Supporting Information for

Targeting of Several Glycolytic Enzymes using RNAi Reveals Aldolase Affects Cancer-Cell Proliferation through a Non-Glycolytic Mechanism

By Carolyn Ritterson Lew and Dean R. Tolan

Optimization of RNAi affects using small-interfering RNAs (siRNA).

Concentration of siRNAs. The optimal concentrations of pooled siRNAs to aldolase were measured by transfecting concentrations varying from 0.5 – 50 nM into mouse fibroblasts (gift from T. Gilmore) using Lipofectamine-2000 as per the manufacturer's instructions. After four days, the remaining specific activity was measured. Fig. S1A shows the results plotted as a fraction of remaining activity relative to mock-transfected cells. The knockdown was achieved at concentrations at and above 0.5 nM siRNA. However, as the concentration of siRNA decreased, the duration of knockdown also decreased.

While 0.5 and 1 nM siRNA knocked down aldolase at 2 d post-transfection, by 6 days after transfection, aldolase activity had recovered to near mock transfection levels. Concentrations up to 10 nM siRNA knocked down aldolase through 4 d, but began to recover by 6 d after transfection. Therefore, siRNAs were used at concentrations ≥25 nM for all subsequent experiments.

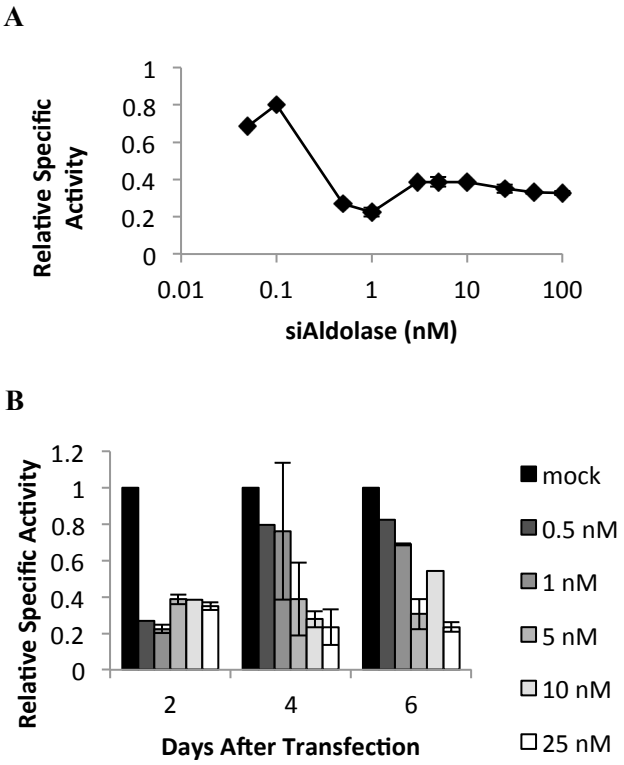

Fig. S1. Titration of pooled aldolase siRNAs. A. Mouse fibroblasts were transfected with pooled aldolase siRNA at various nanomolar concentrations. Relative aldolase activity was measured four days after transfection and is plotted vs. total concentration of pooled aldolase siRNA. Error is expressed as SEM. **B.** Mouse fibroblast cells were mock transfected (black), or transfected with increasing concentrations of siAldolase (decreasing amounts of grey, see key). Aldolase specific activity was measured 2, 4, and 6 days after transfection and normalized to mock transfected cells. Error is shown as SEM. Mock transfection values: Day 2, 0.017 ± 0.0023; Day 4, 0.010; and Day 6, 0.013 U/mg.

Efficacy of aldolase siRNAs. The aldolase siRNAs (25 nM total concentration) in various combinations were transfected into mouse fibroblasts using Lipofectamine-2000. After four days, the cells were lysed, and the specific activity of aldolase was measured. Fig. S2 shows the effects of aldolase knockdown for each individual siRNA (siRNA287, siRNA1171, and siRNA1301) or combinations of siRNAs expressed as a fraction of remaining activity relative to mock-transfected cells. There was no significant difference among any individual siRNA or in any combination of them.

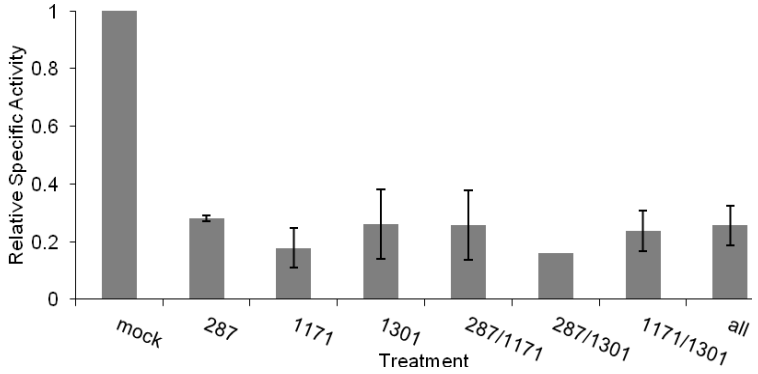

Fig. S2. Effect of pooling aldolase siRNAs. Single, pairs, or all three aldolase siRNAs were transfected into mouse fibroblasts. Residual aldolase activity normalized to mock transfected cells is plotted. The experiment was performed twice, and error bars represent the range of values.

Duration of the knockdown effect. Mouse fibroblasts were transfected with 50 nM siRNAs using Lipofectamine 2000. Cells were lysed at varying times from 1–14 days after transfection, and the specific activity of aldolase was measured. The extent of the knockdown was maximal after four days regardless of each individual siRNA used. The aldolase activity slowly recovered such that by 12-14 days there was no longer any significant knockdown of enzyme activity (Fig. *S3A*). An experiment using 25 nM siRNA to the other enzymes, TIM, GAPDH, and enolase 1 showed a similar effect. By 3-4 days after transfection, there was maximal loss of enzyme activity (Fig. *S3B*).

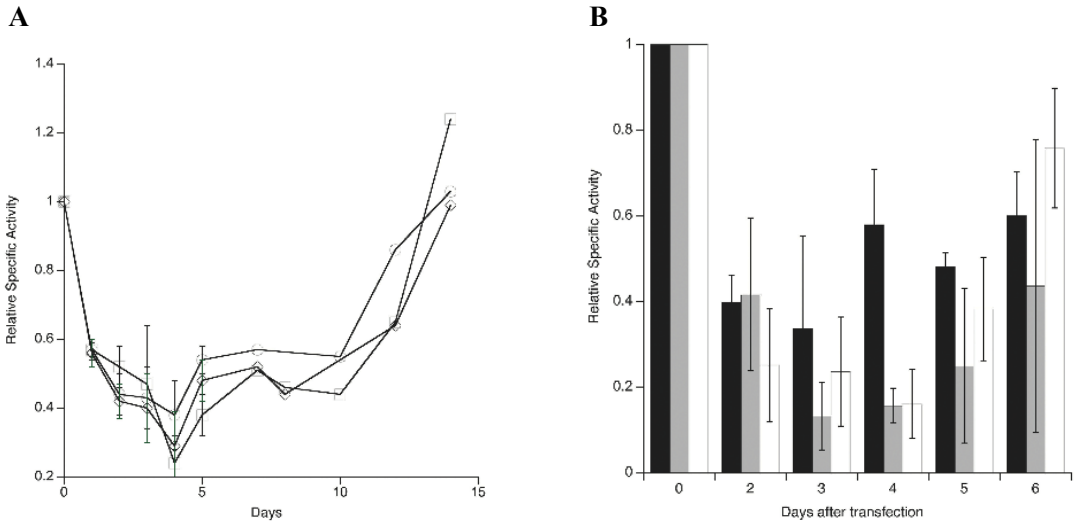

Fig. S3. Determination of maximum attenuation and duration of knockdown using siRNAs to glycolytic enzymes. A. Three different aldolase siRNAs (50 nM), 287 (○), 1171 (□), and 1301 (◇) were transfected into mouse fibroblasts. Relative specific activity was measured each day after transfection and plotted vs. time. Error is represented as SEM. **B.** siRNAs (25 nM) to GAPDH (black), TIM (gray), or enolase (white) were transfected into mouse fibroblasts, and plotted as described in (*A*). Error is represented as SD.

ATP extraction methods.

Several methods were tested for the extraction of ATP from cells:

Freeze/Thaw Deproteinization: Aliquots of the media were removed over 24-36 h and immediately frozen at -80 °C. Samples were incubated at 80 °C, and then centrifuged at 8000x*g* to remove any remaining cellular debris.

Acid/Base Deproteinization: Cells were washed twice with PBS and lysed and deproteinized with 1 M perchloric acid. Acid was neutralized with 3 M potassium phosphate, and the resulting precipitate was removed *via* centrifugation (1000x*g*, 10 min). Alternatively, cells were scraped from their dish and immediately frozen at -80 °C. Samples were incubated at 80 °C, and then centrifuged at 8000x*g* to remove any remaining cellular debris.

Native cell lysates (NCLs): Cells were washed twice in ice-cold PBS, scraped from dish, and pelleted a by brief centrifugation. Cells were resuspended in NCL buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% (v/v) Triton-X 100, 1 mM dithiothreitol (DTT), and 1 μg/μL each leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride (PMSF)) and incubated for 30 min on ice, followed by centrifugation at 20,000x*g* for 1 h.

NCLs/Heat Deproteinization: The NCLs were prepared as descried above, after which samples were incubated at incubated at 80 °C, and then centrifuged at 8000x*g* to remove any remaining cellular debris deproteinized the sample.

These lysates were used in a luciferase-based [ATP] determination assay (Invitrogen). Table S1 shows the luciferase signal obtained for each extraction method as well as the percent error associated with each. The freeze/thaw method was the most robust and precise method, and was used in all subsequent [ATP] determination assays.

Table S1
ATP Luciferase Assay Values for Various Extraction Methods[a]

		Freeze/Thaw	Acid/Base	NCL	NCL/Heat
Mock	**Mean**	3.3±0.65	0.42±0.075	2.1±0.28	5.6±2.0
	% error	19	18	13	35
siAldolase	**Mean**	5.7±0.13	0.13±0.026	1.9±0.65	1.7±1.6
	% error	22	20	35	92

[a]Data was obtained using a luciferase-based assay for 3 trials for each method to calculate the mean and SD. Values ($x10^{-5}$) are arbitrary units based on luminometer readings.

Proliferation rates of glycolytic enzyme-knockdown cells

Proliferation rates were determined for each proliferation assay performed (shown in Fig. 1B-D). Aldolase knockdown caused the greatest decrease in proliferation rate (–80%) of the four enzymes examined using the hemocytometer for direct cell counting. For the aldolase–knockdown cells this decrease in proliferation rate was confirmed using crystal violet and MTS assays.

Table S2

Effect of Glycolytic Enzyme Knockdown on Cell Proliferation Measured by Various Methods

Method[a]	Treatment[a]	Rate[b]	Normalized Rate	Change in Proliferation (%)
Hemocytometer	Mock	0.57	1.0	-
	siAld	0.10	0.2	–80
	siTIM	0.27	0.5	–50
	siGAPDH	0.42	0.7	–25
	siEno	0.47	0.8	–20
Crystal Violet	Mock	0.37	1.0	-
	siAld	0.16	0.4	–60
MTS	Mock	0.41	1.0	-
	siAld	0.13	0.3	–70

[a]Methods and treatments were as described in Experimental Procedures.
[b]Rates of cell proliferation were measured by converting the kinetics of cell proliferation to a log of cells/day and determining the slope. Rates are expressed as ln (cell number)/day.

Glycolytic flux as indicated by glucose consumption.

Glycolytic flux was compared among untreated, mock-transfected, and siRNA to aldolase-transfected Ras-3T3 cells by determining glucose consumption using a glucose oxidase assay according to the manufacturer's instructions (Sigma-Aldrich). Three days after transfection, cells were incubated with 5 mM glucose. Aliquots of media were removed every 4-6 h over a 25-36 h time period. Aliquots were treated as described in Experimental Procedures and used for determination of glucose concentration at each time point. A rate of glucose consumption was determined by plotting the concentration of glucose vs. time and normalized to the number of cells. As shown in Fig. S4, the rate of glucose depletion was not significantly different among all three treatments, as determined by both Student's t-test and ANOVA analysis.

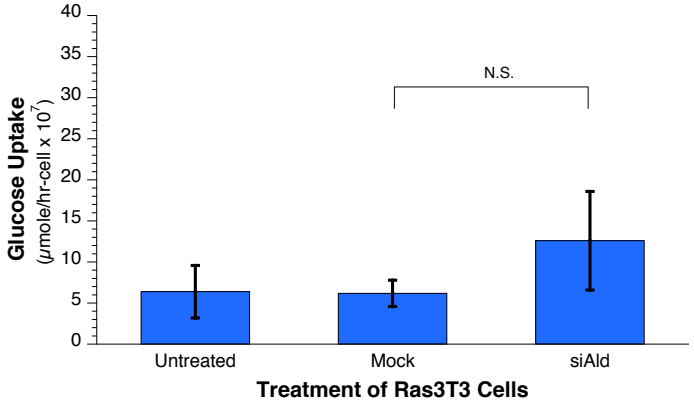

Figure S4. Glucose uptake as an indicator of glycolytic flux. Glucose uptake was measured in Ras-3T3 cells that were untreated, mock-transfected, or siRNA to aldolase-transfected (siAld) using the rate of glucose lost from media measured using glucose oxidase and normalizing to cell number (5-50 x 10^3) counted by hemocytometer. Error is represented as 1SD (n=6). N.S. shows no significant difference ($p>0.05$) between mock and siAld treatments. ANOVA among all three treatments had F-value = 4.62 < F_{crit} of 4.85, not significantly different.

Glycolytic Flux is unaffected in aldolase-, GAPDH-, TIM-, and enolase-siRNA-treated cells.

The effect of aldolase, GAPDH, TIM, and enolase siRNA knockdown on glycolytic flux in both Ras-3T3 and NIH-3T3 cells was examined 4 d after transfection. As shown in Fig. S5, for both NIH-3T3 and Ras-3T3 cells there was no significant change in glycolytic flux following knockdown. Thus, residual activity of these enzymes was sufficient for normal glycolysis.

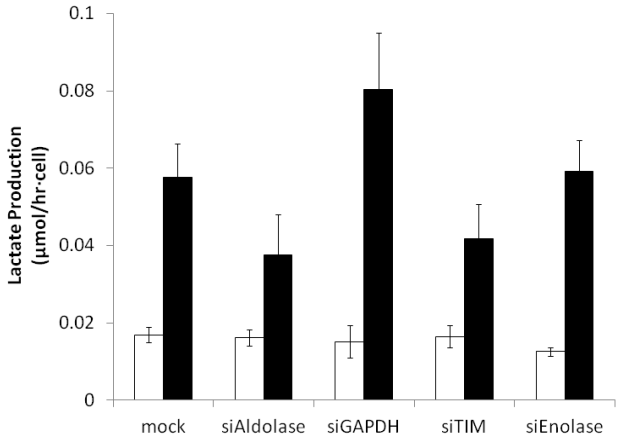

Fig. S5. NIH-3T3 (white) and Ras-3T3 (black) cells were mock-transfected or transfected with 25 nM siRNA to aldolase, GAPDH, TIM or enolase as indicated. Three days post-transfection, cells were starved for 24 h, and then a bolus of 5 mM glucose was added. Lactate production (±SD) was measured as a function of time over 36 h for calculation of the rate of glycolysis as described in Experimental Procedures.