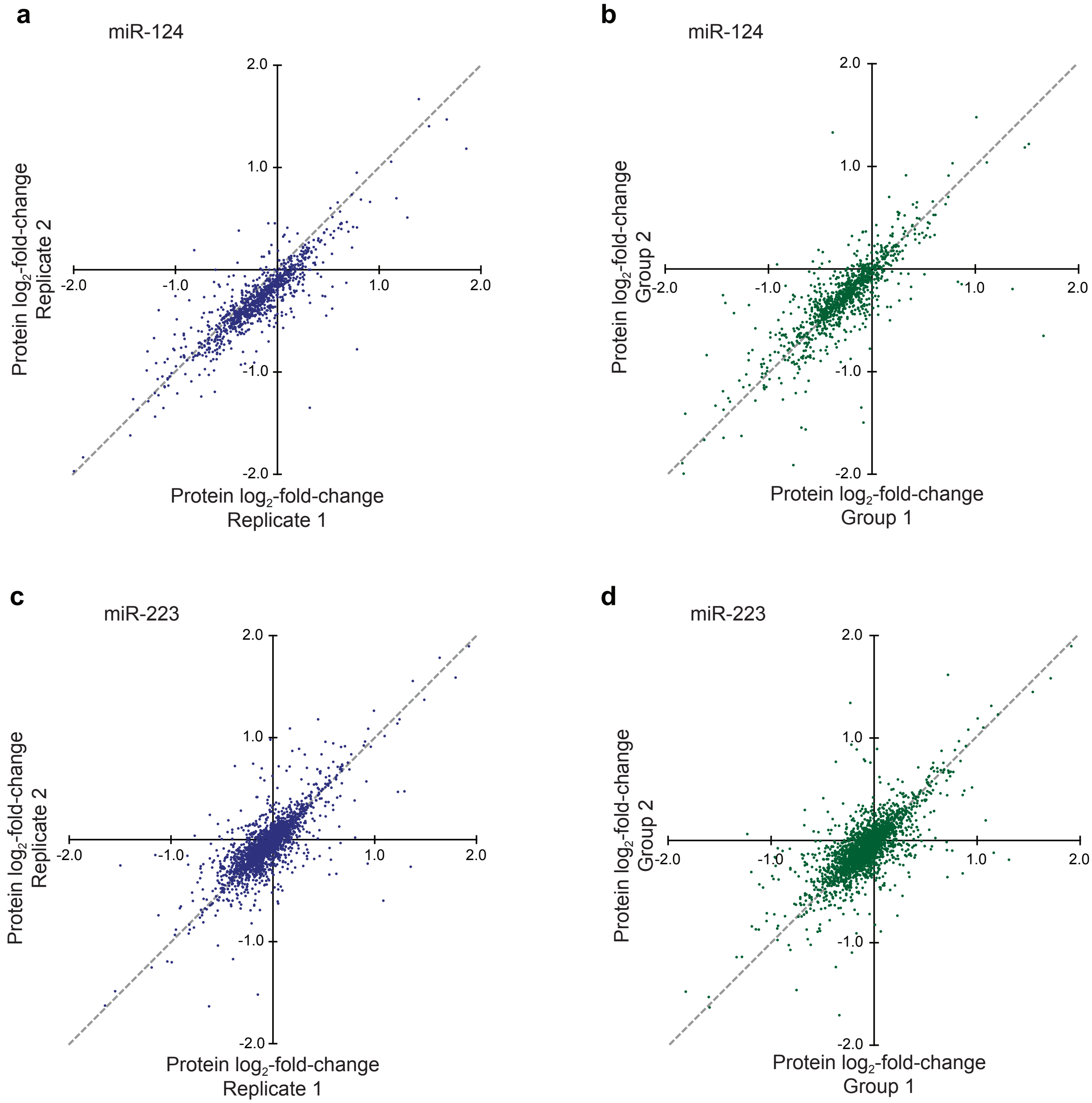
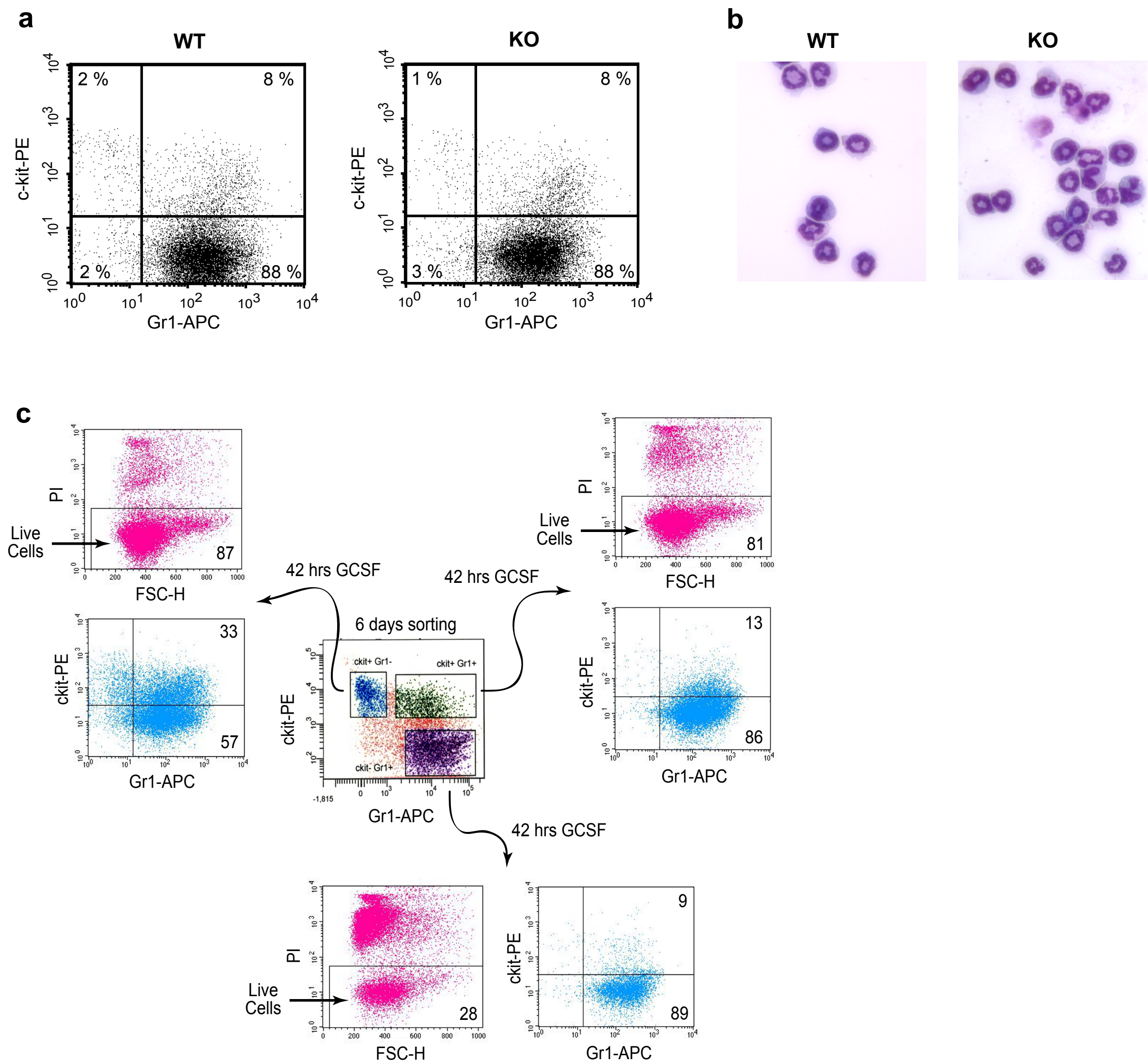
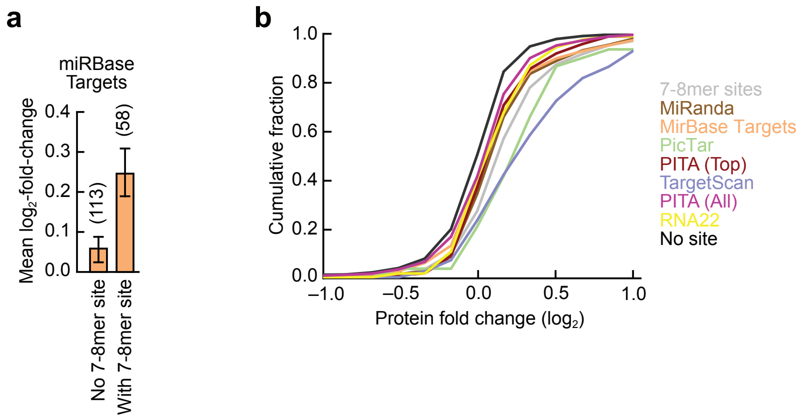


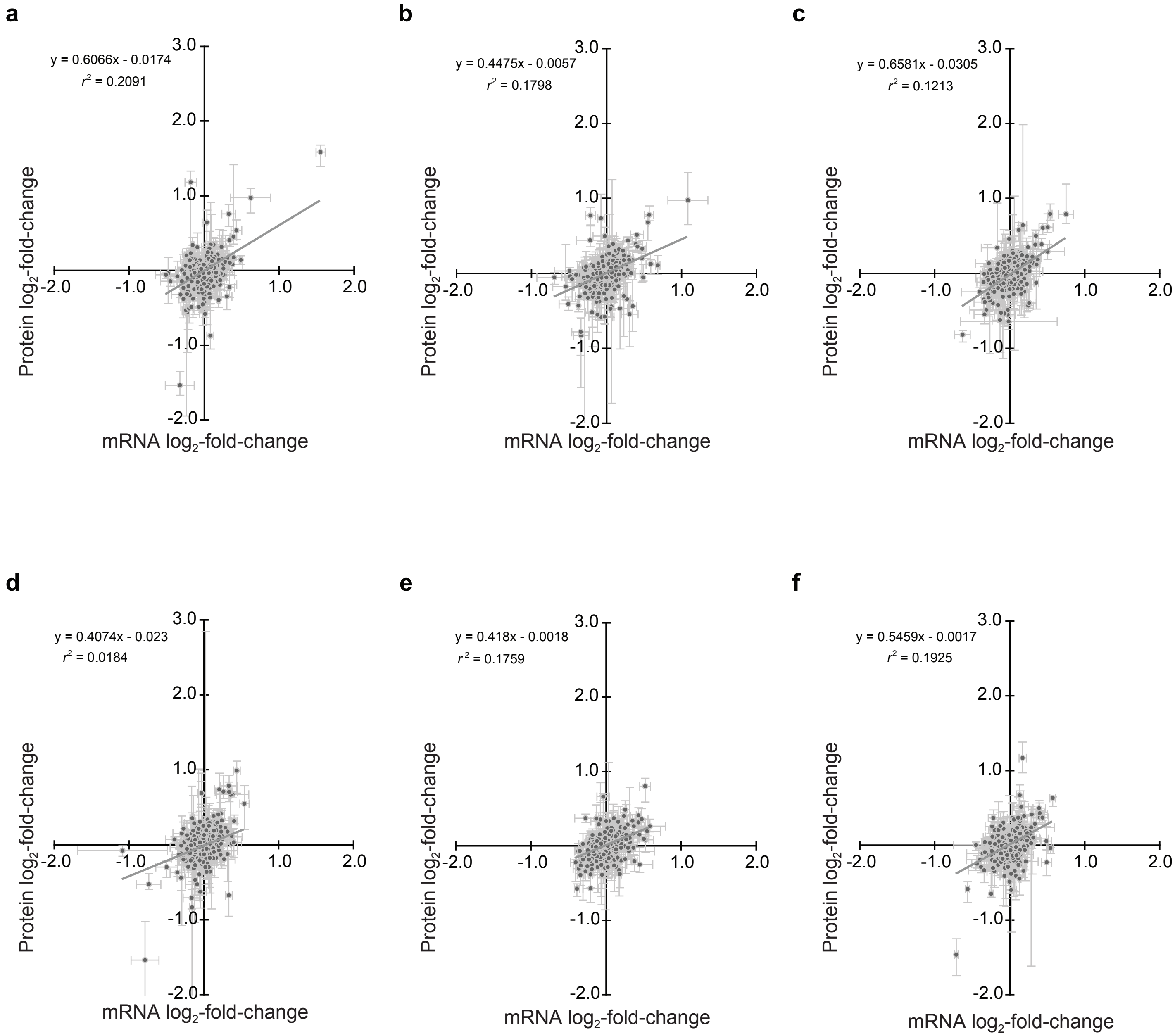
Supplementary Figure 3. Reproducibility of SILAC analyses. a, Comparison of changes quantified in technical replicate 1 with those for technical replicate 2 for the miR-124 experiment. Points represent the median $\log_2$-fold-change for each protein quantified in both replicates. Perfect correlation is represented by the dashed line. $r^2 = 0.72$, Spearman's correlation. **b,** Comparison of quantification when using non-overlapping sets of peptides (group 1 and group 2) for each protein quantified with more than one peptide in the miR-124 experiment. Perfect correlation is represented by the dashed line. $r^2 = 0.65$, Spearman's correlation. **c,** Comparison of changes quantified in technical replicate 1 with those for technical replicate 2 for the miR-223 experiment, otherwise as in panel **a**. $r^2 = 0.58$, Spearman's correlation. **d,** Comparison of quantification when using non-overlapping sets of peptides from the miR-223 experiment, otherwise as in panel b. $r^2 = 0.49$, Spearman's correlation.



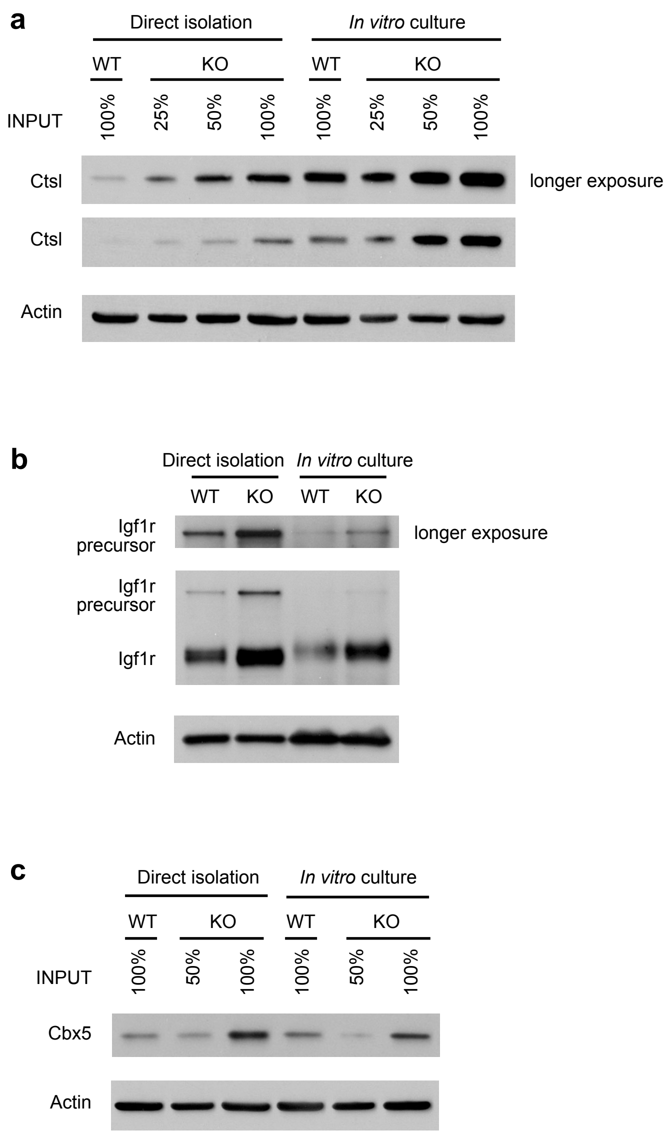
Supplementary Figure 4. Additional characterization of neutrophils differentiated *in vitro*. a, FACS analyses of the cell populations derived from wild-type (WT) and *mir-223* knock-out (KO) mice and cultured for 8 days. These were the populations analyzed by SILAC and as one of the three biological replicates profiled on arrays. Staining with PE-conjugated anti-mouse c-Kit antibody and APC-conjugated anti-mouse Gr-1 antibody indicated that ~88% of the cells cultured and differentiated in vitro had the immunophenotypic hallmarks of mature neutrophils. **b,** Morphological analysis of the cultured cells analyzed in panel **a**. The homogeneity of the population was checked by microscopy after Wright-Giemsa stain of cytospun neutrophils. **c,** Analysis of progenitor, intermediate, and neutrophil subpopulations after additional culture in SCF-minus media. Cells at each developmental stage were sorted for 6 days after culture in media containing both G-CSF and SCF (middle panel, progenitor, intermediate, and neutrophil subpopulations colored in blue, green, and purple, respectively). The sorted subpopulations were cultured in media without SCF for an additional 42 hours, and were examined by FACS analysis (outside panels). Staining with propidium iodide (PI) and forward scatter (FSC-H) showed the percentage of live or dead cells (pink plots). Staining with ckit-PE and Gr1-APC showed the differentiation status of the cells (blue plots). Numbers in each box indicate percentages of each population. The rapid turnover of differentiated cells, with replacement by newly differentiated cells from the progenitor and intermediate pools, resembles that observed *in vivo*.



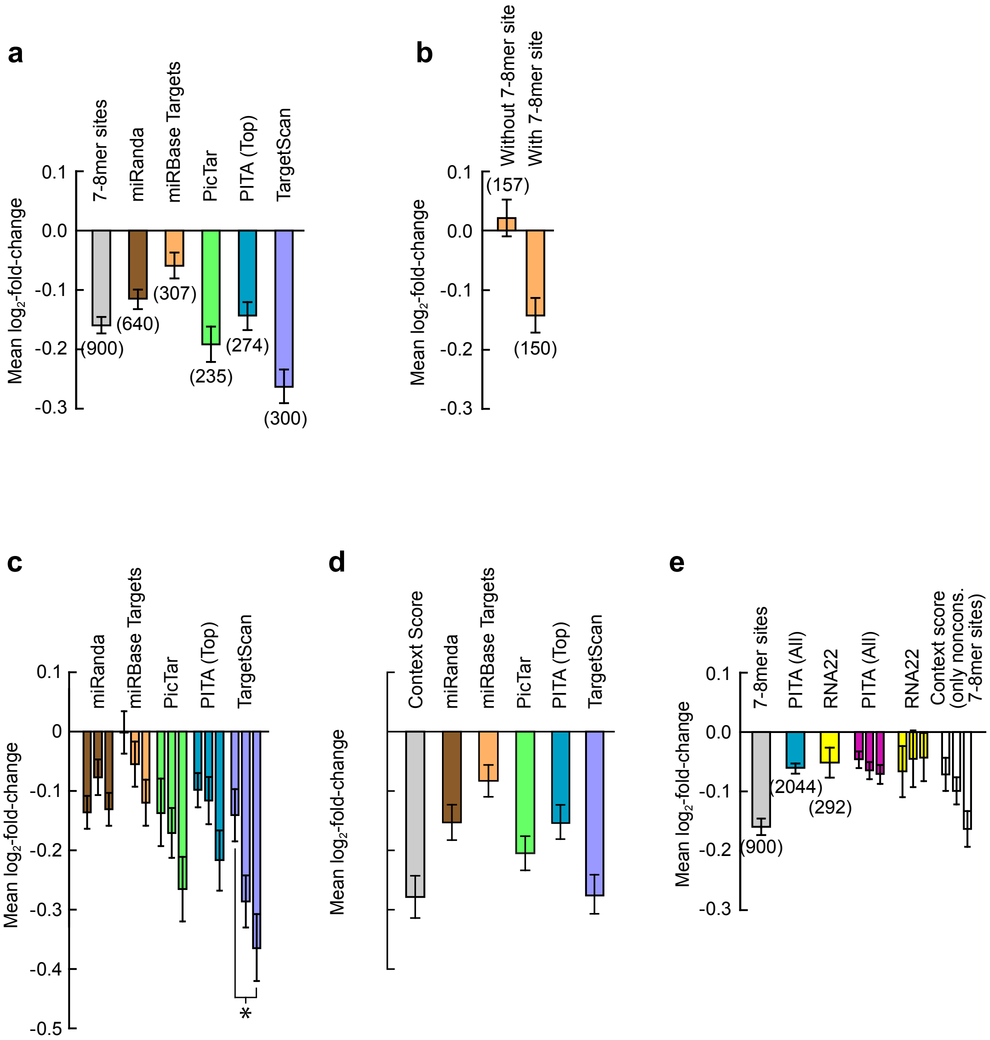
Supplementary Figure 5. Response of predicted targets of miR-223. a, Performance of mirBase Targets (version 5) predictions that contain at least one 7-8mer seed-matched 3'-UTR site, compared to those that do not. Analysis of the transfection datasets gave similar results (Supplementary Fig. 8b) **b,** Cumulative distribution showing the fraction of proteins that change at least to the degree indicated on the x axis, plotted as in Fig. 1c. The maximal cumulative difference from the no-site distribution for the TargetScan predictions was 0.41, with 0.084 of this difference attributed to the bumpiness of the distribution (see methods), thereby indicating that ≥ 33% of the predictions were responsive. Analogous calculations for PicTar predictions indicated that ≥ 34% of the predictions were responsive. Note that these are lower bounds because measurement noise can lessen the cumulative differences. Preferential conservation of 7-8mer sites in orthologous human, mouse, rat and dog UTRs indicates that overall, 55% of these sites are under selective pressure to be maintained, and thus represent biological targets[2]. However, sites for miR-223 are preferentially conserved at a lower rate than sites of most other highly conserved miRNAs (B. Lewis, C. Burge, D.P.B., data not shown), and thus the lower bound of 33% is within range of that inferred from the conservation analysis.



Supplementary Figure 6. Comparison of protein and mRNA changes upon *mir-223* deletion. a-f, Protein and mRNA changes for quantified proteins deriving from messages without 7-8mer 3'-UTR sites. Messages without 7-8mer 3'-UTR sites were randomly assigned to one of seven equally populated cohorts, and then their protein and mRNA changes were plotted as Fig. 4a. The plot for a representative cohort is shown as Fig. 4b, and the others are shown here, with their least-squares regression and r^2 values (Pearson's correlation). A few outliers are not shown in these plots (two in **c** and one in **d**) but were included when performing the regression.



Supplementary Figure 7. Immunoblot analyses of proteins derepressed with loss of miR-223. a, Protein blot of neutrophils isolated directly from wild-type (WT) and *mir-223* knockout (KO) mice and of cells cultured as for the SILAC analysis, probing for Ctsl (cathepsin L) protein. For comparison of derepression, different amounts of input from KO cells were included, and two different exposures are shown. Actin was probed as a loading control. **b,** As in panel **a**, but probing for Igf1r (Insulin-like growth factor receptor 1) protein. **c,** As in panel **a**, but probing for Cbx5 (HP-1α, heterochromatin protein-1α) protein.



Supplementary Figure 8. Response of predicted targets of transfected miRNAs. a, Performance of programs that consider site conservation. Plotted is the average protein change (± standard error) from genes predicted by the indicated programs to be miR-124, miR-1, and miR-181 targets, when considering proteomics data from experiments transfecting miR-124, miR-1, and miR-181, respectively. For comparison, repression of proteins deriving from messages containing at least one conserved or nonconserved 7-8mer 3'-UTR site is also plotted. The number of quantified proteins in each set is indicated in parenthesis. **b,** Performance of mirBase Targets (version 5) predictions that contain at least one 7-8mer seed-matched 3'-UTR site, compared to those that do not. The number of quantified proteins in each set is indicated in parenthesis. **c,** Relationship between the scores of predicted targets and protein repression. Predictions corresponding to quantified proteins were divided into three equal-size bins according to the scores proposed to indicate the quality of the prediction or degree of repression. Average protein downregulation in the respective transfections is plotted (± standard error) for each bin. Statistically significant differences between the bottom and top third are indicated (*, $P < 0.01$, Whitney-Mann *U* test). **d,** Response of the top predictions of each algorithm, plotted as in panel **a**. Considered were the top 87 predictions for miR-1, the top 52 predictions for miR-124, and the top 71 predictions for miR-181, which were the number of predictions from the algorithm with the fewest predictions (PicTar for miR-1 and miR-181, PITA for miR-124). **e,** Performance of programs that do not consider site conservation, displayed as in panels **a** and **c**. For comparison, quantified proteins from messages with nonconserved 7-8mer 3'-UTR sites were also binned based on total context score.