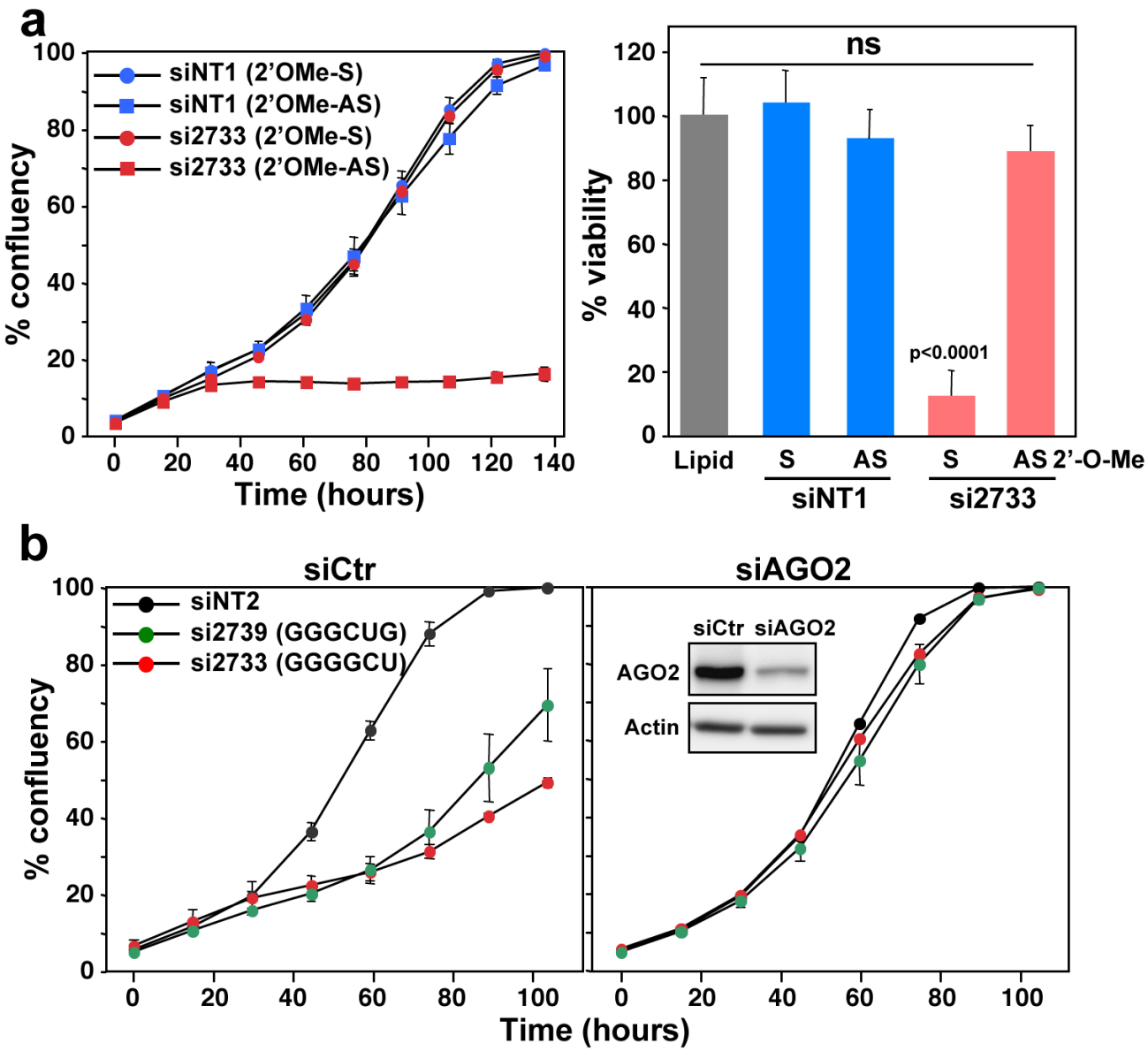
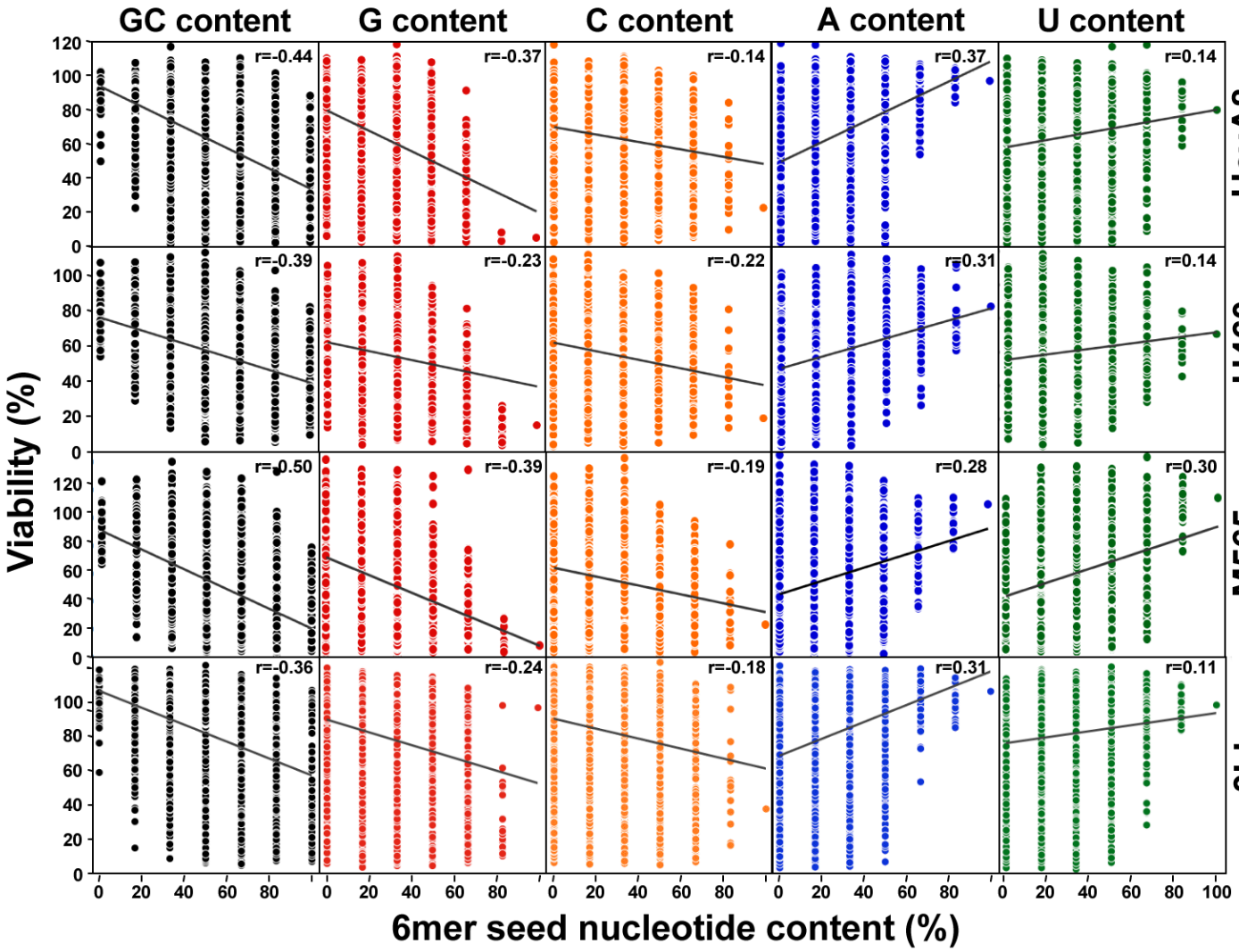
Supplementary Information

6mer seed toxicity in tumor suppressive microRNAs

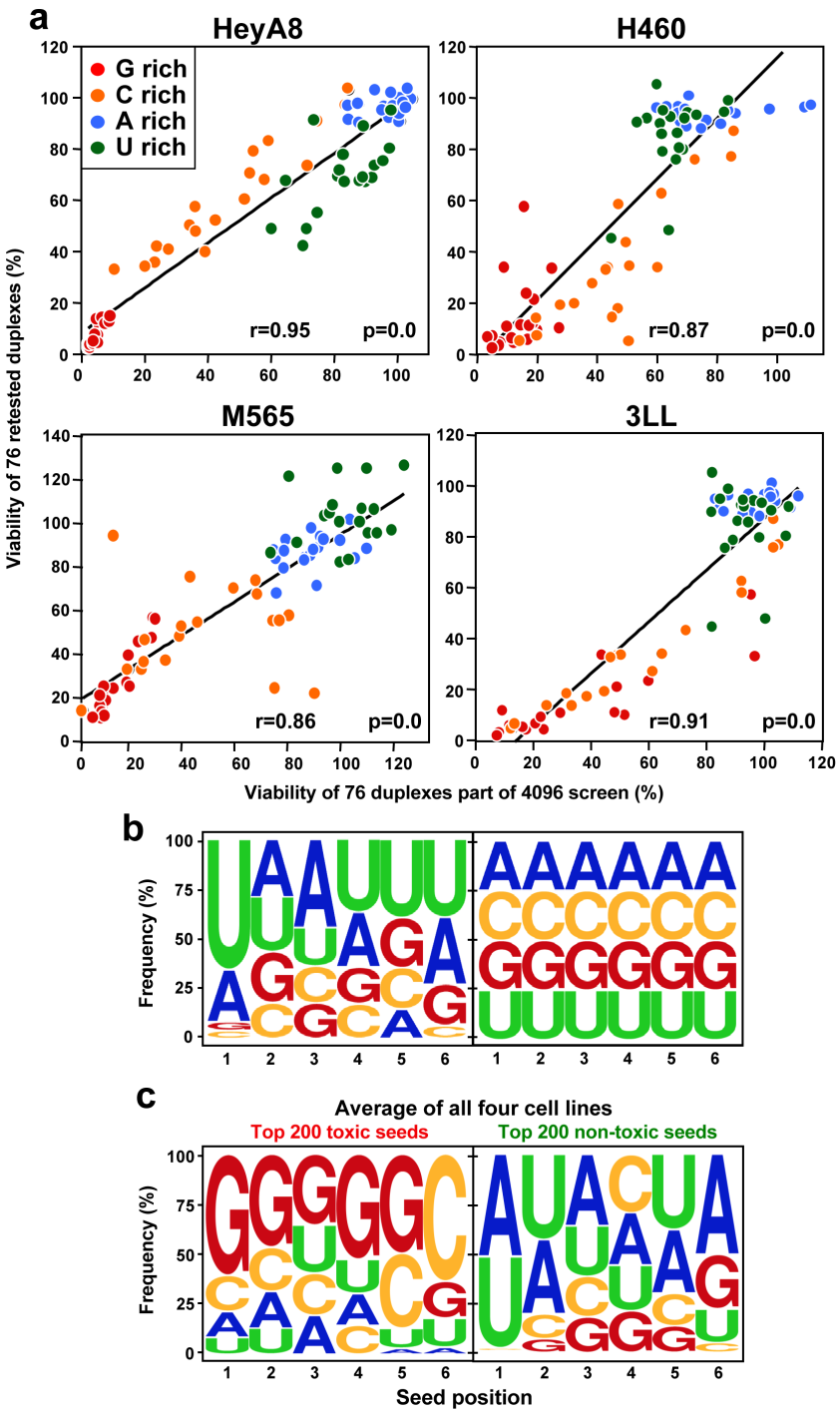
Quan Q. Gao, William E. Putzbach, Andrea E. Murmann, Siquan Chen, Aishe A. Sarshad, Johannes M. Peter, Elizabeth T. Bartom, Markus Hafner, and Marcus E. Peter



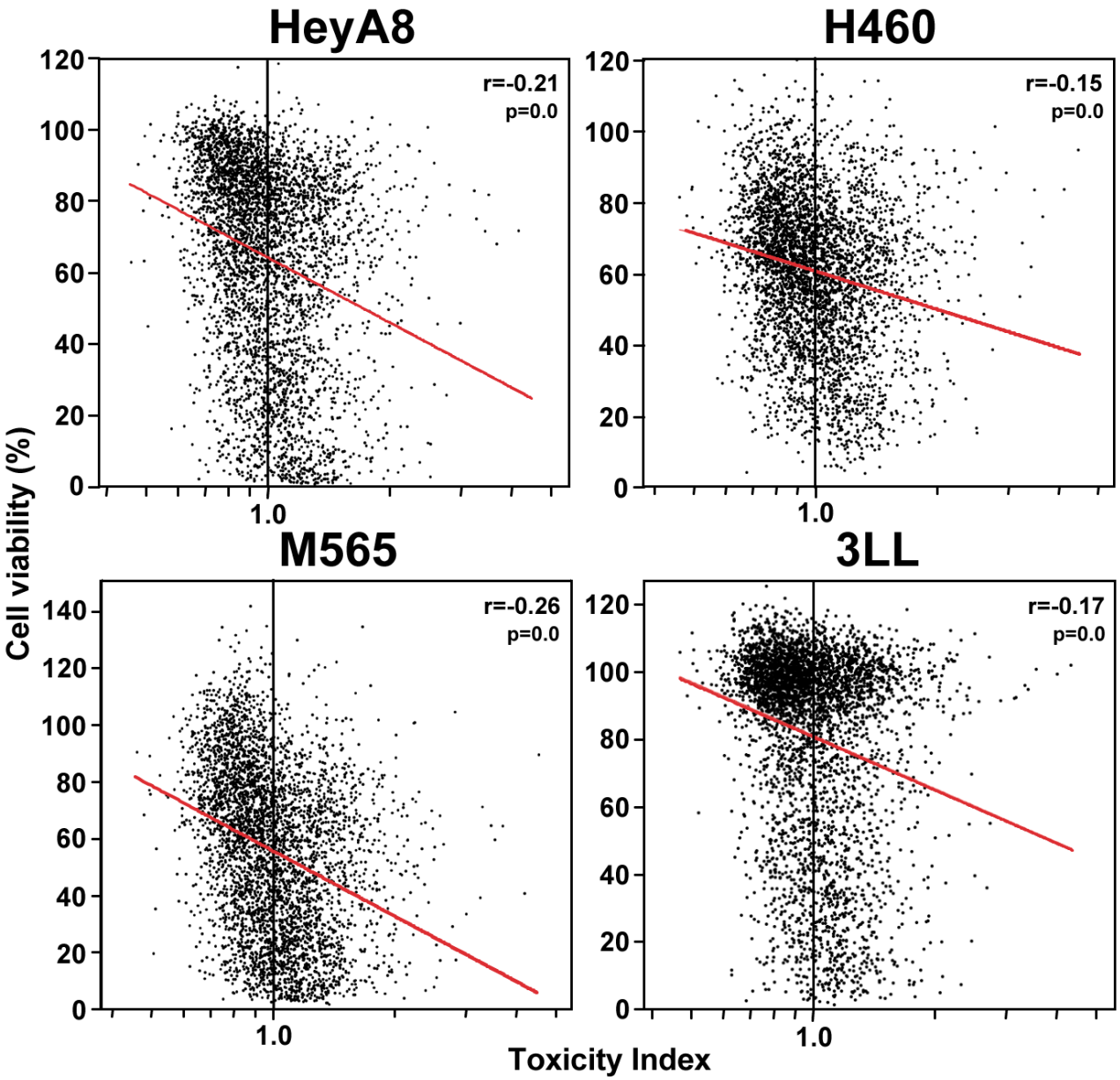
Supplementary Figure 1. Toxic 6mer seeds kill cancer cells through RNAi. **(a)** *Left:* Percent cell confluence over time of HeyA8 cells transfected with 10 nM siNT1 or si2733 with either the guide or the passenger strand modified by 2'-O-methylation in positions 1 and 2. *Right:* Cell viability 96 hrs after transfecting the cells with the siRNAs shown on the left. Cell viability was determined by quantifying ATP content. Change in cell viability was calculated compared to cells treated with lipid only. p-values were calculated using students ttest. ns, not significant. **(b)** Percent cell confluence over time of HeyA8 cells transfected with either nontargeting control (siNT2) or the two highly toxic siRNA duplexes (si2739 and si2733) at 10 nM. Cells were pre-treated with either nontargeting SMARTpool siRNAs (siCtr, left) or AGO2 SMARTpool siRNAs for 24 hours at 25 nM (right). Insert shows Western blot to document AGO2 knockdown efficiency. Each data point represents mean ± SE of three replicates.



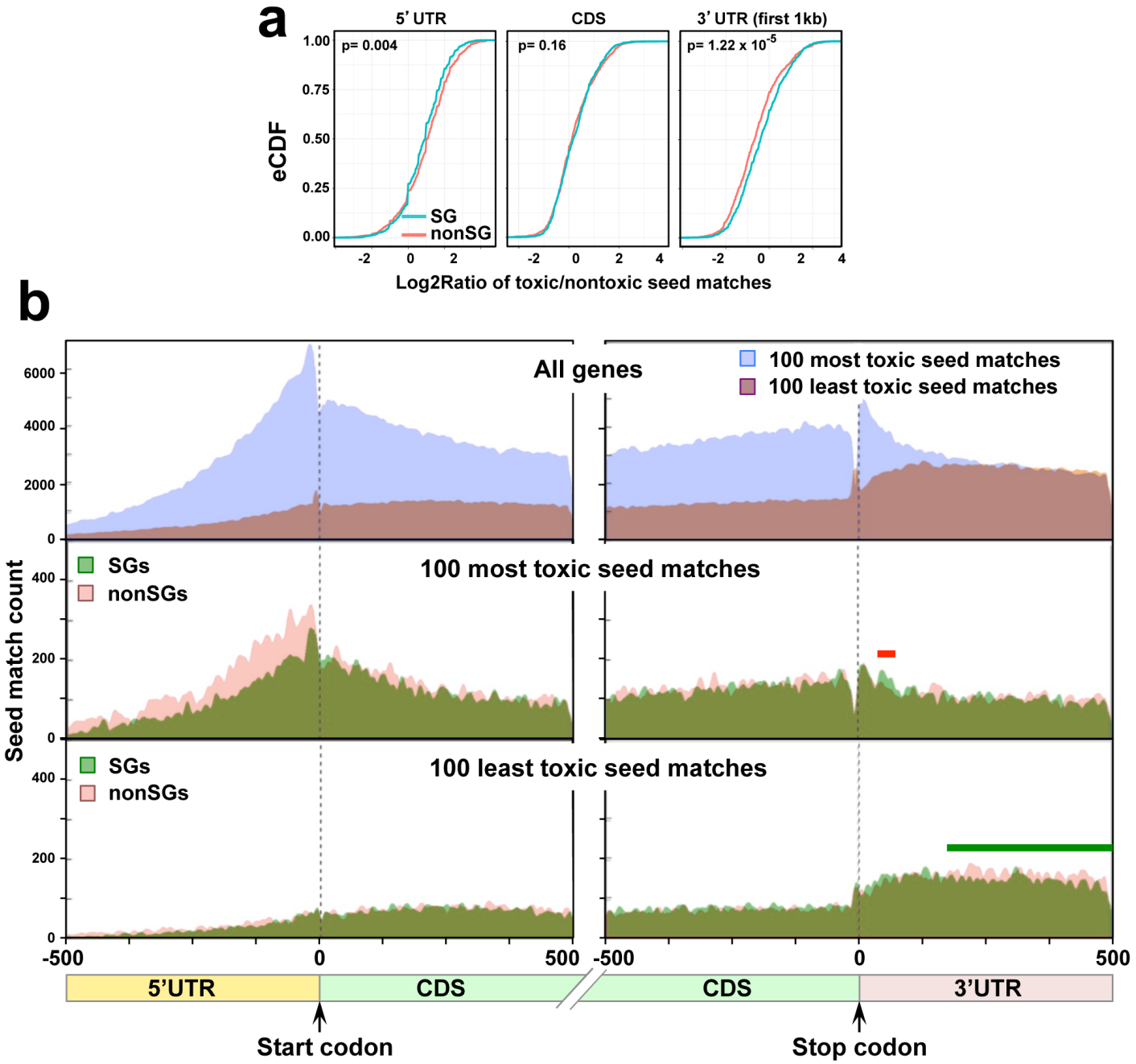
Supplementary Figure 2. 6mer seed toxicity correlates with the nucleotide content of the seed. Regression analysis of cell viability of 6mer seeds in the four cell lines versus GC content or individual nucleotide content (G, C, A, U) of the seeds. All p-values of the Pearson linear correlation coefficients were 0.0.



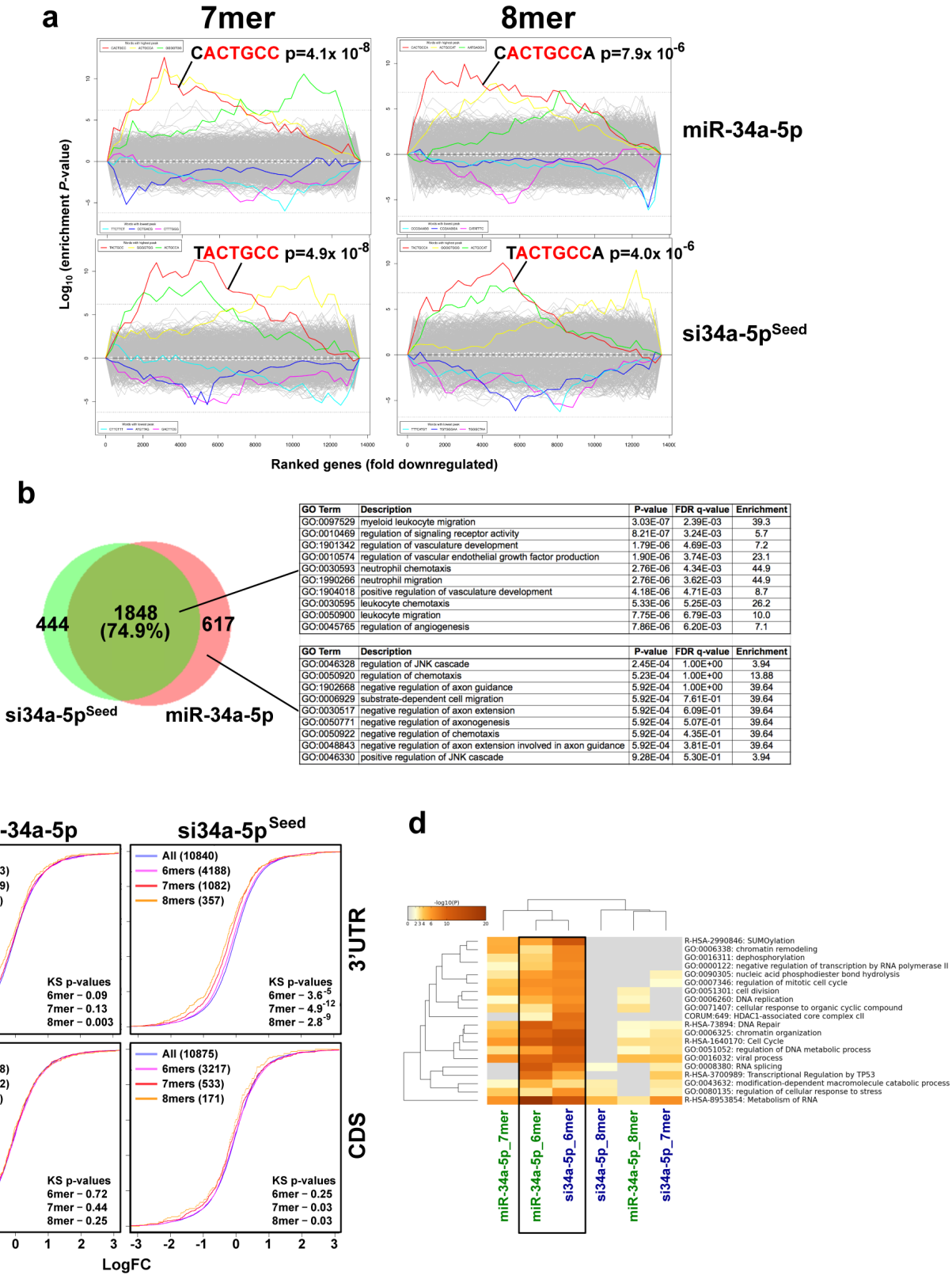
Supplementary Figure 3. Preference for Gs in most toxic siRNAs. (**a**) Regression analysis showing the correlation in cell viability between the original 4096 duplexes screen (x-axis) and the repeat screen with the 76 duplexes with the highest nucleotide content in the seed region in either HeyA8, H460, M565, or 3LL cells. p-values were calculated using Pearson correlation analysis. **(b)** Nucleotide composition at each of the 6 seed positions in a commercial human genome-wide siRNA library (>65,000 siRNAs, left panel) or our balanced 6mer seed duplexes library (right panel). (**c**) Average nucleotide composition at each of the 6 seed positions for the 200 most toxic (left) and 200 least toxic (right) seeds for all four human and mouse cell lines.



Supplementary Figure 4. The toxicity index (TI) correlates with 6mer seed toxicity. Regression analysis showing the correlation between the cell viability and the 6mer toxicity index (TI) (see [1]) in either HeyA8, H460, M565, or 3LL cells.

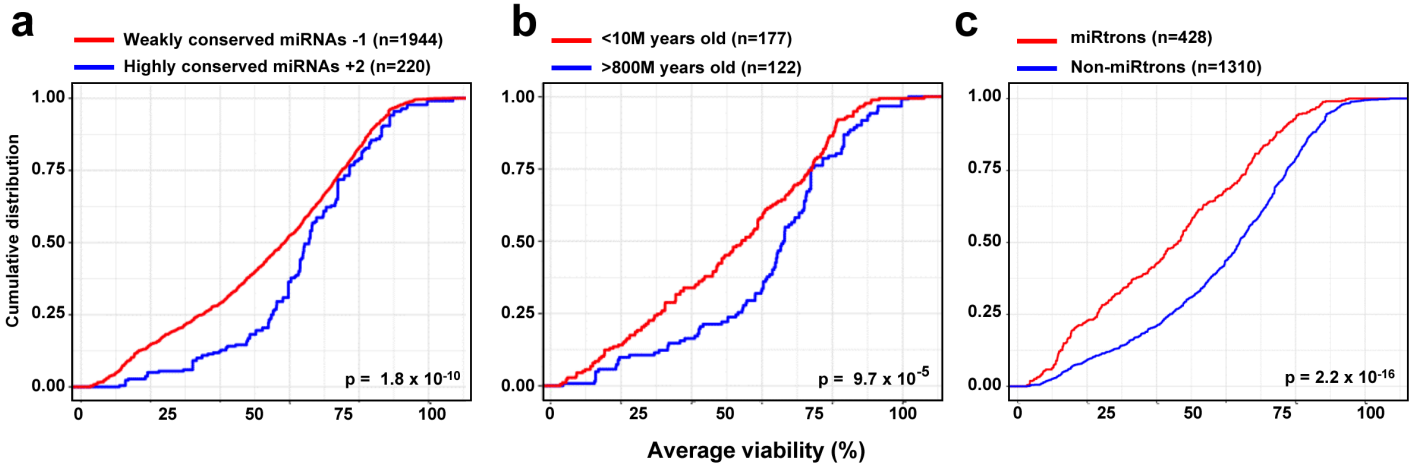


Supplementary Figure 5. Differences in targeting between survival genes and nonsurvival genes by the 100 most and least toxic 6mer seeds. **a** eCDF comparing the ratio of occurrences of the 100 most and least toxic 6mer seed matches in the mRNA elements of two sets of expression matched survival genes and nonsurvival genes. Significance was calculated using a two-sample two-sided K-S test. Significance was higher in the analysis shown with the first 1000 bases of each 3'UTR than with the entire 3'UTR (data not shown). **b** Distribution of the seed matches to the 100 most and least toxic 6mer seeds to human cells in human mRNAs around the boundaries of the 5'UTR and the beginning of the CDS and the end of the CDS and the beginning of the 3'UTR (shown are 500 bases in each direction). Data are shown for either all genes (top) or the expression matched SGs and nonSGs (center and bottom).

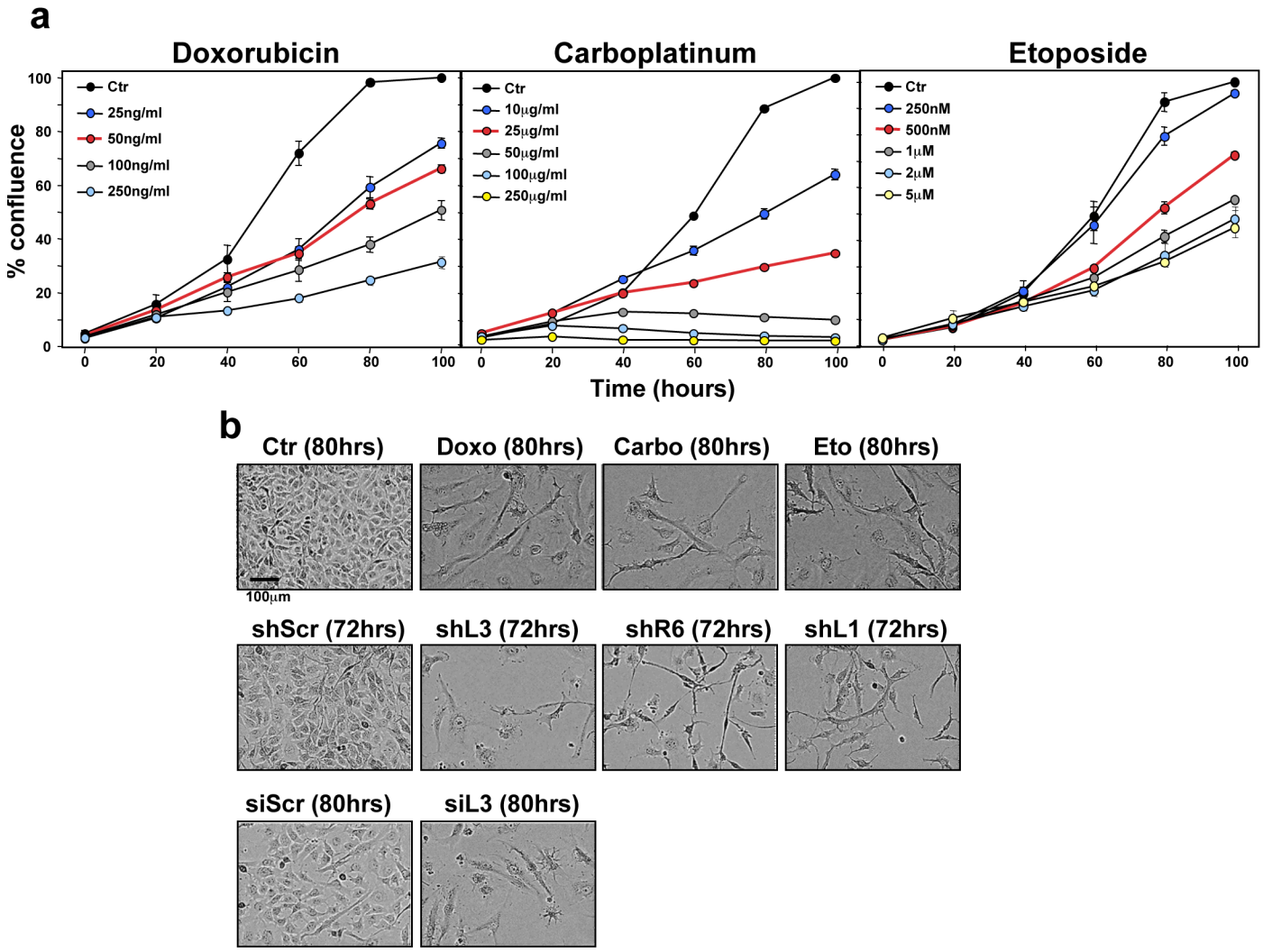


Supplementary Figure 6. Similarities in the effects on cells transfected with miR-34a-5p and si34a-5p. **(a)** Sylamer analysis (7mers and 8mers) for the list of 3'UTRs of mRNAs in cells treated with either miR-34a-5p (top) or si34-5p^{Seed} (bottom) sorted from down-regulated to up-regulated. Bonferroni-adjusted p-values are shown. **(b)** Overlap of RNAs detected by RNA-Seq upregulated in HeyA8 cells (>1.5 fold) 48 hrs after

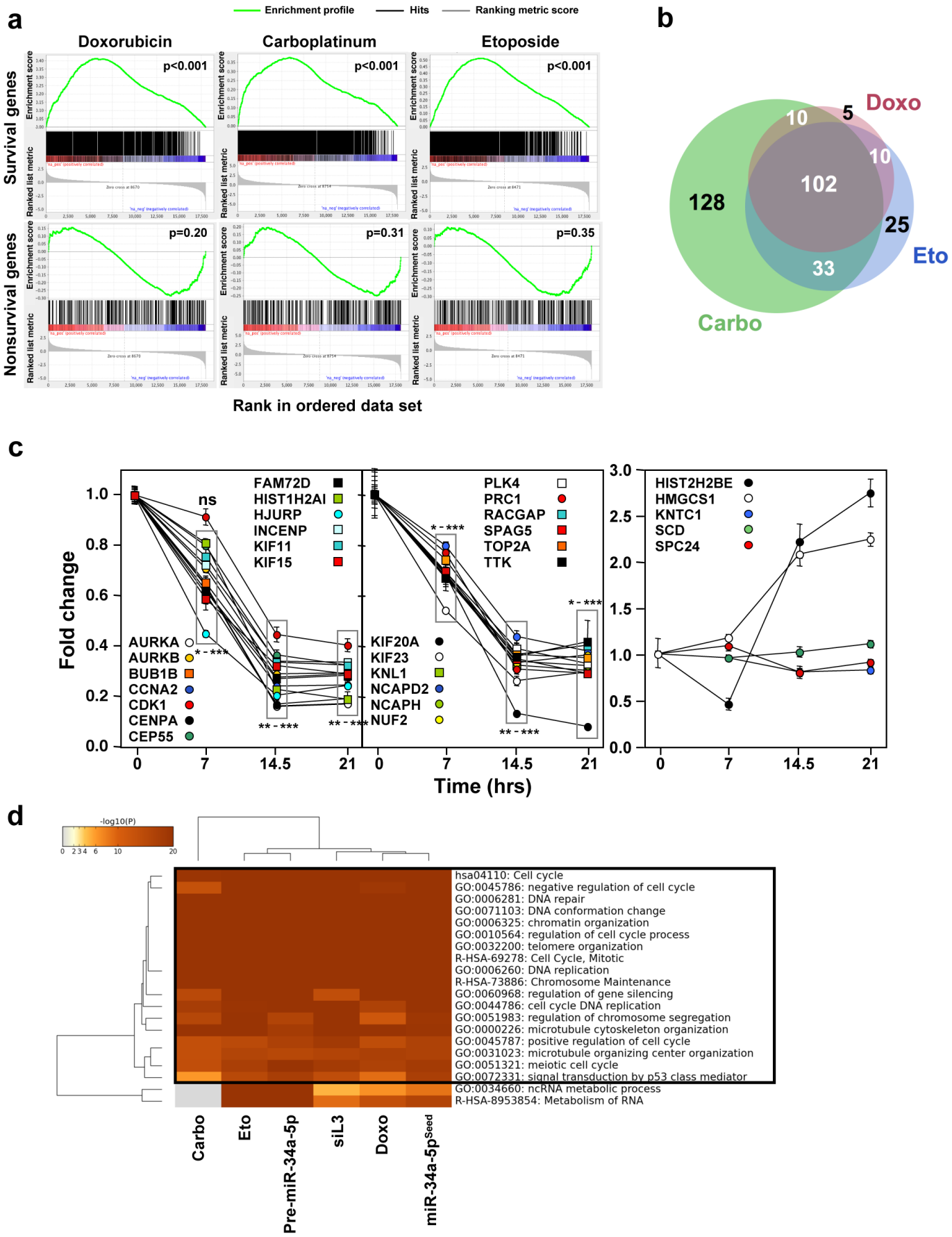
transfection with either si34a-5p^{Seed} or miR-34a-5p when compared to either siNT2 or a nontargeting pre-miR, respectively. *Right*: Results of a GOrilla gene ontology analyses of the genes upregulated in both cells transfected with miR-34a-5p or si34a-5p^{Seed} (top, significance of enrichment $<10^{-6}$), or only in cells transfected with miR-34a-5p (bottom, significance of enrichment $<10^{-4}$). **(c)** eCDF of all mRNAs and mRNAs containing a unique 6mer, 7mer or 8mer in their 3'UTR. Only unique 6, 7 and 8mer seed matches were counted. **(d)** Metascape gene ontology analysis comparing the downregulated genes in cells treated with either miR-34a-5p or si34a-5p^{Seed} containing either unique 6mers, 7mers or 8mers of the respective si/miRNA. GO terms shared by genes downregulated in cells transfected with either siRNA and carrying a unique (not overlapping with either a 7 or 8mer) 6mer are boxed. Note, the downregulated genes that contained unique 7 or 8mers showed no overlap between cells transfected with miR-34-5p or si34a-5p.



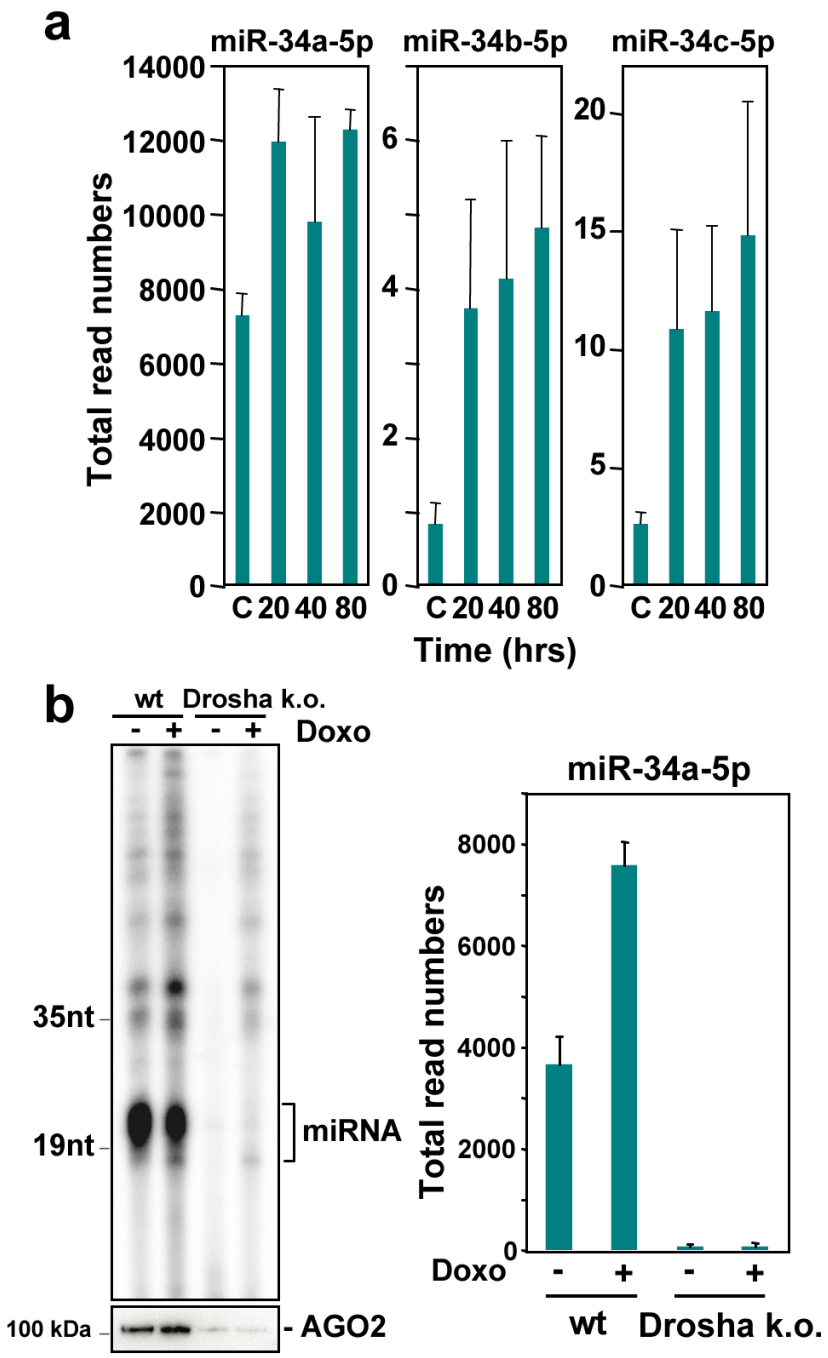
Supplementary Figure 7. Loss of toxic 6mer seed in miRNA during evolution. **(a-c)** Data of **Figs. 4a**, left, **4a**, right and **4c** represented as Empirical Cumulative Density Fraction plots.



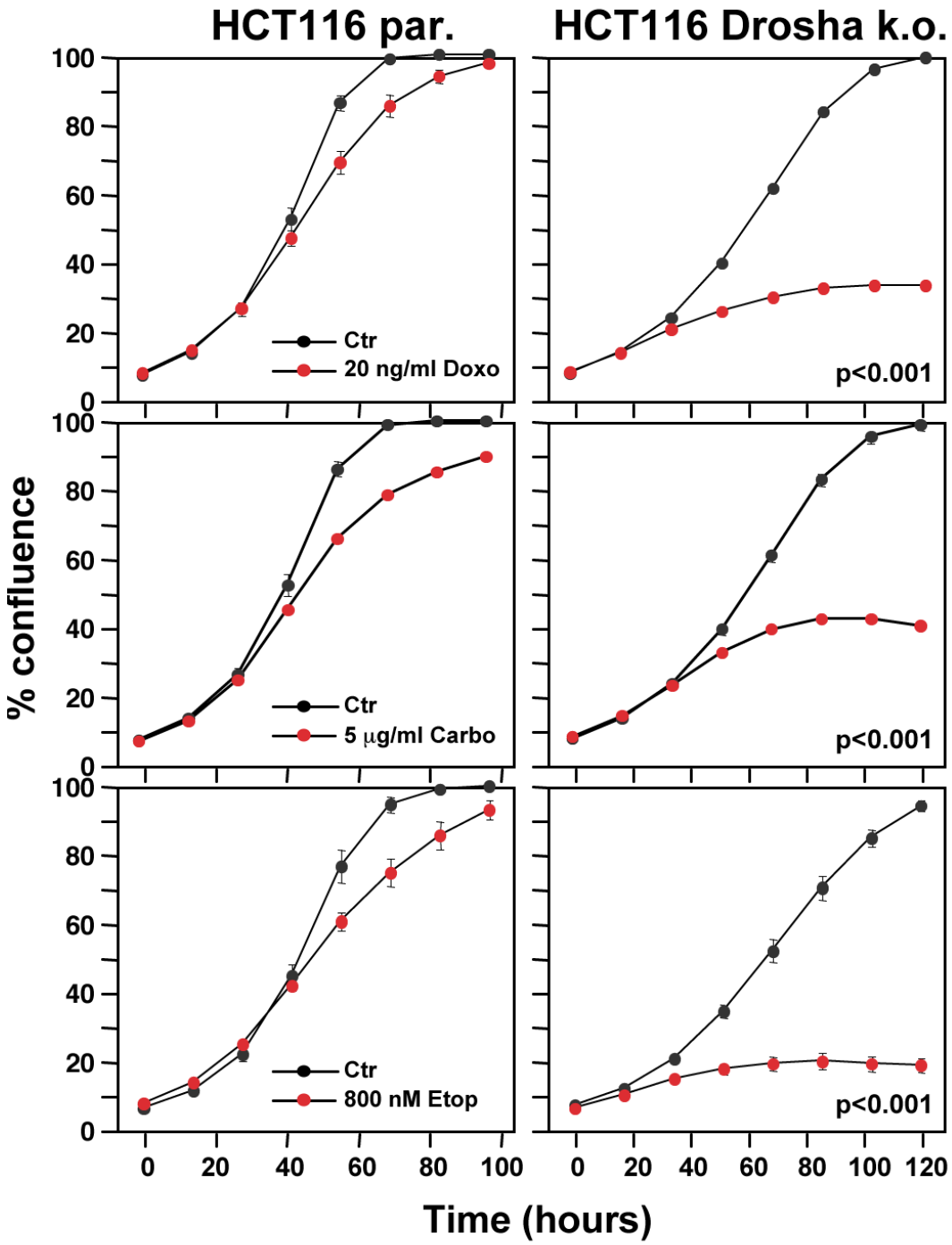
Supplementary Figure 8. Cells treated with genotoxic drugs have features of cells treated with si/shRNAs carrying toxic 6mer seeds. **(a)** Titration of the three genotoxic drugs. Percent cell confluence of HeyA8 cells after treatment with three genotoxic drugs at various concentrations. Medium treated cells were used as control for Carbo and Doxo treated cells. Solvent control treated cells (0.025% DMSO in medium) were used as control for Eto treated cells. Data are representative of three independent experiments. Each data point represents mean ± SE of three replicates. For each treatment the concentration that resulted in the red curve was used for the RNA Seq experiment described in **Fig. S9**. **(b)** Morphological features of HeyA8 cells treated with three genotoxic drugs or control, with the 3 indicated shRNAs (and shScr), or with 10 nM siL3 and siScr for the indicated time.


a
Enrichment profile
Hits
Ranking metric score
Doxorubicin
Carboplatinum
Etoposide
Survival genes
Nonsurvival genes
p<0.001
p<0.001
p<0.001
p=0.20
p=0.31
p=0.35
Enrichment score
Ranked list metric
Rank in ordered data set
b
Doxo
Eto
Carbo
5
10
10
102
128
25
33
c
Fold change
Time (hrs)
ns
FAM72D
HIST1H2AI
HJURP
INCENP
KIF11
KIF15
AURKA
AURKB
BUB1B
CCNA2
CDK1
CENPA
CEP55
PLK4
PRC1
RACGAP
SPAG5
TOP2A
TTK
KIF20A
KIF23
KNL1
NCAPD2
NCAPH
NUF2
HIST2H2BE
HMGCS1
KNTC1
SCD
SPC24
d
-log10(P)
hsa04110: Cell cycle
GO:0045786: negative regulation of cell cycle
GO:0006281: DNA repair
GO:0071103: DNA conformation change
GO:0006325: chromatin organization
GO:0010564: regulation of cell cycle process
GO:0032200: telomere organization
R-HSA-69278: Cell Cycle, Mitotic
GO:0006260: DNA replication
R-HSA-73886: Chromosome Maintenance
GO:0060968: regulation of gene silencing
GO:0044786: cell cycle DNA replication
GO:0051983: regulation of chromosome segregation
GO:0000226: microtubule cytoskeleton organization
GO:0045787: positive regulation of cell cycle
GO:0031023: microtubule organizing center organization
GO:0051321: meiotic cell cycle
GO:0072331: signal transduction by p53 class mediator
GO:0034660: ncRNA metabolic process
R-HSA-8953854: Metabolism of RNA
Carbo
Eto
Pre-miR-34a-5p
siL3
Doxo
miR-34a-5pSeed

Supplementary Figure 9. Similarities between genes downregulated after cell death induction with CD95L and CD95 derived si/shRNAs and cell death induced by genotoxic drugs. **(a)** GSEA analysis showing that ~1800 survival genes are preferentially downregulated in HeyA8 cells treated with all three genotoxic drugs. GSEA analysis using a control set of ~400 genes that are not required for survival (as described in [1] were not enriched in the downregulated genes. **(b)** Venn diagram showing that 102 downregulated survival genes (out of ~1800) (>2 fold, adj p value <0.05) are shared among HeyA8 cells treated with three different genotoxic drugs. A DAVID gene ontology analysis showed that these 102 genes are mostly involved in mitosis and cell cycle (**Supplementary Table 5**). **(c)** 30 of the 102 survival genes in downregulated in all three genotoxic drugs (see C) were chosen for a kinetics study by real-time PCR. 25 of the 30 genes were found to be substantially downregulated at 14.5 hours. 24 of the 25 genes were significantly downregulated as early as 7 hours after Doxo treatment. Five of the 30 genes were found to be either unchanged or up regulated after Doxo treatment. Each data point represents mean ± SD of three replicates. The 20 hr time point was independently repeated. Significance was calculated using a two-sided t-test. *p<0.05, **p<0.001, *** p<0.0001. **(d)** Metascape gene ontology analysis comparing the downregulated genes in cells treated with either siL3, siR-34a-5p^{Seed}, pre-miR34a-5p or the three genotoxic drugs. GO terms shared by all treatments are boxed.



Supplementary Figure 10. Upregulation of Ago-bound miR-34a-5p in cells treated with genotoxic drugs. **(a)** Total read numbers of Ago bound miR-34a (left panel), miR-34b (middle panel), and miR-34C (right panel) in control treated cells or after 20, 40, or 80 hours of Doxo treatment. Shown is variance of two biological replicates. **(b)** Ago pull down of miRNAs from HCT116 wild-type and Drosha k.o. cells treated with Doxo for 80 hrs (left). The images are representative of two biological replicates. Comparison of read numbers of pre-miR-34a-5p bound to Ago proteins between control and Doxo treated samples in wild-type (wt) or Drosha k.o. cells (right). Shown is variance of two biological replicates.



Supplementary Figure 11. Percent cell confluence over time of HCT116 parental or Drosha k.o. cells treated with three genotoxic drugs. P values were calculated using a binomial distribution test and support a difference in sensitivity between parental HCT116 and Drosha k.o. HCT116 cells. Data are representative of two independent experiments. Each data point represents mean ± SE of three replicates.

Supplemental Tables

Supplementary Table 1: Results of the 4096 6mer seed toxicity screen in four cell lines. The top 200 most toxic and bottom 200 least toxic duplexes (for cell lines) are highlighted in red and green, respectively. All data are available at https://6merdb.org.

Supplementary Table 2: The 20 and 100 most and least toxic 6mers to both Hey8 and H460 cells. Nucleotide compositions of the 20 most toxic and 20 least toxic 6mer seed duplexes that was used to perform analyses in **Fig. 3a**.

Supplementary Table 3: Results of RNA Seq analysis of HeyA8 cells transfected with either miR-34a-5p or si34a-5p^{Seed}.

Supplementary Table 4: Ratio of 6mer seed toxicity of the guide strand versus the lesser expressed arm of all miRNAs.

Supplementary Table 5: Downregulated genes in HeyA8 cells treated with either Doxo, Carbo, or Eto and results of DAVID gene ontology analysis of the 102 genes downregulated in HeyA8 cells treated with the three drugs.

Supplementary Table 6: Lists of 938 expression-matched SGs and nonSGs.

REFERENCES

1. Putzbach W, *et al*. Many si/shRNAs can kill cancer cells by targeting multiple survival genes through an off-target mechanism. *eLife* **6**, e29702 (2017).