Supplementary Information

Conditional Antisense Oligonucleotide Triggered by miRNA

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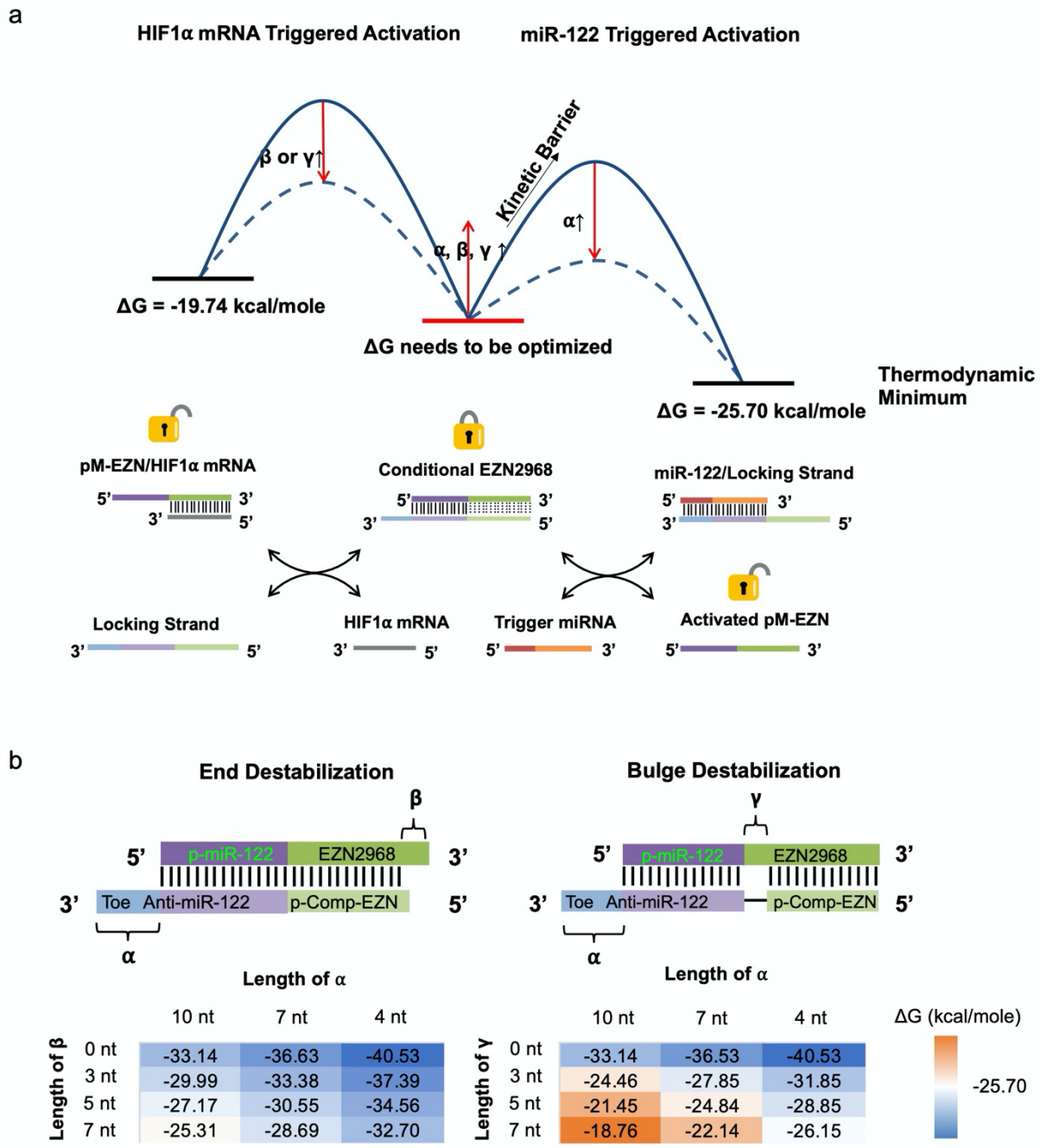

Figure S1. NUPACK prediction of free energy (ΔG) for conditional EZN2968 at 37 ℃. (a) Energy diagram showing displacement of the **pM-EZN** strand by miR-122 or displacement of the locking strand by HIF1α mRNA. The predicted ΔG is -25.70 kcal/mole for miR-122/locking strand duplex, and -19.74 kcal/mole for **pM-EZN**/HIF1α mRNA. The calculation indicates that the conditional ASO binding to miRNA is more favorable than binding to the target mRNA. This design minimizes mRNA-mediated unlocking of the conditional ASO. The toehold length (α, β and γ domains) cannot be independently tuned without also tuning the stability of the conditional ASO. Increasing the length of the β or γ domain destabilizes the conditional ASO and also reduces

the barrier to binding the mRNA target. Likewise, increasing the length of the α domain destabilizes the conditional ASO and lower the barrier to miRNA binding. (b) Predicted ΔG of conditional ASO duplexes with different length of α, β and γ domain. When ΔG > -25.70 kcal/mole, miRNA triggered activation is favorable. These values were calculated using the default parameter set for DNA (SantaLucia, 1998) in NUPACK with 0.15 M NaCl and 10 nM oligonucleotide concentrations at 37°C.

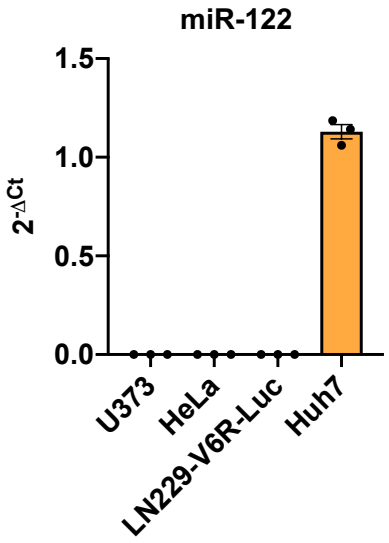

Figure S2. miR-122 expression levels in different cell lines. RNA was isolated from each cell line using miRNeasy Mini Kit (QIAGEN), and reverse-transcribed using miScript II RT Kit (QIAGEN). miR-122 levels were quantified by qPCR with RNU6 as a reference. $\Delta Ct = Ct_{miR\text{-}122} - Ct_{RNU6.}$ The error bars represent SEM from triplicate measurements.

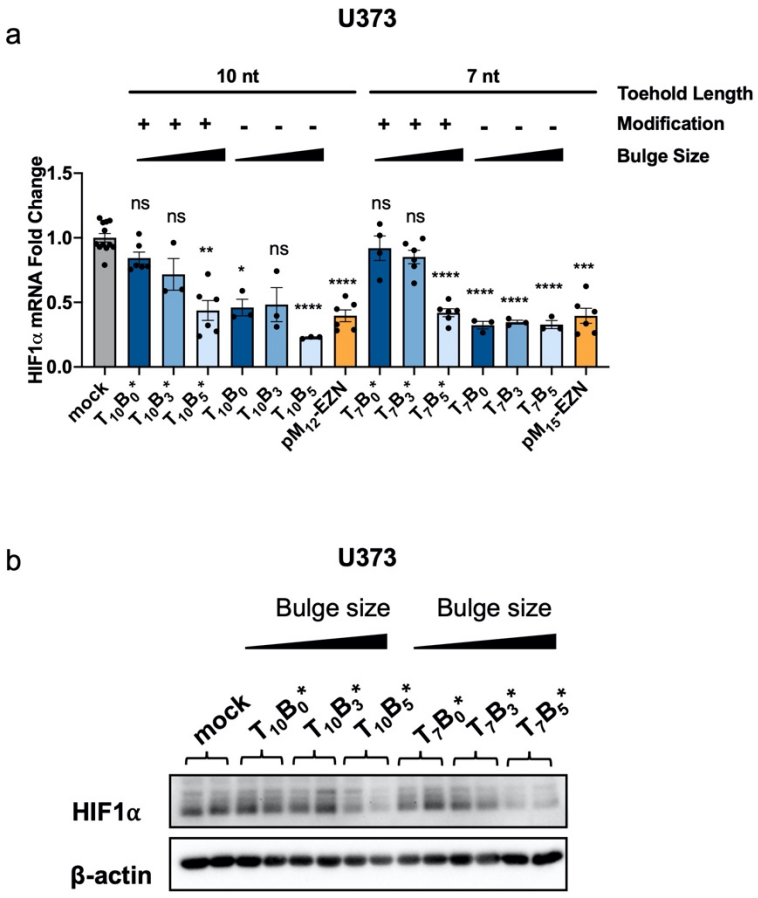

Figure S3. Spontaneous activation of conditional EZN2968 in U373 cells. U373 cells were transfected with 10 nM **conditional EZN2968** using Oligofectamine and incubated for 24 h. (a) HIF1α mRNA levels were quantified by qPCR normalized to 18S. The error bars represent SEM from n = or > 3 measurements. ** p<0.01, *** p<0.001, **** p<0.0001, Brown-Forsythe and Welch ANOVA tests with Dunnett's T3 multiple comparison. (b) Western blot of HIF1α protein with β-actin used as a housekeeping gene.

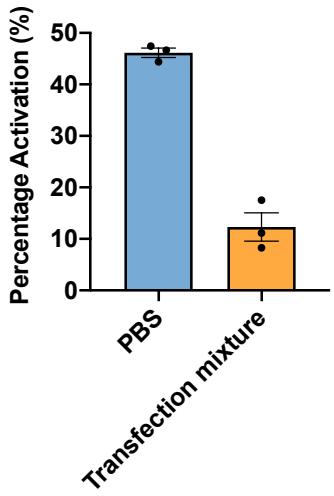

Figure S4. Conditional EZN2968 activation in transfection mixture. 10 nM Cy5/quencher-labeled **T_7B_3*** were incubated with 500 nM miR-122 mimic in PBS or in transfection mixture. For the transfection mixture group, Cy5/quencher-labeled **T_7B_3*** and miR-122 mimic were complexed with Oligofectamine in OptiMEM first and then mixed together, using the same procedure as the *in vitro* transfection experiment.

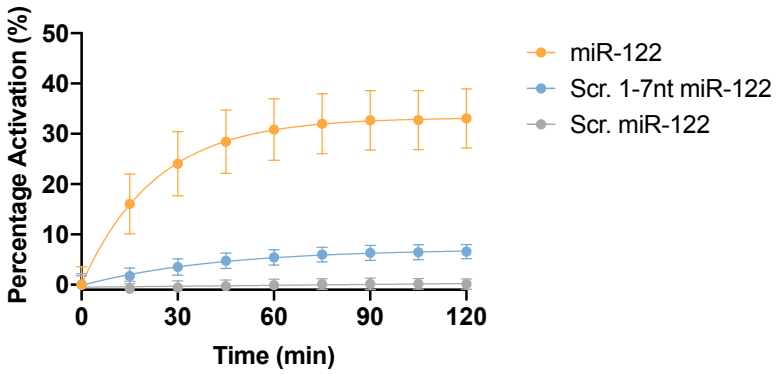

Figure S5. Activation kinetics of T_7B_3* incubated with miR-122, scr. 1-7nt miR-122 or scr. miR-122. 10 nM Cy5/quencher-labeled **T_7B_3*** and 100 nM miR-122, scr. 1-7nt miR-122, or scr. miR-122 were incubated at 37°C for 2h in a plate reader and fluorescence was measured with an interval of 15 min. Percentage activation was determined based on the fluorescence increase. 10 nM Cy5-labeled **pM-EZN** strand was used as a positive control, whose fluorescence intensity represents 100% activation.

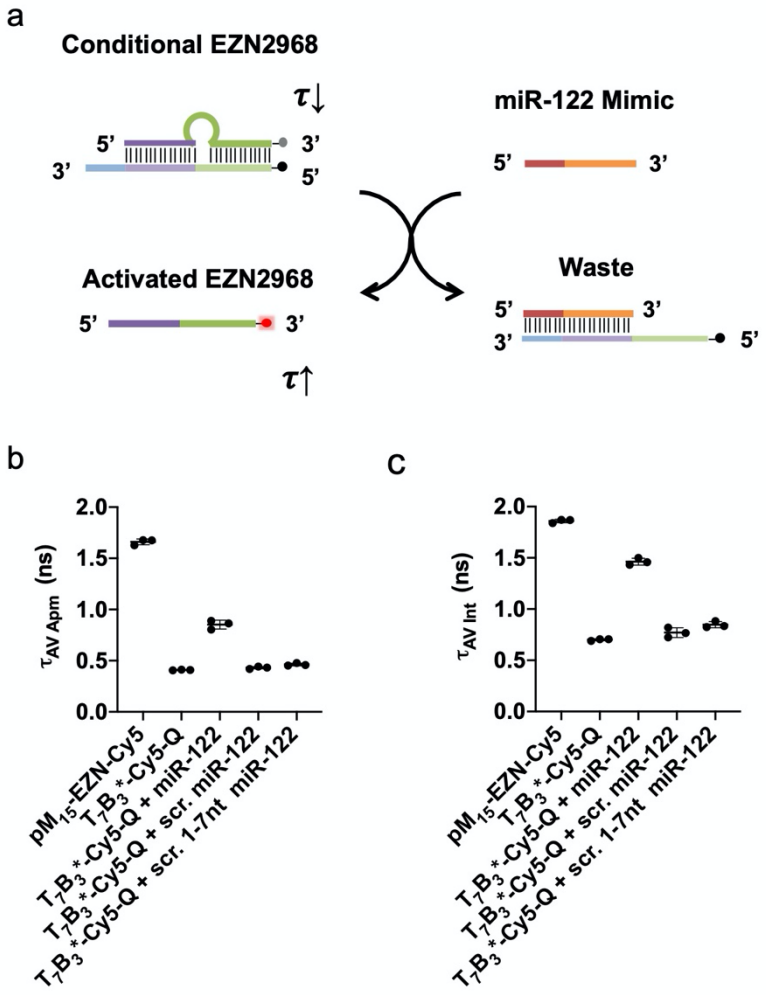

Figure S6. Fluorescence lifetime measurement to evaluate specificity of T_7B_3* to miR-122 in buffer. (a) Scheme showing Cy5 fluorescence lifetime increase due to activation of **T_7B_3*** by miR-122 mimic. (b) Amplitude-averaged and (c) intensity-averaged Cy5 fluorescence lifetime of 10 nM Cy5/Q-labeled **T_7B_3*** solution incubated with 500 nM miR-122, scr. miR-122, or scr. 1-7 nt miR-122 for 2 h at 37 °C in PBS. Unlocked **pM_{15}-EZN**-Cy5 was used as a positive control. The error bars represent SD.

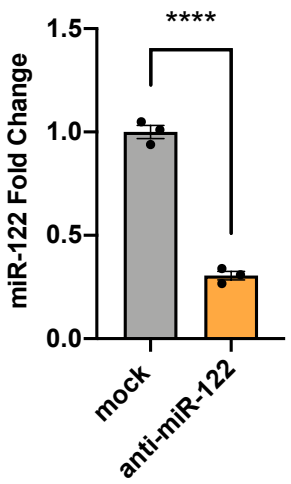

Figure S7. Endogenous miR-122 in Huh7 cells can be knocked down using anti-miR-122. Huh7 cells were transfected with 500 nM anti-miR-122 (Ambion) using Oligofectamine. After 24 h incubation, miR-122 levels were quantified by qPCR normalized to RNU6. **** $p<0.0001$, *t*-test.

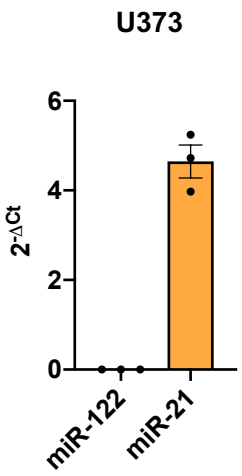

Figure S8. miR-122 and miR-21 expression levels in U373 cells. RNA was isolated from U373 cells using miRNeasy Mini Kit (QIAGEN), and reverse-transcribed using miScript II RT Kit (QIAGEN). miRNA levels were quantified by qPCR with RNU6 as a reference. $\Delta Ct = Ct_{miR\text{-}122} - Ct_{RNU6.}$ The error bars represent SEM.

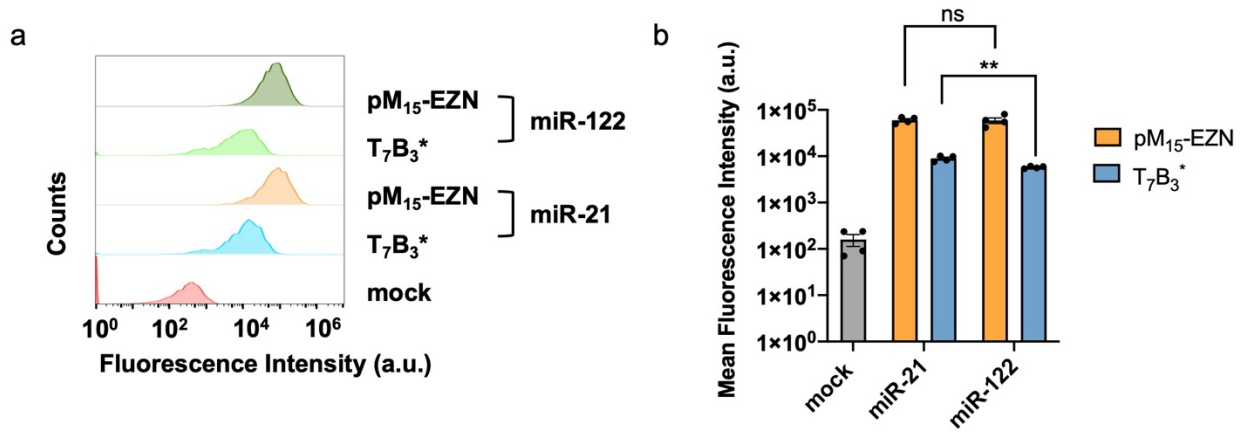

Figure S9. Flow cytometry of U373 cells transfected with miR-21 or miR-122 inducible conditional EZN2968. (a) Histogram and (b) mean fluorescence intensity of U373 cells transfected with 10 nM Cy5/Q-labeled miR-21- or miR-122-inducible **T_7B_3*** and incubated for 24 h. Cells transfected with 10 nM corresponding **pM_{15}-EZN** strands labeled with Cy5 were used as positive controls. The error bars represent SEM. ** $p<0.01$, *t*-test.

Table S1. Oligonucleotide sequences for in buffer testing of leakage activation of bulge and end destabilized conditional EZN2968

/3AmMO/= 3' amino modification; /5IAbRQ/=5' Iowa Black RQ; /5AmMC6/=5' amino modification; "T" indicates a mismatch in the toehold binding region.

ID	Sequence
Unmodified pM_{12}-EZN	CAA TGG TGT TTG TGG CAA GCA TCC TGT A/3AmMO/
Unmodified pM_{15}-EZN	TGA CAA TGG TGT TTG TGG CAA GCA TCC TGT A/3AmMO/
Unmodified pM_{18}-EZN	GTG TGA CAA TGG TGT TTG TGG CAA GCA TCC TGT A/3AmMO/
Unmodified B_0	/5IAbRQ/TA CAG GAT GCT TGC CAC AAA CAC CAT TGT CAC TCT CCA
Unmodified B_3	/5IAbRQ/TA CAG GAT GCT TGC AAA CAC CAT TGT CAC TCT CCA
Unmodified B_5	/5IAbRQ/TA CAG GAT GCT CAA ACA CCA TTG TCA CTC TCC A
Unmodified B_7	/5IAbRQ/TA CAG GAT GCA AAC ACC ATT GTC ACT CTC CA
Unmodified E_3	/5IAbRQ/AG GAT GCT TGC CAC AAA CAC CAT TGT CAC TCT CCA
Unmodified E_5	/5IAbRQ/GA TGC TTG CCA CAA ACA CCA TTG TCA CTC TCC A
Unmodified E_7	/5IAbRQ/TG CTT GCC ACA AAC ACC ATT GTC ACT CTC CA

Table S2. Oligonucleotide sequences for *in vitro* testing of conditional EZN2968

"+" = LNA modification; "*" = PS modification; /3AmMO/ = 3' amino modification; "T" indicates a mismatch on the toehold binding region.

ID	Sequence
EZN2968	+T*+G*+G* C*A*A* G*C*A* T*C*C* +T*+G*+T* A
EZN3088	+C*+G*+T* C*A*G* T*A*T* G*C*G* +A*+A*+T* C
Modified pM_{12}-EZN	C*A*A* T*G*G* T*G*T* T*T*G* +T*+G*+G* C*A*A* G*C*A* T*C*C* +T*+G*+T* A/3AmMO/
Modified pM_{15}-EZN	T*G*A* C*A*A* T*G*G* T*G*T* T*T*G* +T*+G*+G* C*A*A* G*C*A* T*C*C* +T*+G*+T* A/3AmMO/
Modified B_0 (B_0*)	/5IAbRQ/+T*+A* +C*A*G* G*A*T* G*C*T* T*G*C* C*A*C* A*A*A* C*A*C* C*A*T* T*G*+T* +C*+A*C* A*C*T* C*C*A
Modified B_3 (B_3*)	/5IAbRQ/*T*A* C*A*G* G*A*T* G*C*+T* +T*+G*+C* +A*+A*A* C*A*C* C*A*T* T*G*T* C*A*C* A*C*T* C*C*A
Modified B_5 (B_5*)	/5IAbRQ/*T*A* C*A*G* G*A*T* +G*+C*+T* +C*+A*+A* A*C*A* C*C*A* T*T*G* T*C*A* C*T*C* T*C*C* A
B_3* without toehold	/5IAbRQ/T*A* C*A*G* G*A*T* G*C*+T* +T*+G*+C* +A*+A*A* C*A*C* C*A*T* T*G*T* C*A
miR-122 mimic	+T*G*G* +A*G*T* +G*T*G* +A*C*A* +A*T*G* +G*T*G* +T*T*T* +G
Scrambled miR-122	+G*A*A*+G*T*A*+T*G*T*+G*G*T*+G*A*T*+T*G*C*+G*T* G*+T
Scr. 1-7nt miR-122	+G*G*G*+T*T*G*+A*T*G*+A*C*A*+A*T*G*+G*T*G*+T*T* T*+G

Table S3. Oligonucleotide sequences for miR-21-inducible EZN2968

"+" = LNA modification; "*" = PS modification; /3AmMO/ = 3' amino modification

ID	Sequence
pM_{15}-EZN for miR-21-inducible EZN2968	T*C*A* G*A*C* T*G*A* T*G*T* T*G*A* +T*+G*+G* C*A*A* G*C*A* T*C*C* +T*+G*+T* A/3AmMO/
B_3* for miR-21-inducible EZN2968	/5IAbRQ/*T*A* C*A*G* G*A*T* G*C*+T* +T*+G*+T* +C*+A*A* C*A*T* C*A*G* T*C*T* G*A*T* A*A*G* C*T*A

Table S4. Primer Sequences.

Primer	**Sequence (5'→3')**
HIF1α Forward	TATGAGCCAGAAGAACTTTTAGGC
HIF1α Reverse	CACCTCTTTTGGCAAGCATCCTG
18S Forward	AGGAATTGACGGAAGGGCACCA
18S Reverse	GTGCAGCCCCGGACATCTAAG