RESEARCH ARTICLE

Figures and figure supplements

Differential translation of mRNA isoforms underlies oncogenic activation of cell cycle kinase Aurora A

Roberta Cacioppo *et al*.

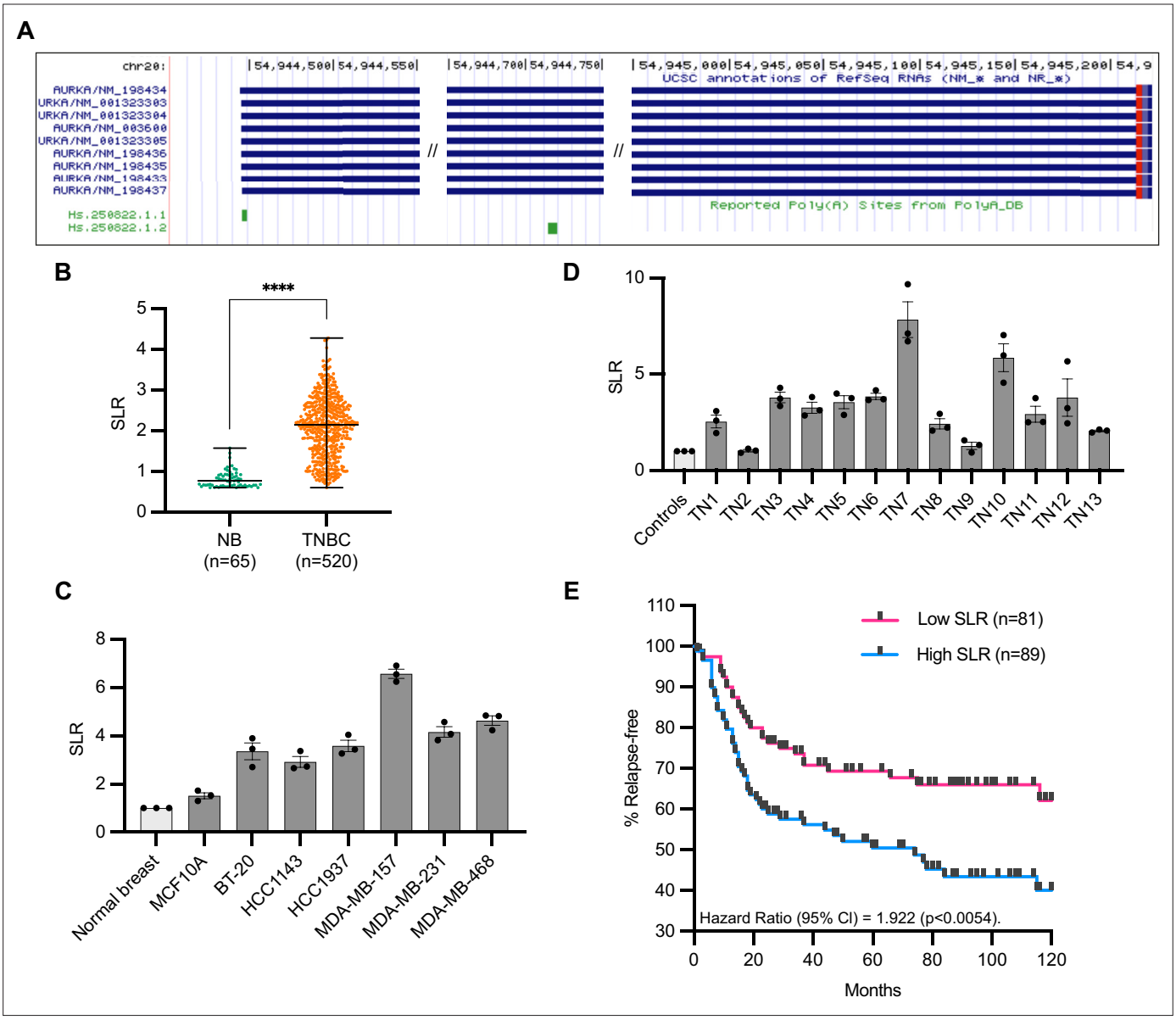


Figure 1. Increased short/long ratio (SLR) of Aurora Kinase A (AURKA) alternative polyadenylation (APA) isoforms in triple-negative breast cancer (TNBC). (**A**) AURKA transcript isoforms (USCS Genome Browser). AURKA gene is located on (-) strand. (**B**) Median and range of SLR values for AURKA 3'UTR obtained using APADetect. Mann–Whitney test; ****p<0.0001. (**C**), (**D**) RT-qPCR analysis of SLR of AURKA 3'UTR in TNBC cell lines (**C**) and patient samples (**D**). SDHA used as reference gene. TN, tissue number. (**E**) Relapse-free survival rates of TNBC patients with high (highest 25%) or low (lowest 25%) AURKA SLRs. p-value determined by log-rank test.

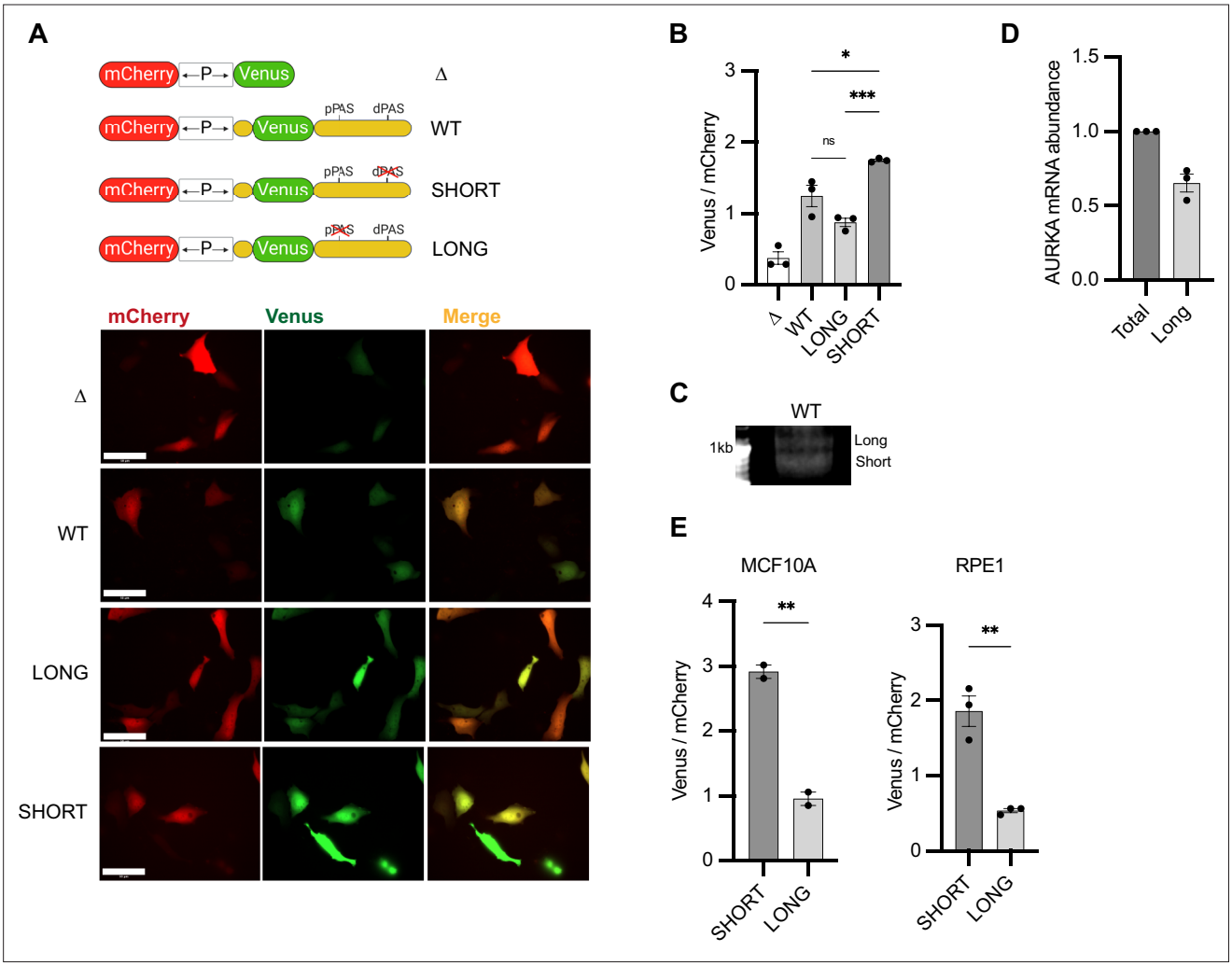
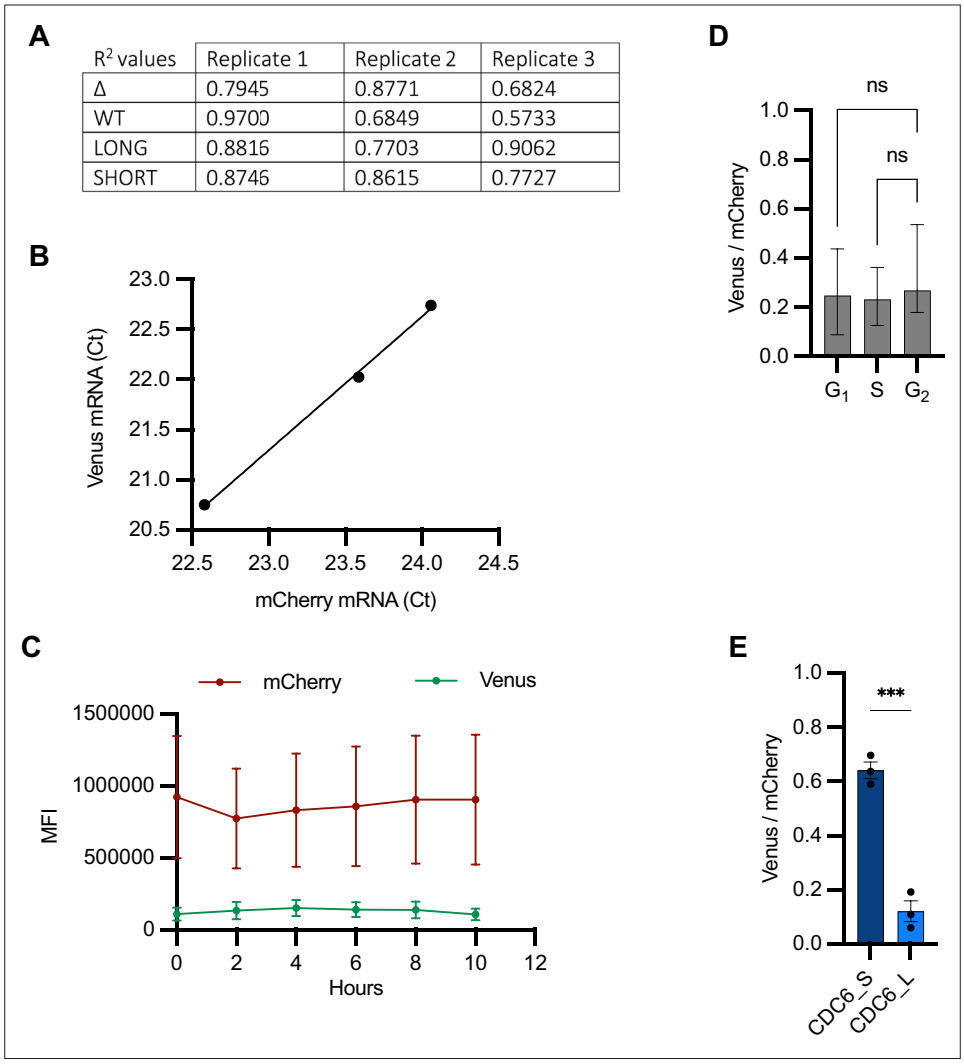


Figure 2. Aurora Kinase A (AURKA) shows 3'UTR isoform-dependent protein expression. (**A**) Top: UTR-dependent protein expression reporters. Venus coding sequence (CDS) is flanked by AURKA 5'UTR and 3'UTR, WT or polyadenylation signal (PAS)-mutated. Bottom: representative snapshots of transfected U2OS cells. Scale bar 50 µm. (**B**) Mean and SEM of median Venus/mCherry mean fluorescence intensity (MFI) ratios from transfected U2OS cells from three biological replicates. n ≥ 129 cells per condition. Ordinary one-way ANOVA with Tukeys multiple-comparisons test. (**C**) 3'RACE of endogenous AURKA APA isoforms. (**D**) RT-qPCR of endogenous AURKA short/long ratio (SLR) in U2OS cells. Long isoform abundance plotted as fold change over total AURKA mRNA. 18S rRNA used as reference target. (**E**) Same as (**B**) but in MCF10A (left) and RPE1 (right) cells. n ≥ 55 cells per condition. Unpaired *t*-test. ns, not significant; *p<0.05; **p<0.01; ***p<0.001.



R^2 values	Replicate 1	Replicate 2	Replicate 3
Δ	0.7945	0.8771	0.6824
WT	0.9700	0.6849	0.5733
LONG	0.8816	0.7703	0.9062
SHORT	0.8746	0.8615	0.7727

Figure 2—figure supplement 1. Validation of bidirectional reporter. (**A**) R^2 (coefficient of determination) values to indicate goodness of fit of a simple linear regression between mCherry and Venus single-cell mean fluorescence intensity (MFI) values. Data relative to ***Figure 2B***. (**B**) RT-qPCR of mCherry and Venus mRNAs from three RNA extracts of U2OS cells transfected with Δ reporter. Ct values are mean between three technical replicates of the amplification reaction. (**C**) Quantification of mCherry and Venus MFI from U2OS cells transfected with the Δ reporter and imaged at the indicated time points. Mean and SEM (n = 8 cells) shown at each time point. (**D**) Single-cell Venus/mCherry MFI values from U2OS^{CDK2} cells transfected with Δ reporter grouped by intervals of CDK2 activity (***Figure 4A***, right) and plotted as median and 95% CI. n = 43 cells analyzed. Kruskal–Wallis with Dunnett's multiple-comparisons test; ns, not significant. (**E**) Mean and SEM of median Venus/mCherry MFI ratios from U2OS cells transfected with constructs containing long or short CDC6 3'UTR from three biological replicates. n ≥ 119 cells per condition. Unpaired *t*-test; ***p=0.0005.

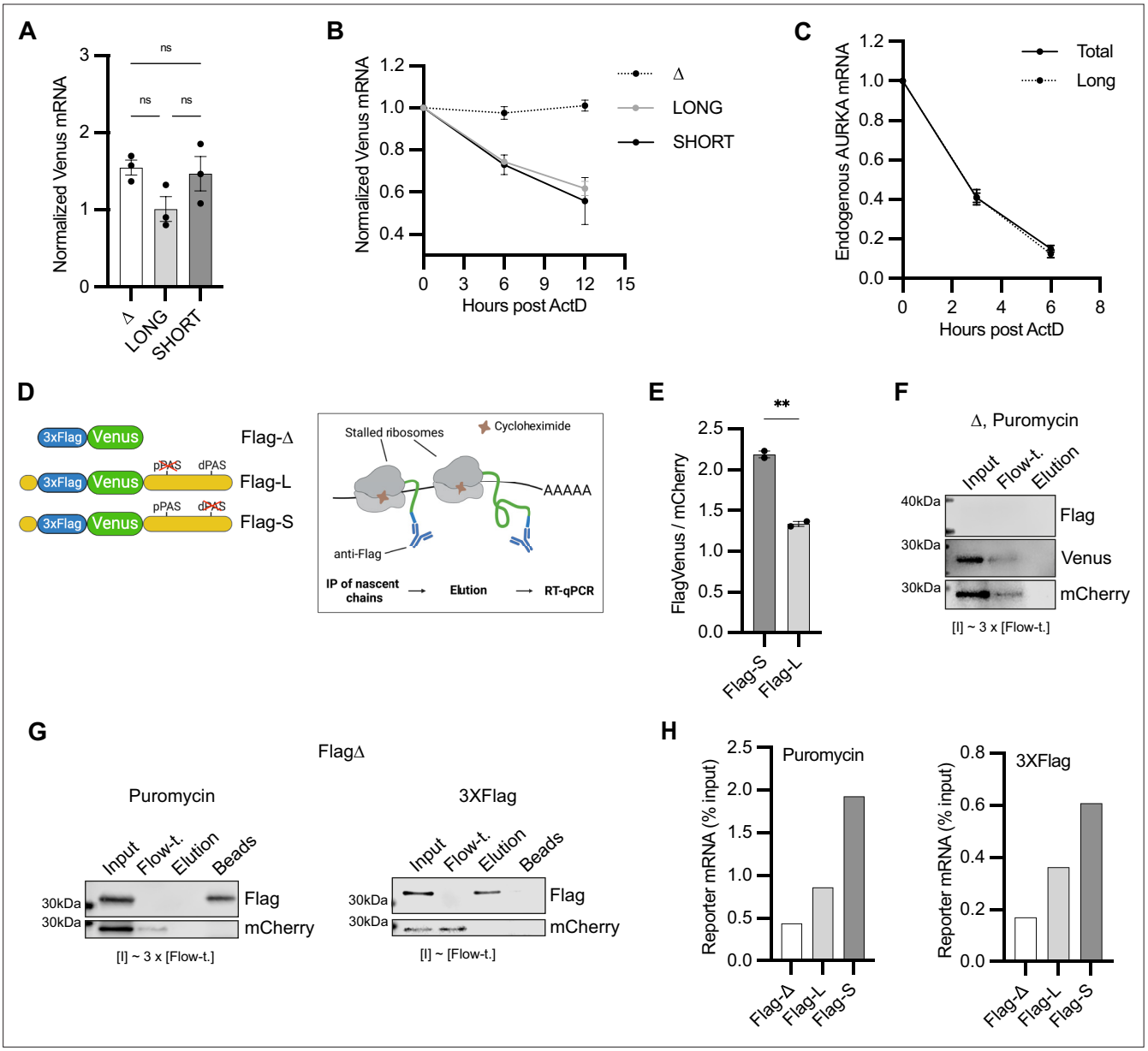


Figure 3. Aurora Kinase A (AURKA) alternative polyadenylation (APA) isoforms are translated with different efficiency. (**A**, **B**) RT-qPCR of reporter mRNAs abundance (**A**) and decay rate (**B**) from transfected U2OS cells. mCherry mRNA used as reference target. Ordinary one-way ANOVA with Tukey's multiple-comparisons test; ns, not significant. (**C**) Decay rate of endogenous AURKA mRNA as in (**B**). 18S rRNA used as reference target. Abundance of long isoform plotted as fold change over total AURKA mRNA. (**D**) Design of the nascent chain immunoprecipitation (NC IP) reporters and assay. (**E**) Mean and SEM of median FlagVenus/mCherry mean fluorescence intensity (MFI) ratios from transfected U2OS cells from two biological replicates. n ≥ 160 cells per condition. Unpaired *t*-test; **p<0.005. (**F**), (**G**) Immunoblots of NC IP fractions using Δ (**F**) or Flag-Δ (**G**) reporter. mCherry used as negative control. (**H**) RT-qPCR of eluted reporter mRNAs. Results representative of three biological replicates.

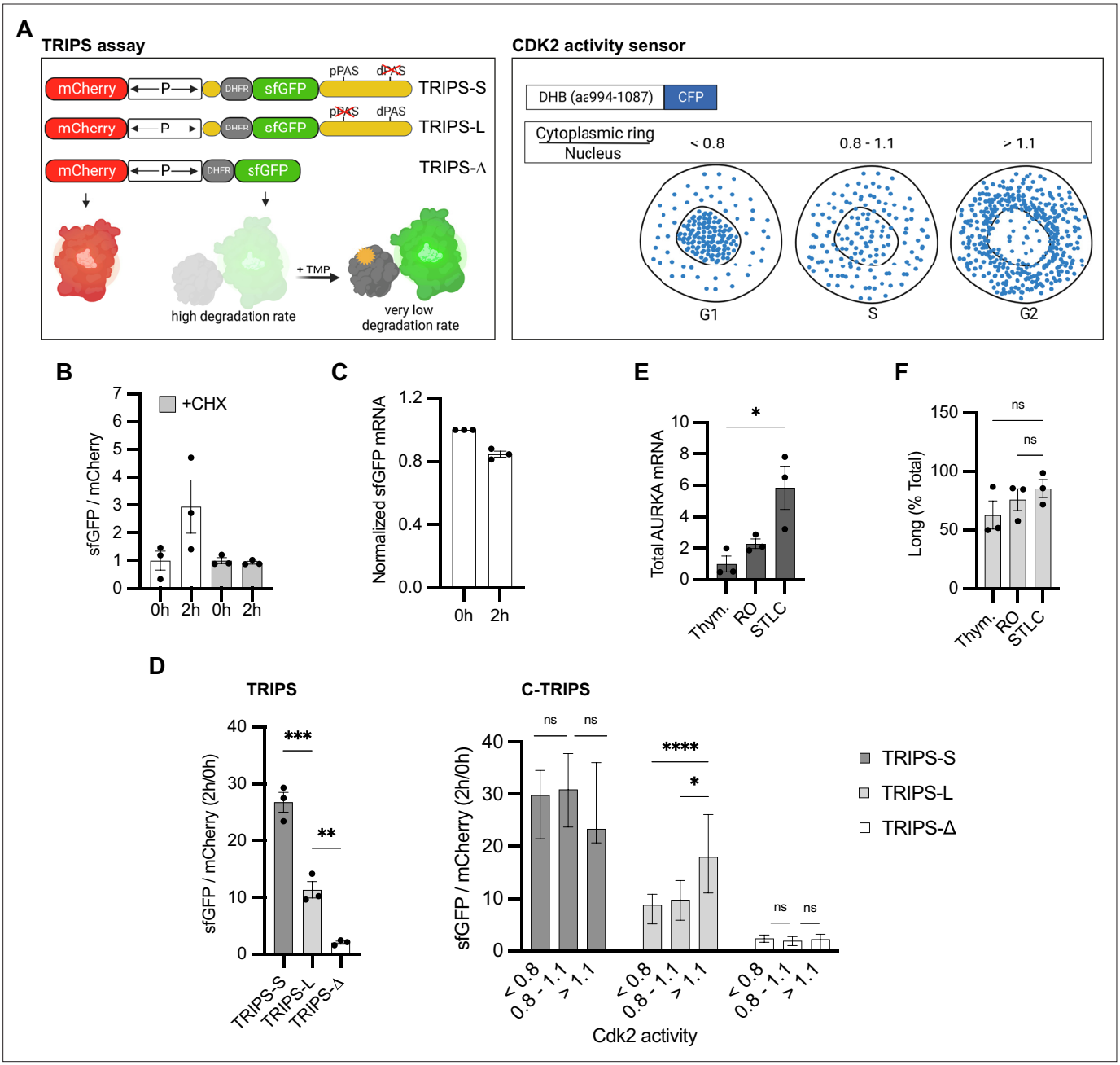


Figure 4. Translation rate of Aurora Kinase A (AURKA) alternative polyadenylation (APA) isoforms follows different cell cycle periodicity. (**A**) Design of the translation rate imaging by rate of protein stabilization (TRIPS) reporters and assay (left) and CDK2 activity sensor (right). (**B**) Mean and SEM of median sfGFP/mCherry mean fluorescence intensity (MFI) ratios from U2OS cells transfected with TRIPS-Δ and imaged at 0 hr and 2 hr of 50 μM trimethoprim (TMP) treatment, with or without 0.1 mg/ml cycloheximide (CHX), from three biological replicates. Baseline at 0 hr. n ≥ 55 cells per condition. (**C**) RT-qPCR of sfGFP mRNA from U2OS cells transfected with TRIPS-Δ, at 0 hr and 2 hr of 50 μM TMP treatment. mCherry mRNA used as reference target. RNA extracts at 0 hr of treatment used as reference sample. (**D**) TRIPS (left) and C-TRIPS (right) assays in transfected U2OS^{CDK2} cells. n ≥ 200 cells per condition. Left: mean and SEM from three biological replicates. Right: median and 95% CI of pooled data from left. Kruskal–Wallis with Dunnett's multiple-comparisons test. (**E**) RT-qPCR of endogenous AURKA mRNA in U2OS cells. 18S rRNA used as reference target. Baseline at G_1/S. (**F**) Endogenous AURKA long isoform as in (**E**) plotted as percentage of total AURKA mRNA. (**D**) Left, (**E**, **F**) Ordinary one-way ANOVA with Tukey's multiple-comparisons test. ns, not significant; *p<0.05; **p<0.01; ***p<0.0005; ****p<0.0001.

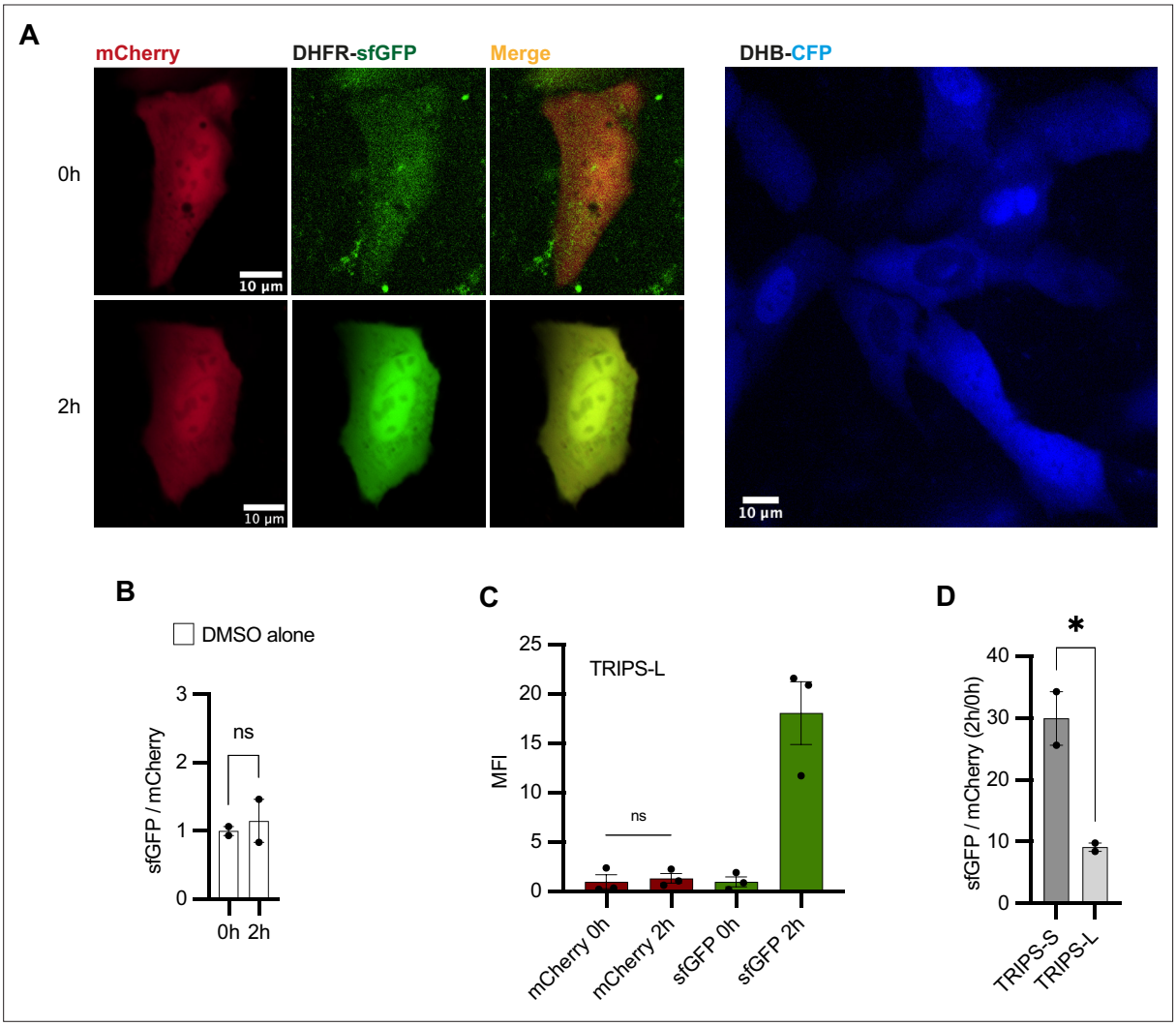


Figure 4—figure supplement 1. Validation of TRIPS assay. (**A**) Left: representative U2OS cells transfected with TRIPS-S and imaged at 0 hr and 2 hr of 50 µM trimethoprim (TMP) treatment. Right: representative U2OS^{CDK2} cells. (**B**) TRIPS assay performed in U2OS cells transfected with TRIPS-Δ and treated with DMSO for 2 hr. Mean and SEM of median sfGFP/mCherry ratios from two biological replicates, with baseline at 0 hr. n = 95 cells analyzed. (**C**) Graph using data from ***Figure 4D*** showing mean fluorescence intensity (MFI) of mCherry and sfGFP at 0 hr and 2 hr of TMP treatment. Mean and SEM of median MFI values from three biological replicates. (**D**) TRIPS assay performed in transfected U2OS cells and imaged at 0 hr and 2 hr of 50 µM TMP treatment. Ratios between the median of single-cell sfGFP/mCherry ratios at 2 hr and that at 0 hr of TMP treatment were calculated for two biological replicates and are shown as mean and s.e.mSEM. n ≥ 343 cells analyzed per condition. (**B–D**) Unpaired *t*-test. ns, not significant; *p<0.05.

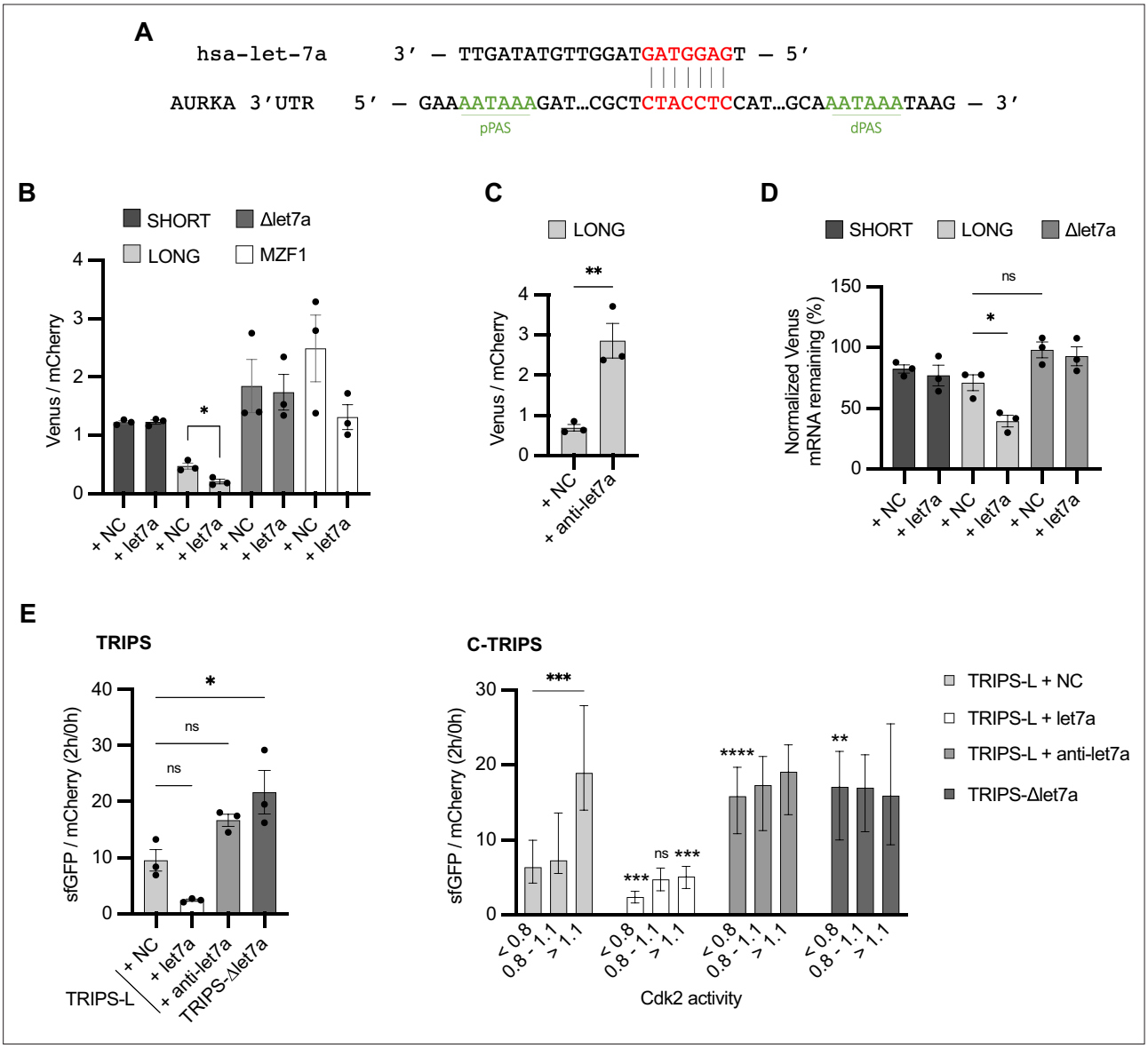


Figure 5. Translational periodicity of long 3′UTR isoform is regulated by *hsa-let-7a* miRNA. (**A**) Complementarity of *hsa-let-7a* binding to Aurora Kinase A (AURKA) 3′UTR. (**B**) Mean and SEM of median Venus/mCherry mean fluorescence intensity (MFI) ratios from U2OS cells co-transfected with 250 nM *hsa-let-7a* or a negative control (NC) miRNA from three biological replicates. n ≥ 182 cells per condition. Unpaired *t*-test. (**C**) Same as (**B**) but co-transfecting 300 nM *anti-let-7a* or NC. n ≥ 94 cells per condition. Unpaired *t*-test. (**D**) RT-qPCR of reporter mRNAs abundance from U2OS cells transfected as (**B**), at 8 hr of 10 µg/ml ActD. mCherry mRNA used as reference target. Ordinary one-way ANOVA with Tukey's multiple-comparisons test. (**E**) Translation rate imaging by rate of protein stabilization (TRIPS) (left) and C-TRIPS (right) assays in transfected U2OS^{CDK2} cells. n ≥ 162 cells per condition Left: mean and SEM from three biological replicates. Ordinary one-way ANOVA with Dunnett's multiple-comparisons test vs. NC. Right: median and 95% CI of pooled data from left. Kruskal–Wallis with Dunnett's multiple-comparisons test vs. NC of the respective phase. ns, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

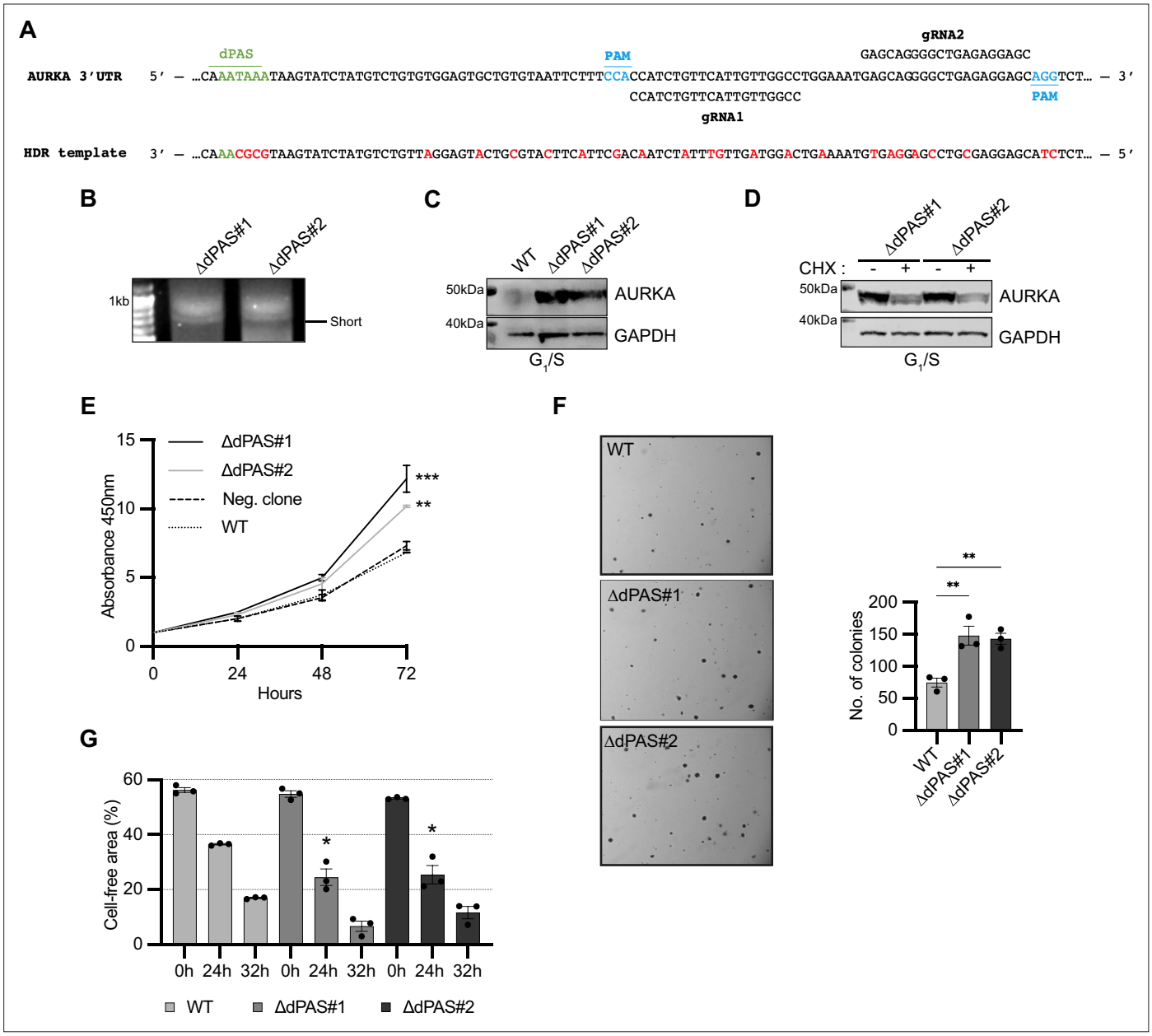


Figure 6. Increased Aurora Kinase A (AURKA) short/long ratio is sufficient to disrupt cell behavior. (**A**) Design of CRISPR editing. Nucleotide substitutions in red. (**B**) 3'RACE of endogenous AURKA alternative polyadenylation (APA) isoforms. (**C**), (**D**) Western blot after G_1/S enrichment (**C**) and with or without 6 hr treatment with 0.1 mg/ml cycloheximide (CHX) (**D**). Blots representative of three biological replicates. (**E**) CCK8 assay. A nonparental WT U2OS cell line used as negative control. Ordinary one-way ANOVA with Dunnett's multiple-comparisons test vs. WT; **p<0.005; ***p<0.001. (**F**) Left: representative images of cells grown in soft agar. Right: mean number of clones and SEM of three biological replicates. (**G**) Measurement of migration rate. (**F**, **G**) Ordinary one-way ANOVA with Dunnett's multiple-comparisons test vs. WT; *p<0.05; **p<0.01.

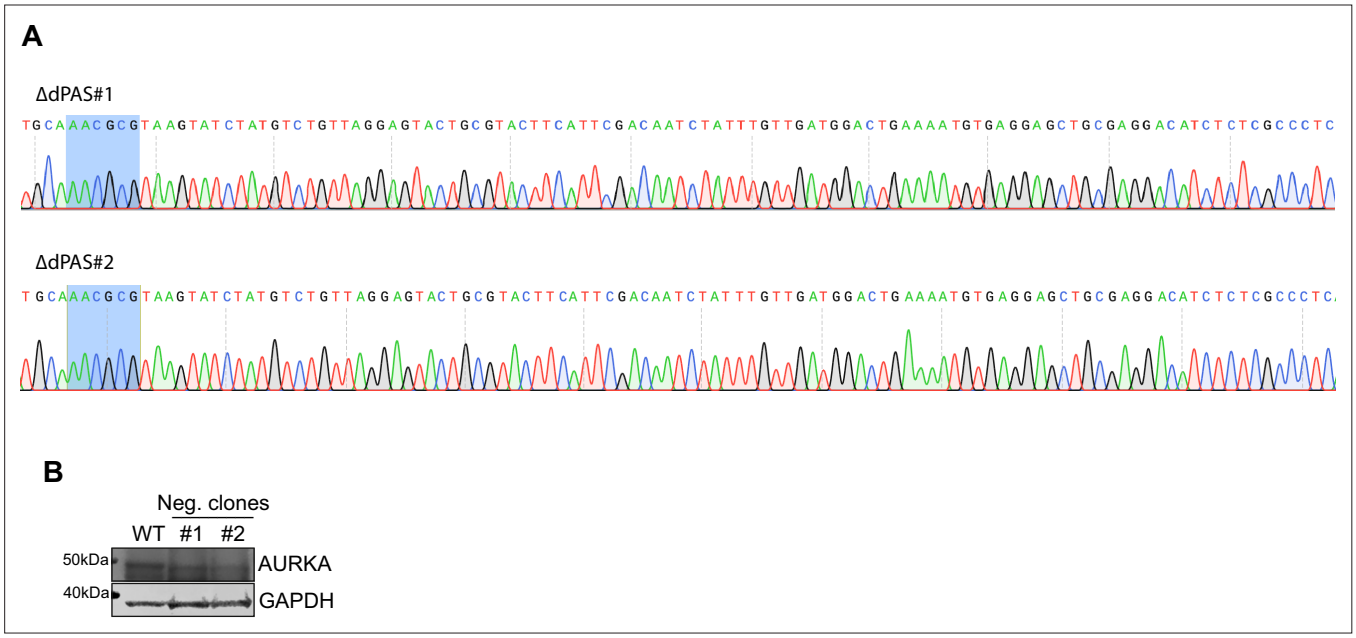
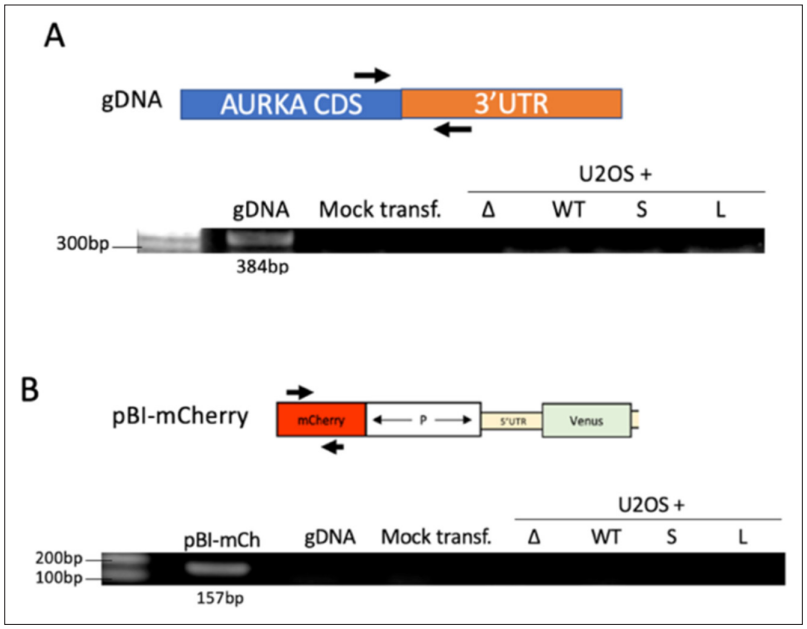
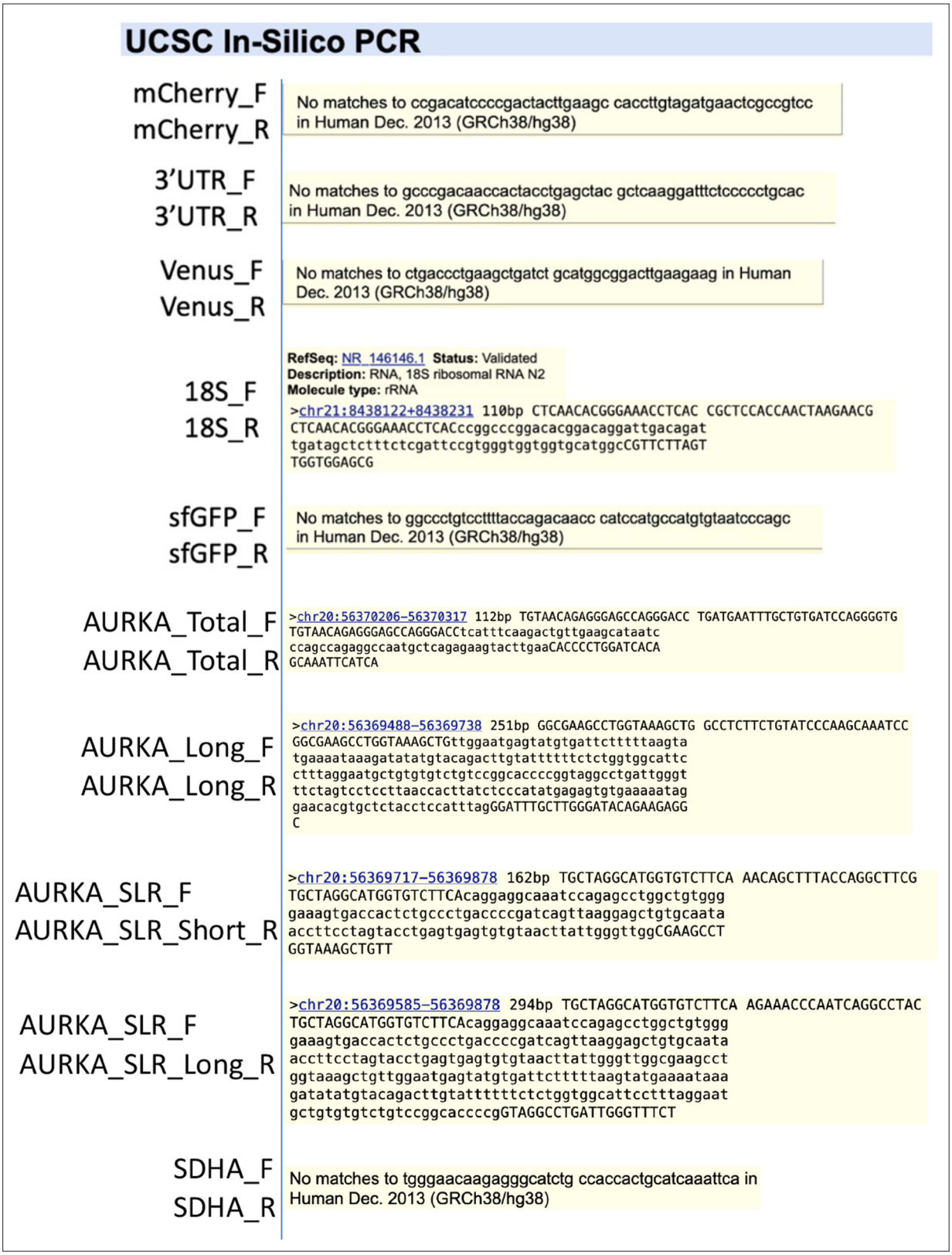


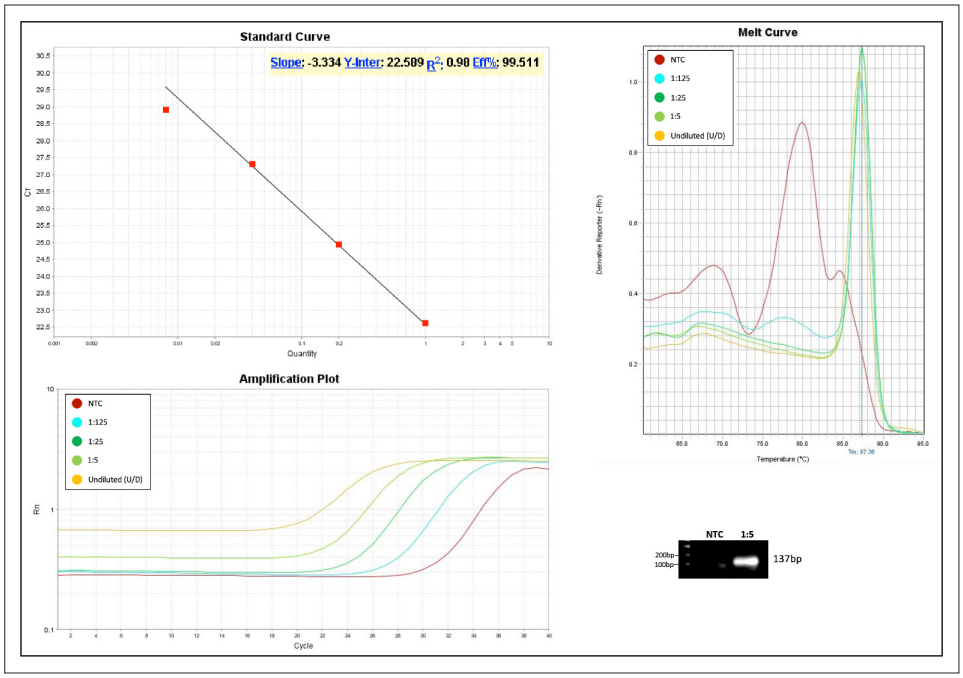
Figure 6—figure supplement 1. Validation of mutated cell lines. (**A**) Sanger sequencing validation of the mutated dPAS element in two cell lines. (**B**) Western blot showing similar Aurora Kinase A (AURKA) protein expression in unsynchronized WT or U2OS cell lines that were not mutated following CRISPR editing (#1, #2).



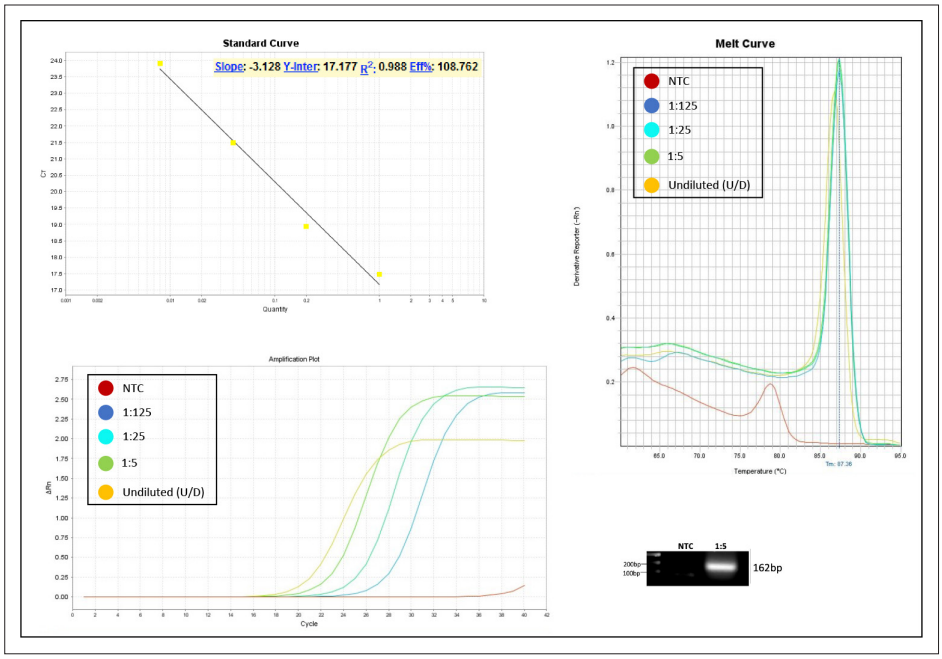
Appendix 1—figure 1. Assessment of DNA contamination of RNA extracts. (**A**) Lack of genomic DNA (gDNA) contamination in RNA samples was assessed by PCR using Aurora Kinase A (AURKA)-specific primers. RNA extracts from U2OS cells electroporated with the indicated reporters (***Figure 2A***) were used as template for the amplification reaction. An RNA extract from mock transfected U2OS cells used as negative control. gDNA from untransfected U2OS cells used as positive control. (**B**) Lack of plasmid DNA contamination in RNA samples was assessed by PCR using mCherry specific primers. RNA extracts from U2OS cells electroporated with the indicated reporters (***Figure 2A***) were used as template for the amplification reaction. An RNA extract from mock transfected U2OS cells and gDNA from untransfected U2OS cells used as negative controls. pBI-mCherry plasmid (pBI-mCh) used as positive control template.



Appendix 1—figure 2. Predicted targets on human genome of primer pairs used in this study. Analysis performed using UCSC In-Silico PCR tool (https://genome.ucsc.edu/cgi-bin/hgPcr). Genomic PCR product of SDHA primer pairs is above >850 bp length set for the in silico search.



Appendix 1—figure 3. Validation of primer pairs used in RT-qPCR. Standard curve, melt curve, and amplification plot of amplification reactions of serial dilutions (1:5) are shown. Sample reactions were loaded on gel to validate amplicon size. NTC, non-template control.



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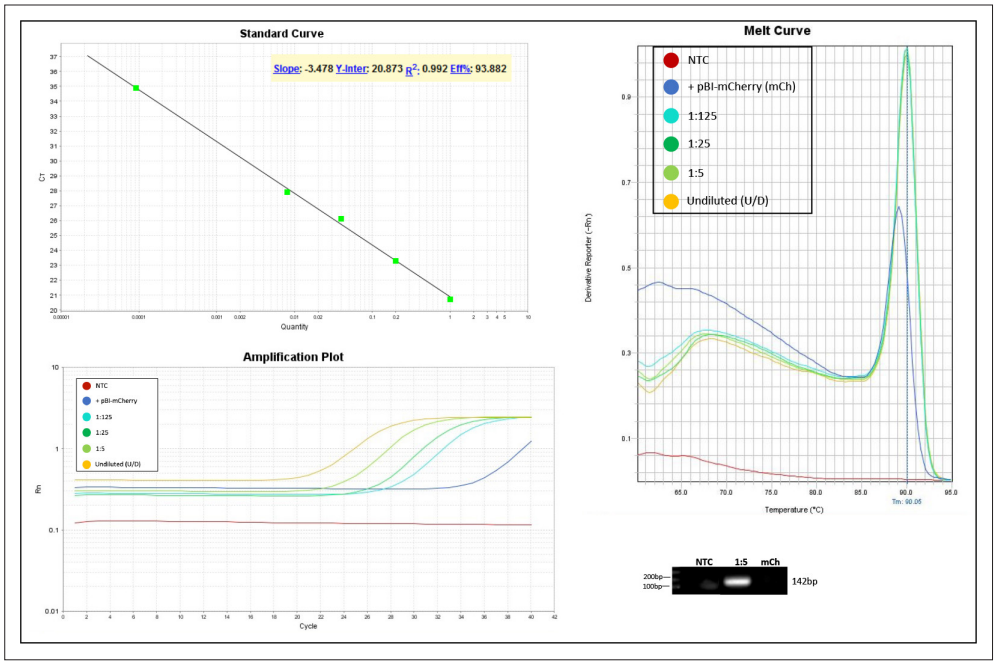
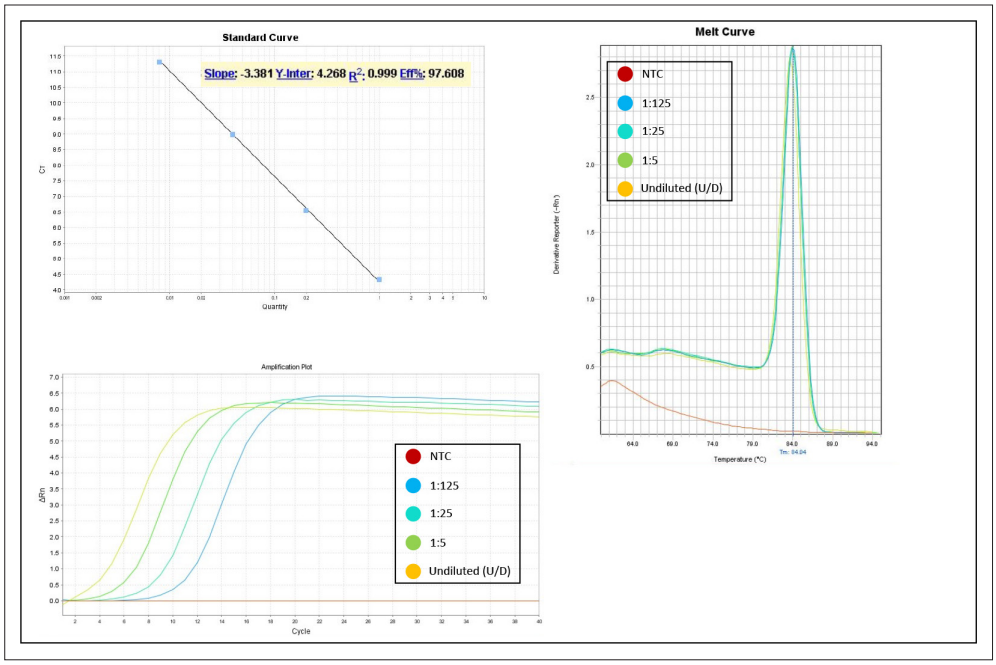
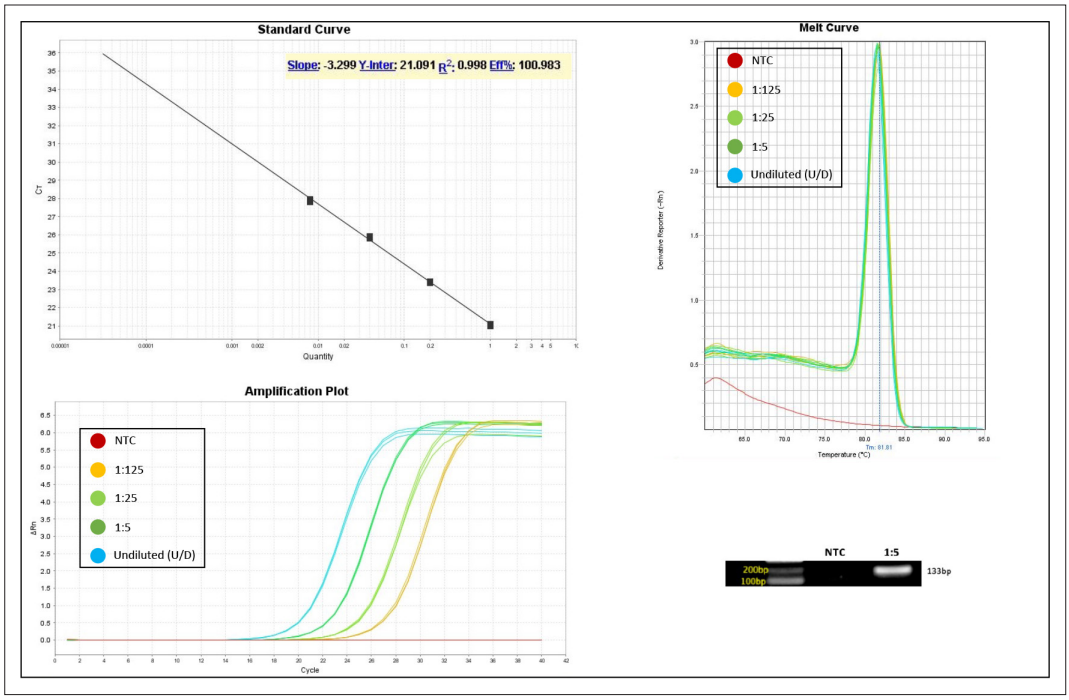


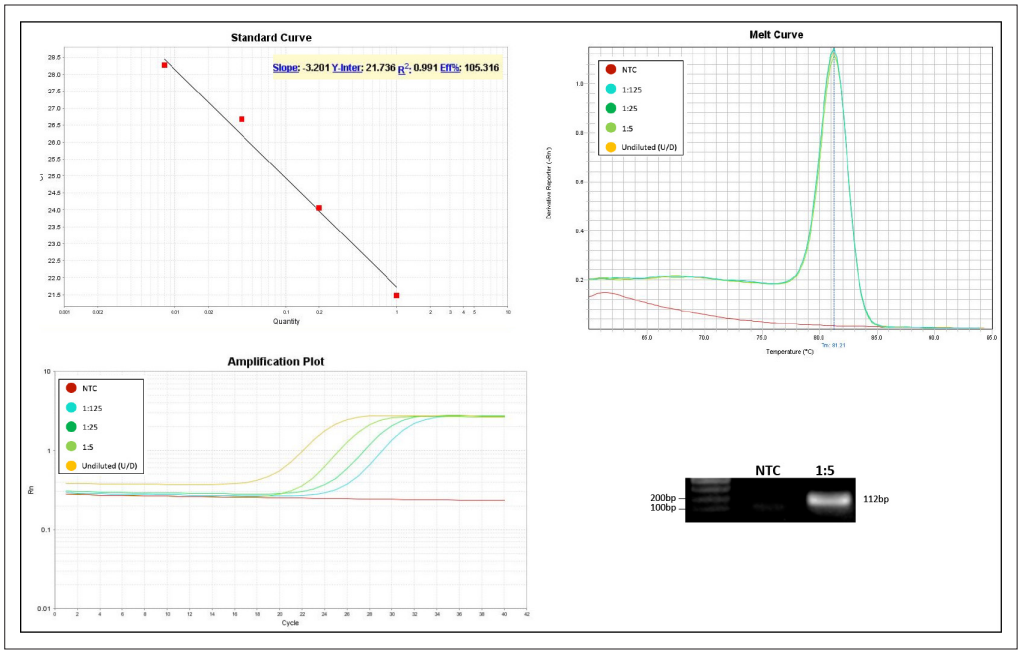
Appendix 1—figure 5. Validation of primer pairs used in RT-qPCR. Standard curve, melt curve, and amplification plot of amplification reactions of serial dilutions (1:5) are shown. Sample reactions were loaded on gel to validate amplicon size. The target was also amplified from RNA extracts of cells transfected with the pBI-mCherry construct as neg. control (mCh). NTC, non-template control.



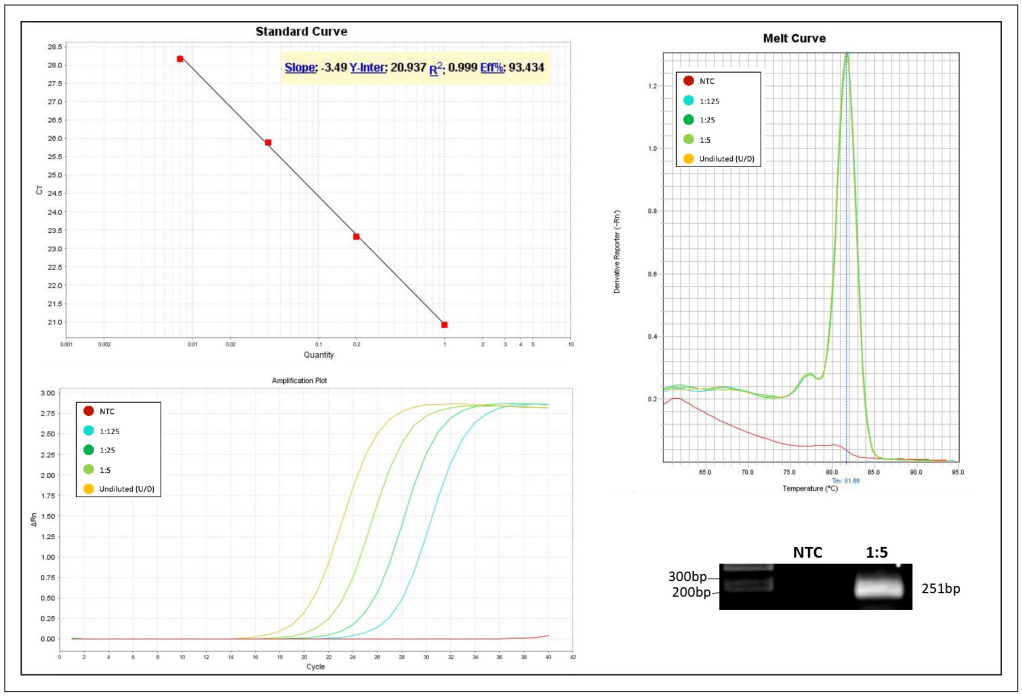
Appendix 1—figure 6. Validation of primer pairs used in RT-qPCR. Standard curve, melt curve, and amplification plot of amplification reactions of serial dilutions (1:5) are shown. NTC, non-template control.



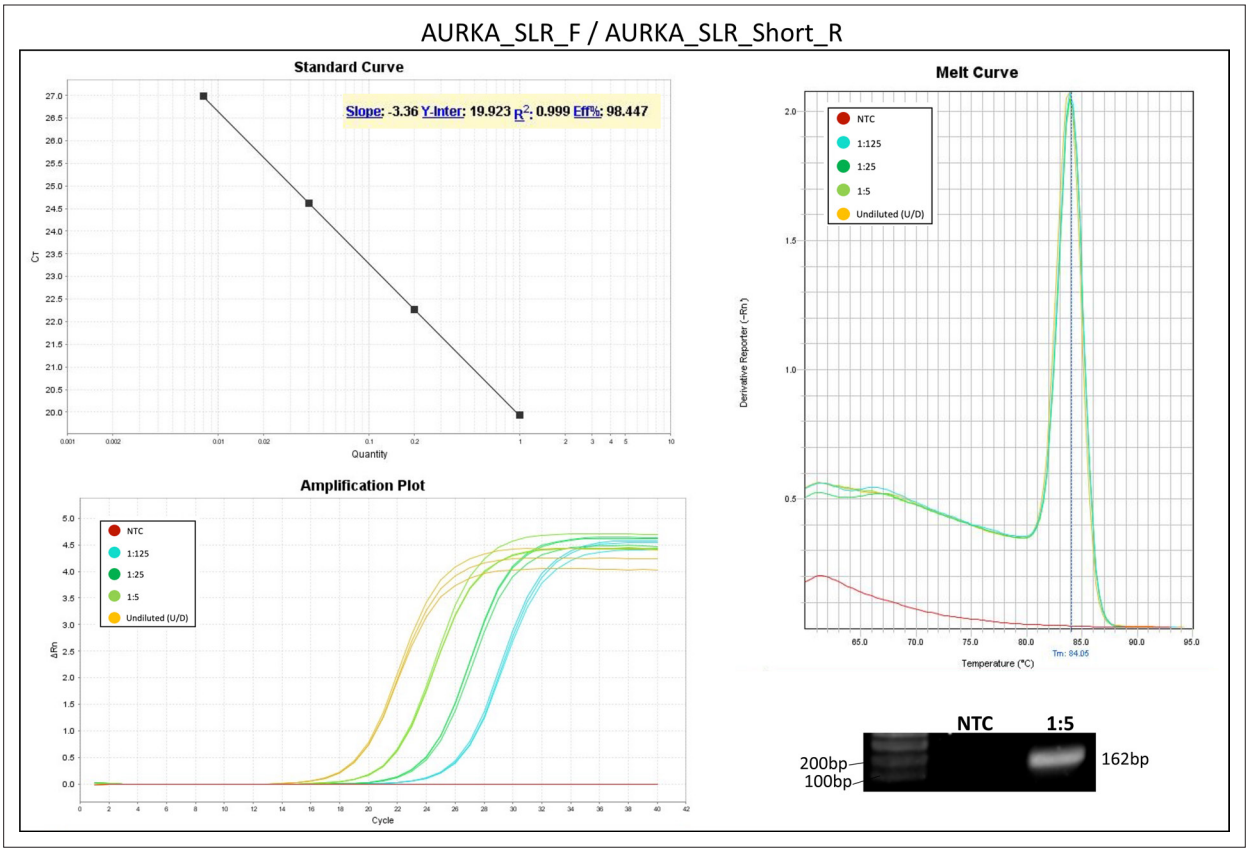
Appendix 1—figure 7. Validation of primer pairs used in RT-qPCR. Standard curve, melt curve, and amplification plot of amplification reactions of serial dilutions (1:5) are shown. Sample reactions were loaded on gel to validate amplicon size. NTC, non-template control.



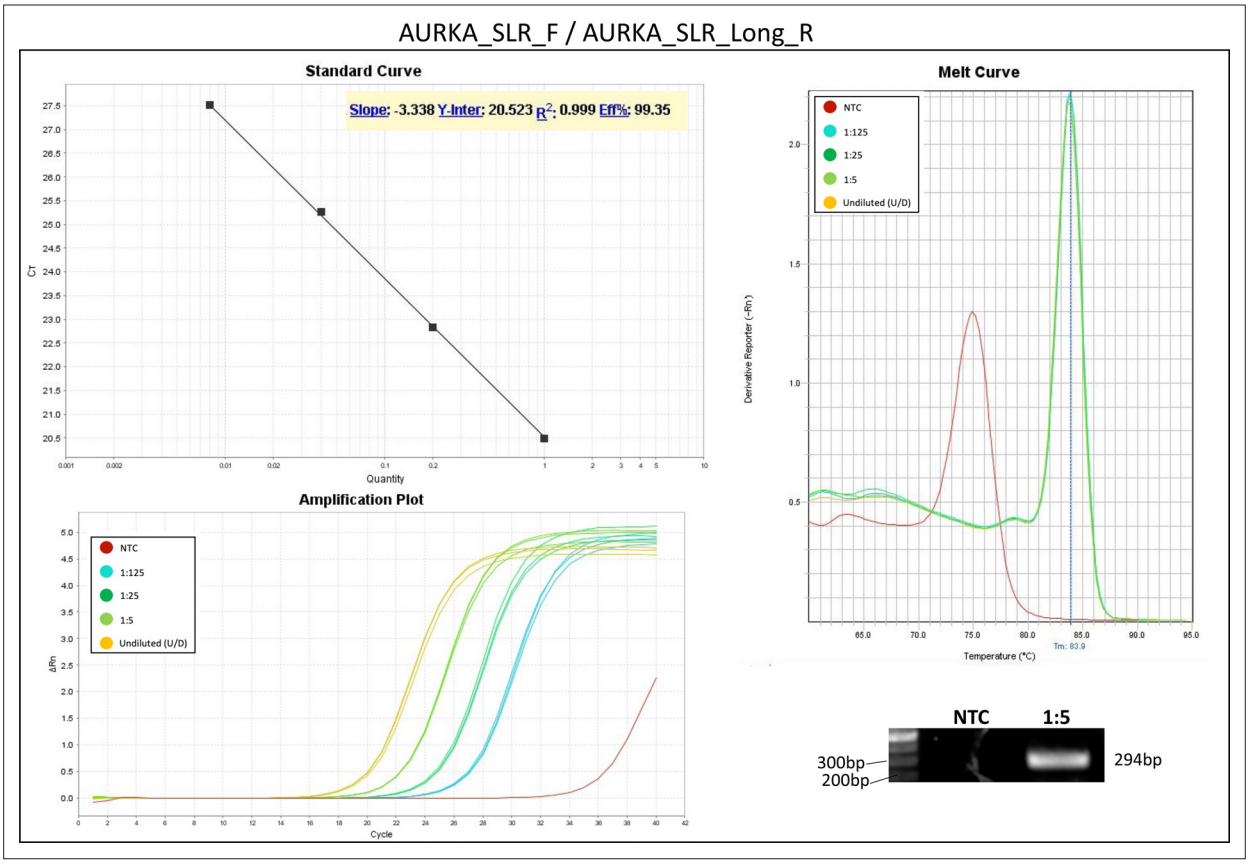
Appendix 1—figure 8. Validation of primer pairs used in RT-qPCR. Standard curve, melt curve, and amplification plot of amplification reactions of serial dilutions (1:5) are shown. Sample reactions were loaded on gel to validate amplicon size. NTC, non-template control.



Appendix 1—figure 9. Validation of primer pairs used in RT-qPCR. Standard curve, melt curve, and amplification plot of amplification reactions of serial dilutions (1:5) are shown. Sample reactions were loaded on gel to validate amplicon size. NTC, non-template control.



Appendix 1—figure 10. Validation of primer pairs used in RT-qPCR. Standard curve, melt curve, and amplification plot of amplification reactions of serial dilutions (1:5) are shown. Sample reactions were loaded on gel to validate amplicon size. NTC, non-template control.



Appendix 1—figure 11. Validation of primer pairs used in RT-qPCR. Standard curve, melt curve, and amplification plot of amplification reactions of serial dilutions (1:5) are shown. Sample reactions were loaded on gel to validate amplicon size. NTC, non-template control.