Supplementary Methods

Synchronization of mouse N2a cells

N2a cells were synchronized through mitotic shake-off. Mitotic cells were harvested by gently tapping on the Petri dishes containing logarithmically growing cells and subsequently released into fresh media.

Protein structure prediction

The predicted structures of SAF-A isoforms were obtained from the AlphaFold Protein Structure Database (https://alphafold.com). And the protein structure images were made using PyMOL (http://www.pymol.org/pymol).

Intrinsically disordered region prediction

Protein disorder estimation was performed using PONDR server (https://www.pondr.org), using both PONDR-VSL2 and PONDR-VL3 prediction algorithms.

Tissue expression analyses

To analyze the expression levels of SAF-A across various tissues, transcripts per million (TPM) values were obtained from the Genotype-Tissue Expression (GTEx) database (http://gtexportal.org).

Table S1 Primers and oligos used in this study

Name	Sequence (5' to 3')	Description
ASAT siRNA_1	UUUUGAAACACUCUUUUUGUTT	siRNA for α-satellite RNA knockdown
ASAT siRNA_2	AGAAUCUGCAAGUGGAUAUUTT	siRNA for α-satellite RNA knockdown
Hs-a-sate_qF	CATTCTCAGAAACTTCTTTG	qPCR primer
Hs-a-sate_qR	AGCGCTCCAAATATCCACT	
GAPDH-qF	CTTCATTGACCTCAACTACATGG	qPCR primer
GAPDH-qR	CTCGCTCCTGGAAGATGGTGAT	
Hs-U6-qF	CGCTTCGGCAGCACATATAC	qPCR primer
Hs-U6-qR	CTCGCTCCTGGAAGATGGTGAT	
Hs-U5-qF	TGGTTTCTCTTCAGATCGCATA	qPCR primer
Hs-U5-qF	CAAAGCAAGGCCTCAAAAA	
ACTB-qF	CCAACACAGTGCTGTCTGG	qPCR primer
ACTB-qR	GAGTACTTGCGCTCAGGAG	
B1-ASAT-S1	gAggAgggCAgCAAACgggAAgAgTCTTC CTTTACgATATTCATTCTCAGAAACTT CTTTGTGATGTTTGCATTCAACTCACA GAGTTGAAATATAgCATTCTTTCTTgAg gAgggCAgCAAACgggAAgAg	smFISH probe
B1-ASAT-S2	gAggAgggCAgCAAACgggAAgAgTCTTC CTTTACgATATTAACATTCCTTTTCATA GAGCAGTTTTGAAACACTCTTTTTGT AGAATCTGATATAgCATTCTTTCTTgAg gAgggCAgCAAACgggAAgAg	smFISH probe
B1-ASAT-S3	gAggAgggCAgCAAACgggAAgAgTCTTC CTTTACgATATTAGTGGATATTTGGAGC GCTTTGAGGCCTTCGTTTGAAACGGG AATATCTTATATAgCATTCTTTCTTgAgg AgggCAgCAAACgggAAgAg	smFISH probe
HS-a-satellite-PD-S	TTCAACTCACAGAGTTGAACCTTCCC TTTG	5'-biotin-labeled α-satellite RNA pull-down probe
HS-a-satellite-PD-AS	GCGCTCCAAATATCCACTTGCAGATTC TAC	5'-biotin-labeled α-satellite RNA pull-down probe
ASAT-CHIPR-1	ATTCCCGTTTCAAACGAAGG	ChIPR-seq probe pool odd
ASAT-CHIPR-3	GCAGATTCTACAAAAAGAGTG	
ASAT-CHIPR-5	GTTCAACTCTGTGAGTTGAAT	
ASAT-CHIPR-2	AAGCGCTCCAAATATCCACT	ChIPR-seq probe pool even
ASAT-CHIPR-4	CAAAACTGCTCTATGAAAAGG	
ASAT-CHIPR-6	CAAAGAAGTTTCTGAGAATGC	
MS-major-sat-PD-S	ACACACTTTAGGACGTGAAATATGGC GAGG	5'-biotin-labeled major satellite RNA pull-down

		probe
MS-major-sat-PD-AS	CCATATTCCACGTCCTACAGTGGACATTTC	5’-biotin-labeled major satellite RNA pull-down probe
MS-minor-sat-PD-S	CCACACTGTAGAACATATTAGATGAGTGAG	5’-biotin-labeled minor satellite RNA pull-down probe
MS-minor-sat-PD-AS	CTGTTCTACAATGCCGGTTTCCAACGAATG	5’-biotin-labeled minor satellite RNA pull-down probe
PD-scramble	TTCTCCGAACGTGTCACGTTCGAACGTGTC	5’-biotin-labeled RNA pull-down control probe
Ms-U5-qF	CACCGCAACAGGAATCATCCTTCAG	qPCR primer
Ms-U5-qR	ACTCTGGTTTCTCTTCAGATCGT	
Ms-U6-qF	GTGCTCGCTTCGGCAGC	qPCR primer
Ms-U6-qR	AAAAATATGGAACGCTTCACGAAT	
Ms-minor-qF	CATGGAAAATGATAAAAACC	qPCR primer
Ms-minor-qR	CATCTAATATGTTCTACAGTGTGG	
Ms-major-qF	ACACACTTTAGGACGTGAAA	qPCR primer
Ms-major-qR	CCATATTCCACGTCCTACAG	
18S-qF	CGGCGACGACCCATTCGAAC	qPCR primer
18S-qR	GAATCGAACCCTGATTCCCCGTC	
T7-sense probe-F	TAATACGACTCACTATAGGGAGCATTCTCAGAAACTTCTT	PCR primer for Northern blot
T7-sense probe-R	TTATGTGAAGATATTTCCTT	
T7-antisense probe-F	TAATACGACTCACTATAGGGTTATGTGAAGATATTTCCTT	PCR primer for Northern blot
T7-antisense probe-R	AGCATTCTCAGAAACTTCTT	

Table S2 Plasmids used in this study

Name	Description	Source
shSAFA-1	shRNA vector for SAF-A knockdown	MISSION shRNA library ID: TRCN0000001298
shSAFA-2	shRNA vector for SAF-A knockdown	MISSION shRNA library ID: TRCN0000001299
ShCtrl	shRNA control vector	MISSION shRNA control vector shC002
pET28-SAFA-iso-a	*E. coli* expression of SAF-A	This study
pET28-SAFA-iso-a	*E. coli* expression of SAF-A	This study
pET28-hnrnpA1	*E. coli* expression of hnrnpA1	This study
p3XFLAG-Myc-CMV-SAFA-FL	Expression of full-length SAF-A	This study
p3XFLAG-Myc-CMV-SAFA-dN1	Expression of domain truncated SAF-A	This study
p3XFLAG-Myc-CMV-SAFA-dN2	Expression of domain truncated SAF-A	This study
p3XFLAG-Myc-CMV-SAFA-dC1	Expression of domain truncated SAF-A	This study
p3XFLAG-Myc-CMV-SAFA-dC2	Expression of domain truncated SAF-A	This study
p3XFLAG-Myc-CMV-SAFA-iso-b	Expression of SAF-A isoform b	This study
pGEM-T-ASAT-S	In vitro transcription of α-satellite RNA	This study
pGEM-T-ASAT-AS	In vitro transcription of α-satellite RNA	This study

Table S3 Antibodies used in this study

Antibody	Catalog No.	Supplier	Dilution
Anti-SAF-A	Ab180952	Abcam	1:100 for IF, 1:50 for IP
Anti-SAF-A	Ab20666	Abcam	1:1000 for WB
Anti-Tubulin	HC101	Transgene	1:1000 for WB
Anti-β-Tubulin	Ab6046	Abcam	1:200 for IF
Anti-β-Actin	HC201	Transgene	1:1000
Anti-GAPDH	60004-1-Ig	Proteintech	1:5000 for WB
Anti-Rabbit IgG	L3031	Signalway	1:5000
Anti-Mouse IgG	L3032	Signalway	1:5000
Anti-CREST	HCT-0100	Bioss	1:100
Anti-Rabbit IgG (Alexa Fluor 488)	Ab150077	Abcam	1:100
Anti-Human IgG (Alexa Fluor 647)	A-21445	Invitrogen	1:100
Anti-Histone H3S10p	Ab5176	Abcam	1:2000 for WB; 1:100 for IF
Anti-ArkA T288p/ArkB T232p/ArkC T198p	2914	CST	1:500
Anti-dsRNA clone rJ2	MABE1134	Merck	1:200 for RIP
Anti-Lamin	Ab108922	Abcam	1:100 for IP, 1:2000 for WB, 1:100 for IF
Anti-LAP2	PA5-52519	Invitrogen	1:2000 for WB, 1:100 for IF

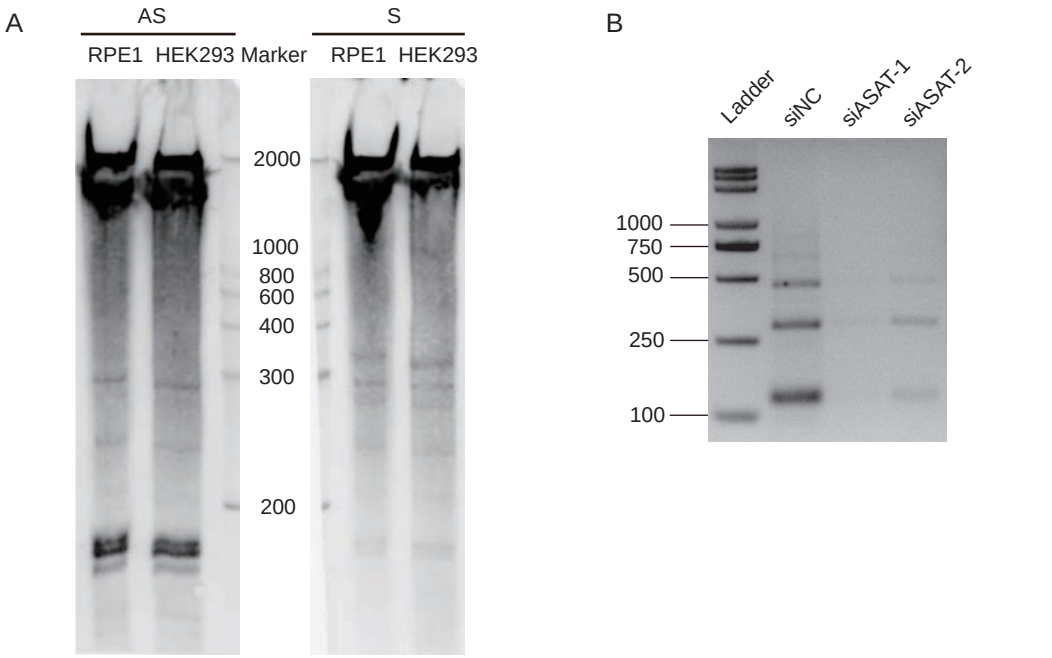
Table S4 Putative α-satellite RNA binding protein identified by mass spectrometry

Name	Protein Mass	Uniprot No.	No. of peptide hit	Probability
HNRNPK	51229.50	P61978	5	99.0%
SAF-A	91269.27	Q00839	3	99.0%
TPM3	32986.81	P06753	3	99.0%
PPB1	74018.88	P05187	3	99.0%
PCBP2	34120.54	F8VZX2	3	99.0%
TPM2	32944.63	P07951	2	99.0%
PYC	130292.91	P11498	2	97.9%
PCBP1	3798712	Q15365	2	93.5%
DHX36	115600.27	Q9H2U1	2	92.3%

Table S5 Motif analysis for αSAT ChIRP-seq and SAF-A CUT&Tag

Async				Sync(M)			
with SAF-A		without SAF-A		with SAF-A		without SAF-A	
Motif	E-value	Motif	E-value	Motif	E-value	Motif	E-value
ZN768	0.018084	CENPB	0.000422	NR1H2	0.002189	CENPB	0.000341
		ZN232	0.003001	RXRG	0.037309	PRDM4	0.000944
		OZF	0.003820	NR4A3	0.049904	ZN768	0.002114
		ZN768	0.004905			ZN232	0.002671
		ZN502	0.007144			GLI1	0.004416
		FOXH1	0.007536			ZN502	0.004487
		NR1H2	0.008092			SNAI1	0.005378
		PRDM4	0.008710			ZN250	0.008848
		NF2L2	0.011175			ZN528	0.009663
		SRF	0.011633			FOXO6	0.019468
		HSF1	0.012195			RXRG	0.020965
		CPEB1	0.013037			THA11	0.021602
		NFAC1	0.018490			NR4A3	0.024058
		STAT6	0.018917			ZN121	0.025085
		NFAC2	0.020727			ZN410	0.026126
		NFAC3	0.022426			IRF9	0.029966
		ZN568	0.022577			ZFP28	0.030209
		ZNF8	0.026733			ZN143	0.031224
		SNAI1	0.027841			REST	0.033024
		ZN436	0.029106			BCL6B	0.036241
		FOXJ3	0.035444			ZSCA4	0.039639
		FOXF2	0.036943			CR3L1	0.042849
		ZN410	0.037776				
		HXB13	0.041031				
		RXRG	0.044517				
		SOX15	0.047817				

Figure S1



C

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ASAT-C1          ---------GTGAAGATATTTCCTTTTCCACCACAGGCCCCAAACTGATCCAAATATCCA  51
ASAT-C2          ---------GTGAAGATATTTCCTTTTCCACCACAGGCCCCAAACTGATCCAAATATCCA  51
ASAT-C3          ---------GTGAAGATATTTCCTTTTCCACCACAGGCCTCAAAGCGCTCCAAATGTCCA  51
ASAT-C4          ------CTCAGAAACTTCTTTGTGATGTGTGTACTCAATTAACAGAGTTGAACTTTTCTT  54
ASAT-C5          ------CTCAGAAACTTCTTTGTGATGTGTGCATTCAGCTCACAGAGTTGAACCTTTCTT  54
ASAT-consensus   AGCATTCTCAGAAACTTCTTTGTGATGTGTGCATTCAACTCACAGAGTTGAACCTTCCCT  60
                             **  * ***    *      *        * *  * *  *  *  *

ASAT-C1          CATG--CAGATCCTTCAAAAGA-AGTGTTTCAAAACTGTTCGATCAAAAGAAAGGTTCAA  108
ASAT-C2          CATG--CAGATCCTTCAAAAGA-AGTGTTTCAAAACTGTTCGATCAAAAGAAAGGTTCAA  108
ASAT-C3          CTTG--CAGATTCTATGAAAAG-AGAGTTTCAAAACCGCTCAATCAAAAGAAAGGTTTAA  108
ASAT-C4          TTGATAGAGCTGTTTTGAAACACACTTTTTGTAAAATCTGCAAGTG---CATATTTGGAT  111
ASAT-C5          CTGGTAGACCAGTTTTAAAACACCCTTTTTGTAGAATCTGCAAGTA---GATATTTGGAA  111
ASAT-consensus   TTGATAGAGCAGTTTTGAAACACTCTTTTTGTAGAATCTGCAAGTG---GATATTTGGAG  117
                       *     *   ***       ***  * *     * *       * *  *  *

ASAT-C1          TTCTGTGAGATGAAT---------GC---ACACATCACAAAGAAGTTTCTGAG-      149
ASAT-C2          TTCTGTGAGATGAAT---------GC---ACACATCACAAAGAAGTTTCTGAG-      149
ASAT-C3          CTCTGTGAGATGAAT---------GC---ACACATCACAAAGAAGTTTCTGAG-      149
ASAT-C4          ATCTTTGAGGATTTCATTGGAAAAGGAAATATCTTCAC----------------      149
ASAT-C5          AGCTTTGAAGCCTATGGTGGAAAAGGAAATATCTTCAC----------------      149
ASAT-consensus   CGCTTTGAGGCCTTCGTTGGAAAAGGAAATATCTTCACATAAAAACTAGACAGA      171
                   ** ***              *       * ****
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Figure S1 Detection of α-satellite RNA. (A) Northern blot of α-satellite RNA in RPE1 and HEK293 cells. AS, antisense transcripts; S, sense transcripts. (B) PCR products of α-satellite RNA primers were tested in cells transfected with siASATs. (C) Alignment of α-satellite amplicons with the consensus α-satellite sequence.

Figure S2

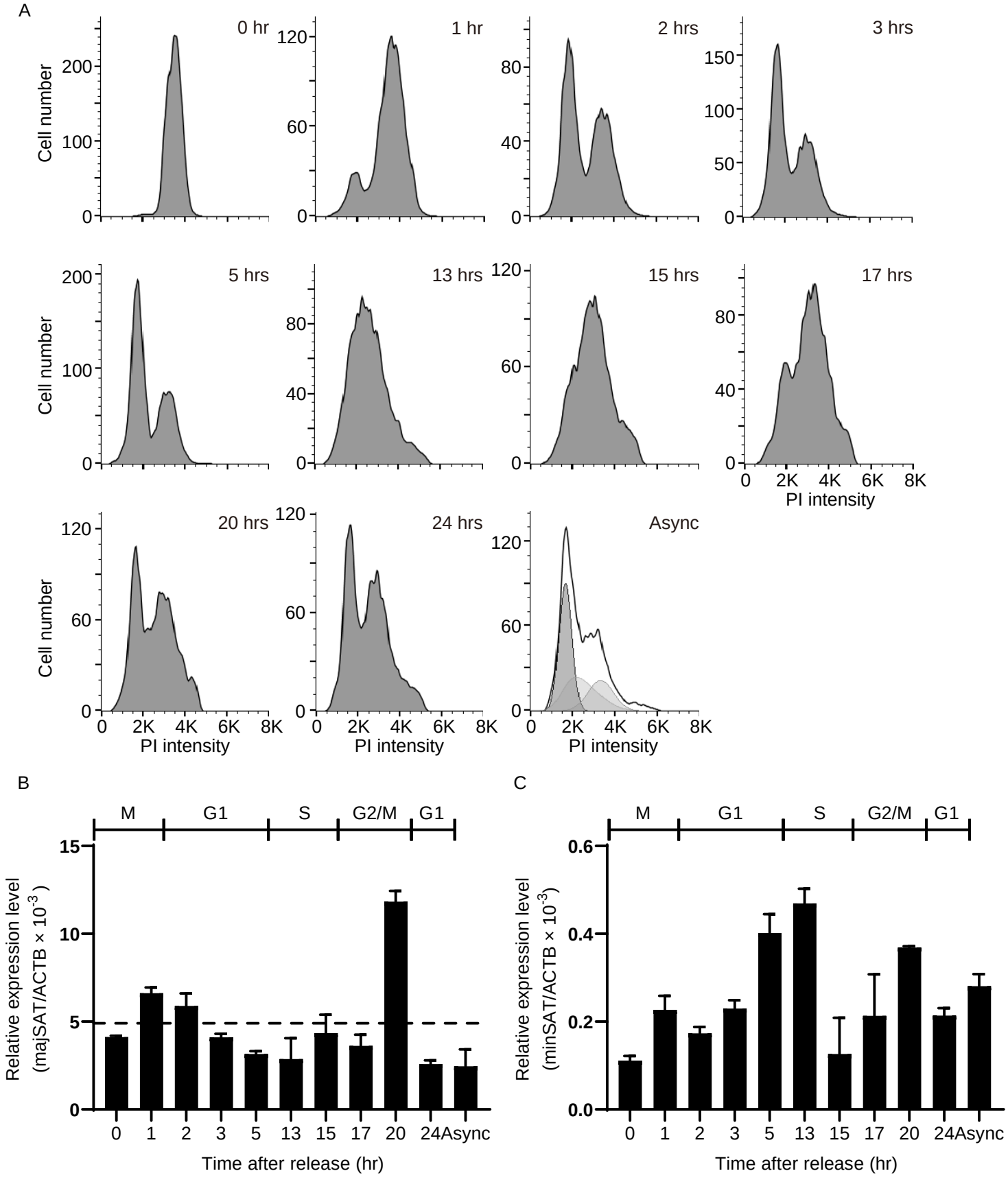

Figure S2 Cell cycle expression profiles of mouse major satellite RNAs and minor satellite RNAs. (A) Flow cytometry analysis showing cell cycle distribution of N2a cells released from mitotic shake-off at indicated time points using PI staining. Asynchronous cells (Async) serve as the control. (B)-(C) RT-qPCR showing expression levels of mouse (B) major satellite RNA and (C) minor satellite RNA in cells at indicated time points after release, normalized to β-actin (ACTB). The predominant cell cycle stage of each time point was indicated on top. Dash line indicate a threshold (fold change =2 relative to Async). The error bars represent SD of three independent experiments.

Figure S3

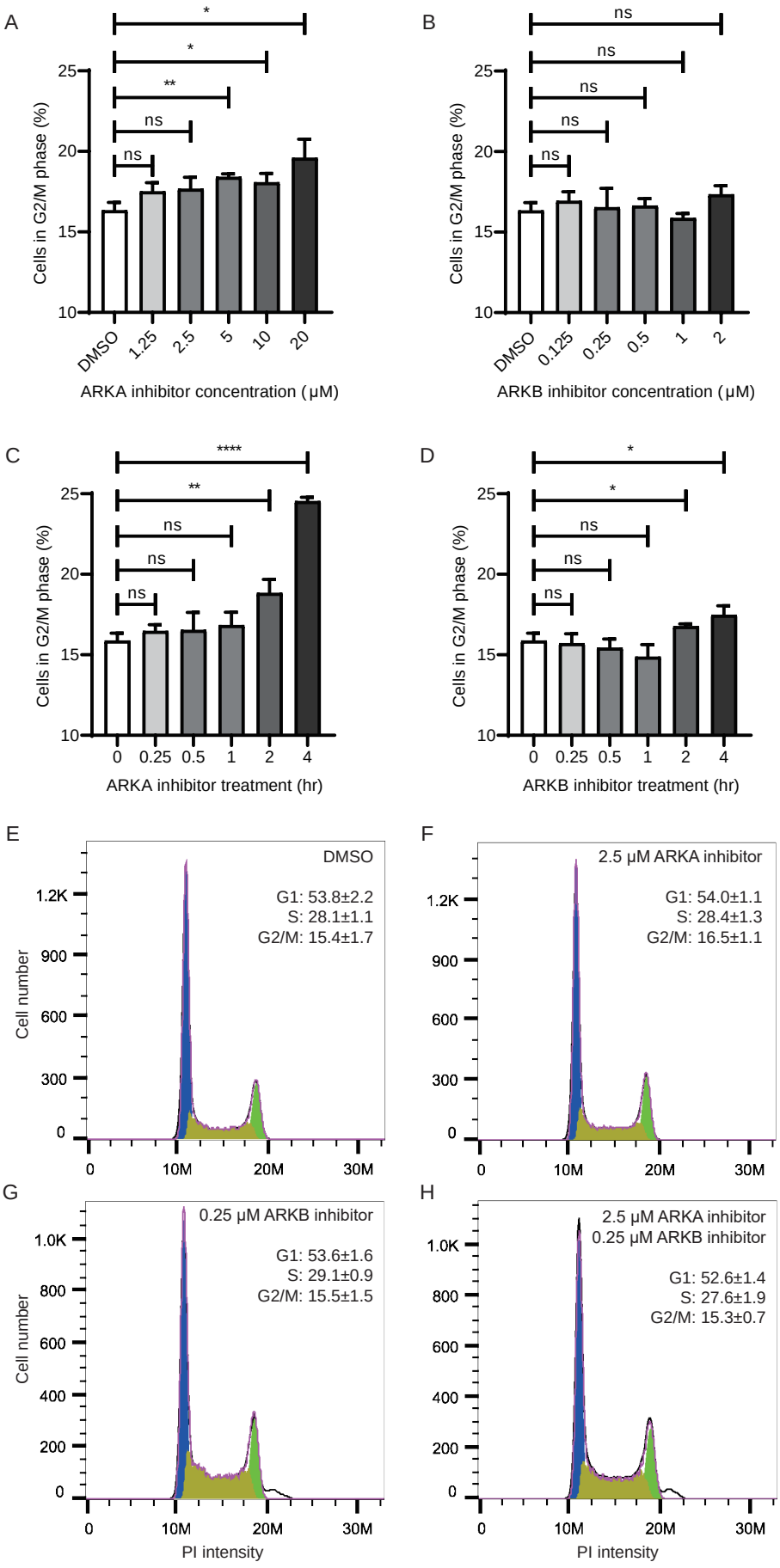

Figure S3 Impact of Aurora kinase inhibitors on cell cycle progression. (A)-(B) Concentration test for AURKA inhibitor (A) and AURKB inhibitor (B). (C)-(D) Treatment time test for AURKA inhibitor (C) and AURKB inhibitor (D). Percentage of G2/M phase cells were quantified by flow cytometry after PI staining. Statistical evaluation was performed by unpaired t-test and is reported as $P > 0.05$ ns, $P < 0.05$ *, $P < 0.01$ **, $P < 0.0001$ ****. Error bars represent SD, n=3. (E)-(H) Representative flow cytometry graphs of cell cycle upon Aurora kinase inhibitor treatment for 30 min. PI, propidium iodide. Percentage of cells at G1, S, G2/M stages shown as the mean ± SD, n=3.

Figure S4

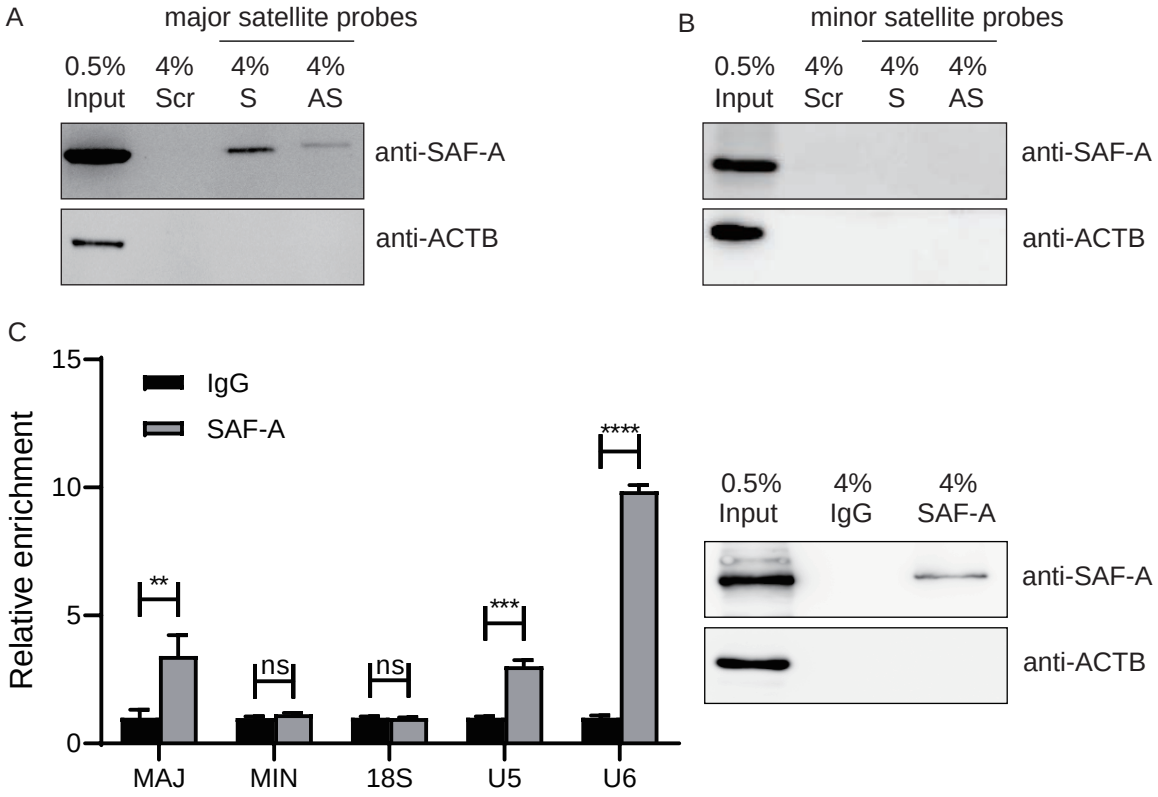

Figure S4 SAF-A interacts with mouse major satellite RNA but not minor satellite RNA. (A)-(B) RNA pull-down in N2a cells using (A) major satellite RNA and (B) minor satellite RNA targeting probes. Scr, scramble; S, sense; AS antisense. (C) RNA immunoprecipitation with SAF-A antibody and non-specific IgG antibody. Relative enrichments of RNA transcripts were quantified using RT-qPCR. 18S is a negative control; U5 and U6 are positive controls. The error bars represent SD, n=3. Statistical significance is calculated using unpaired t-tests and is reported as $P < 0.01$ **, $P < 0.001$ ***, $P < 0.0001$ ****, $P > 0.05$ ns.

Figure S5

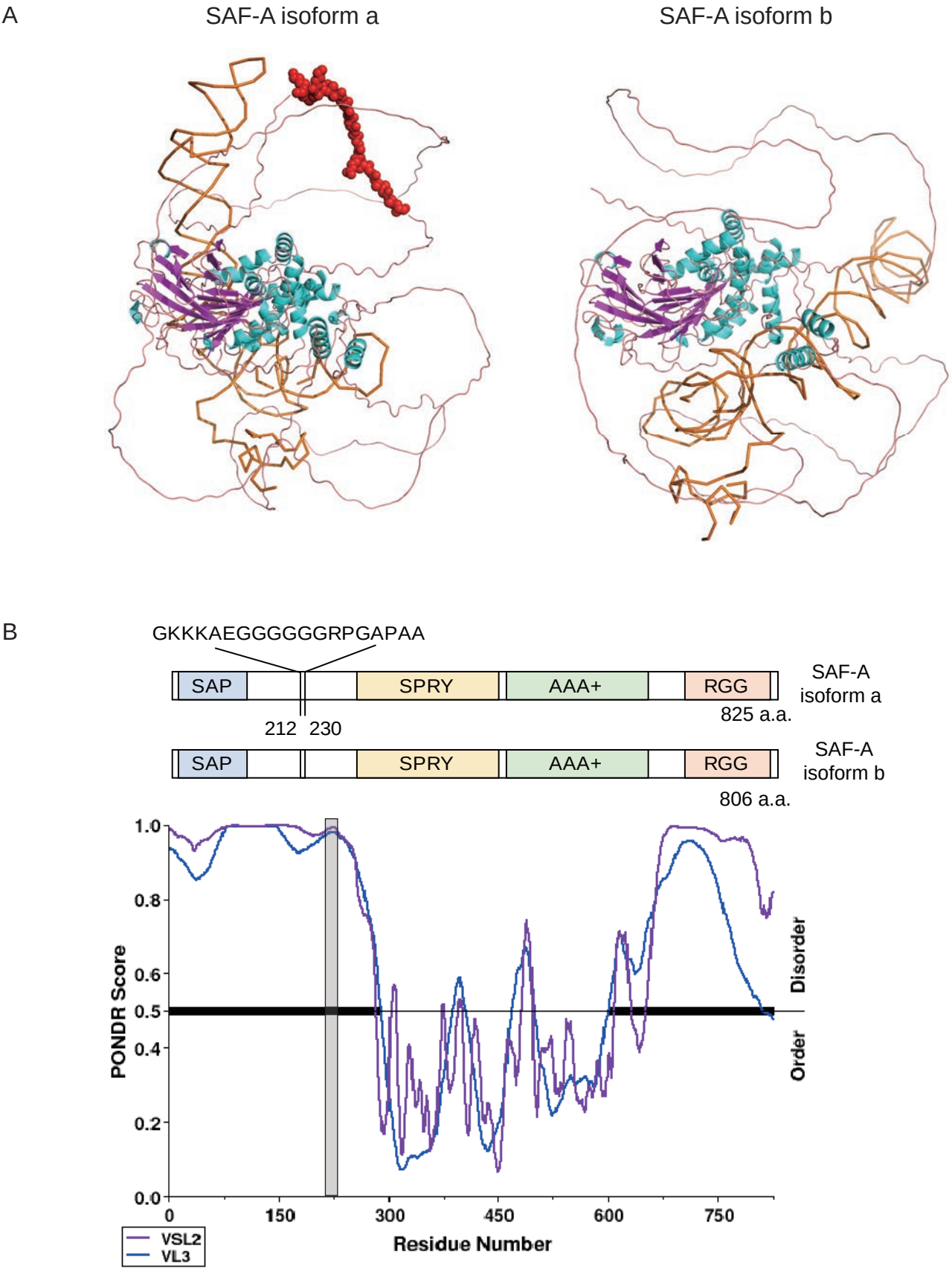

Figure S5 Structural prediction of SAF-A isoforms. (A) Protein structure of SAF-A isoforms predicted by AlphaFold. Secondary structure elements are color-coded as follows: blue, helices; magenta, sheets; pink, loops. Red spheres highlights the 19 amino acid residues that differ between the two isoforms. (B) Prediction of intrinsic disorder regions in SAF-A. The grey box highlights amino acid 212-230, which differ between SAF-A isoform a and b.

Figure S6

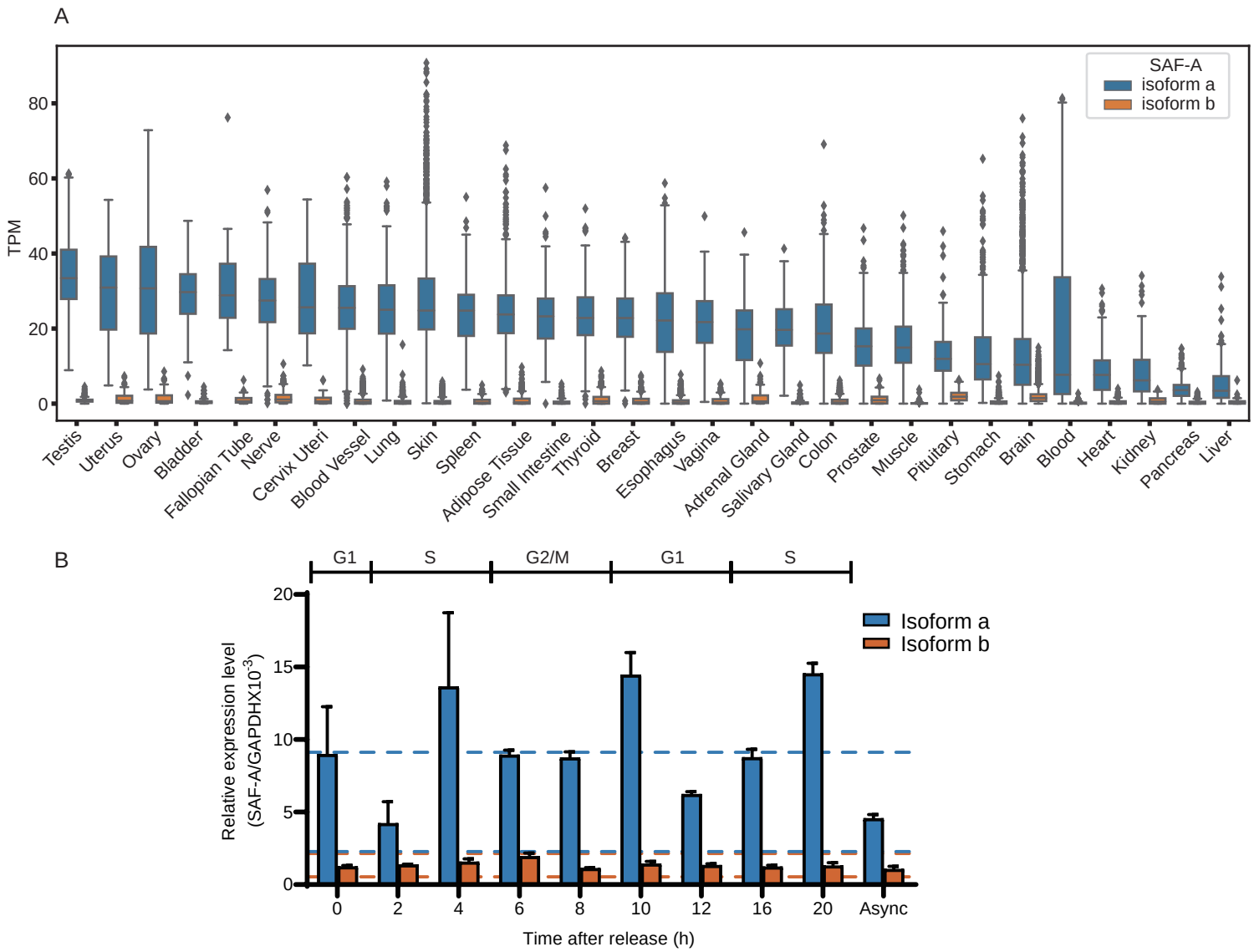

Figure S6 Expression profiles of SAF-A isoforms. (A) Expression levels of SAF-A isoforms in human tissues based on GTEx datasets. Total n=19788, n≥29 for each tissue type. (B) Cell cycle expression profiles of SAF-A isoforms in RPE1 cells. Dash lines, fold change of 2 relative to the level in asynchronous cells. The error bars represent SD, n=6.

Figure S7

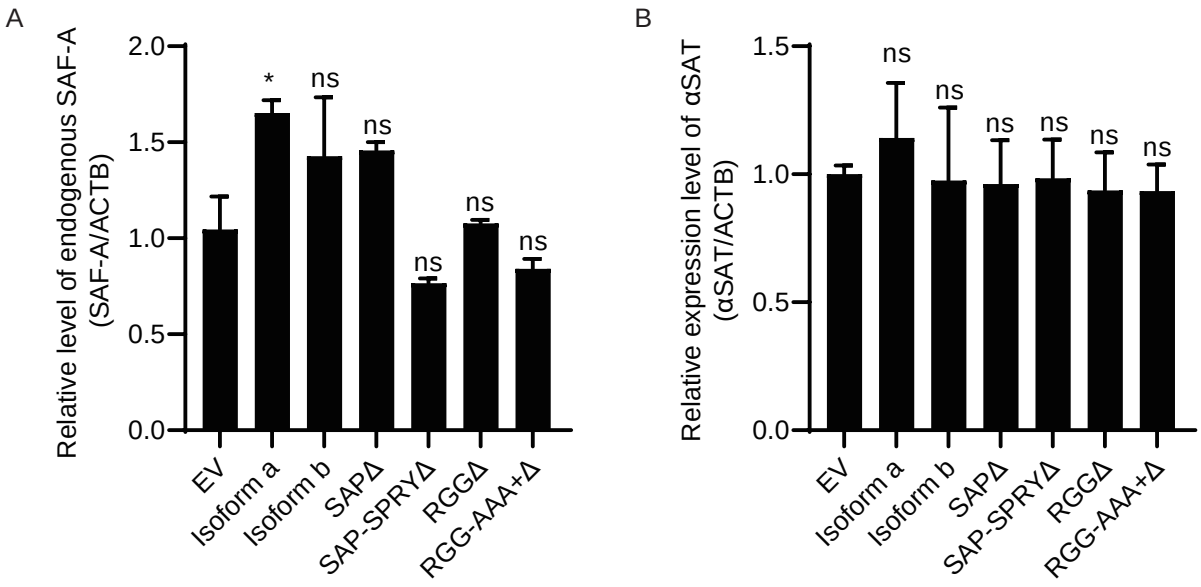

Figure S7 Impact of ectopic expression of FLAG-tagged SAF-A truncations on endougenous SAF-A and α-satellite RNA. (A) Quantification of endogenous SAF-A levels upon transfection of FLAG-SAF-A constructs. (B) Relative expression levels of α-satellite RNA. ACTB, internal control. Protein and RNA levels are normalized to cells transfected with EV, empty vectors. The error bars represent SD, n=3. Statistical significance is calculated using unpaired t-tests and is reported as $P < 0.05$, *, $P > 0.05$ ns.

Figure S8

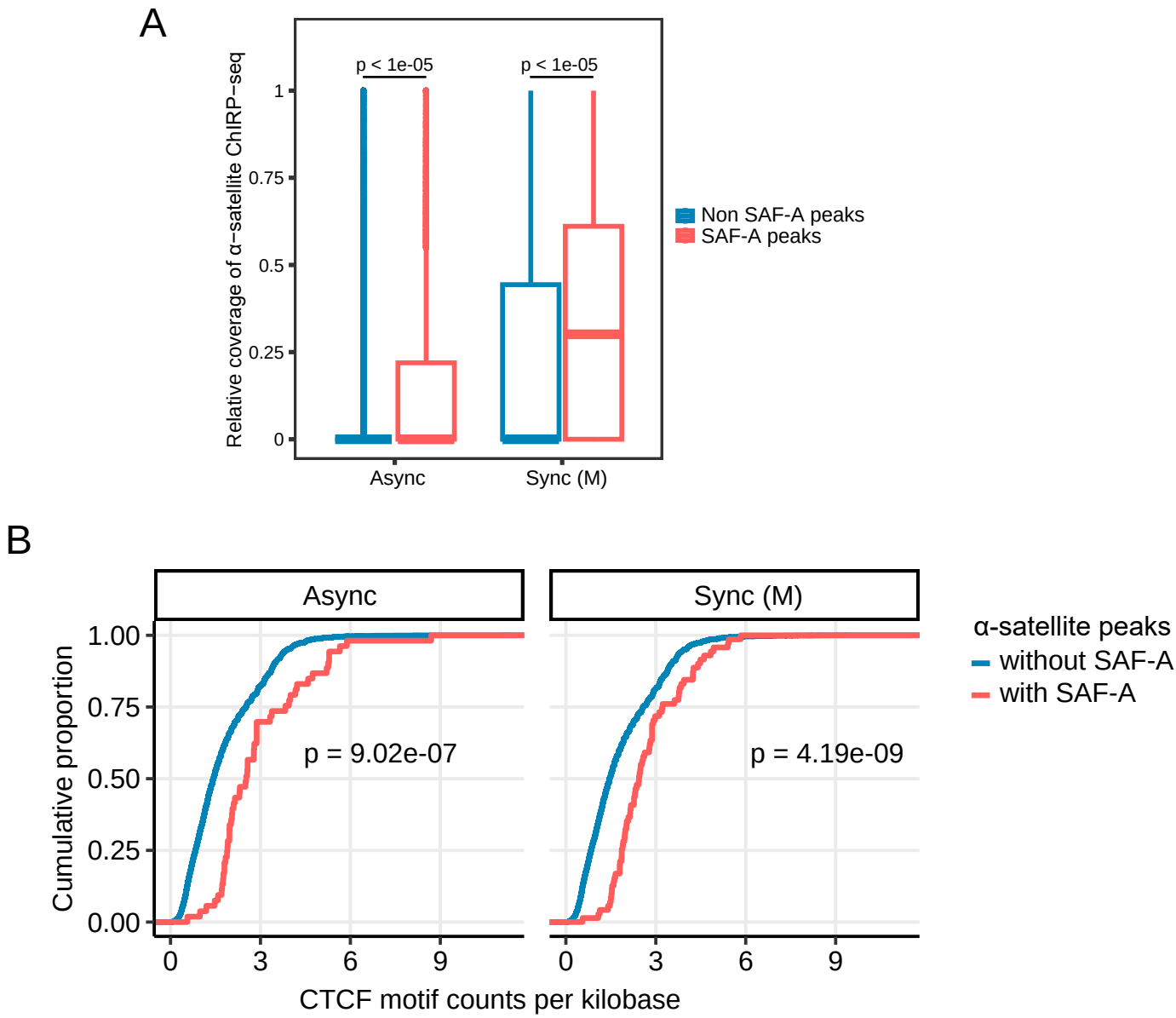

Figure S8 Colocalization of α-satellite RNA and SAF-A. (A) Colocalization of α-satellite RNA ChIRP-seq peaks and SAF-A CUT&Tag peaks, calculated using 100,000 times permutation test. (B) CTCF motif counts per kilobases in genomic regions covered by α-satellite RNA ChIRP-seq peaks with or without SAF-A CUT&Tag peaks.

Figure S9

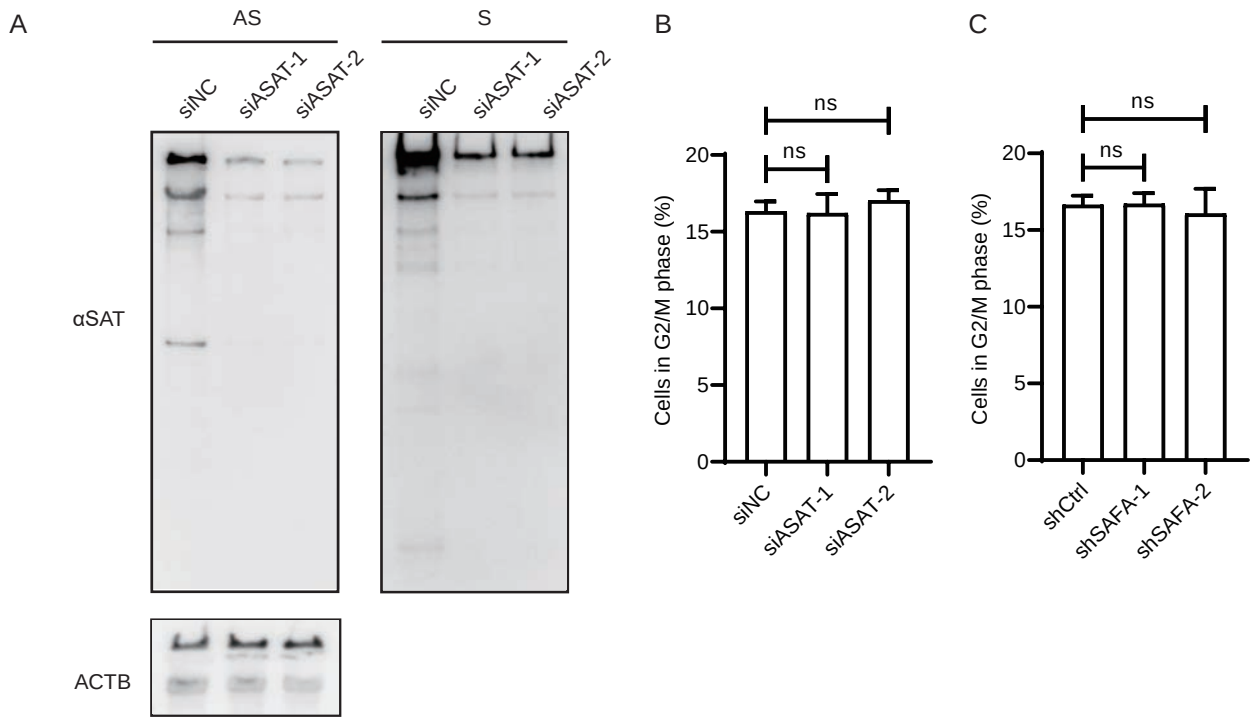

Figure S9 Impact of α-satellite RNA and SAF-A knockdown on cell cycle progression. (A) Northern blots demonstrates the knockdown of α-satellite RNA by siASATs. (B) Ratio of G2/M phase cells in siASAT knockdown cells. (C) Ratio of G2/M phase cells in shSAFA knockdown cells. Error bars represents SD, n=3. Statitical significance is calculated using unpaired t-tests and is reported as $P > 0.05$ ns.

Figure S10

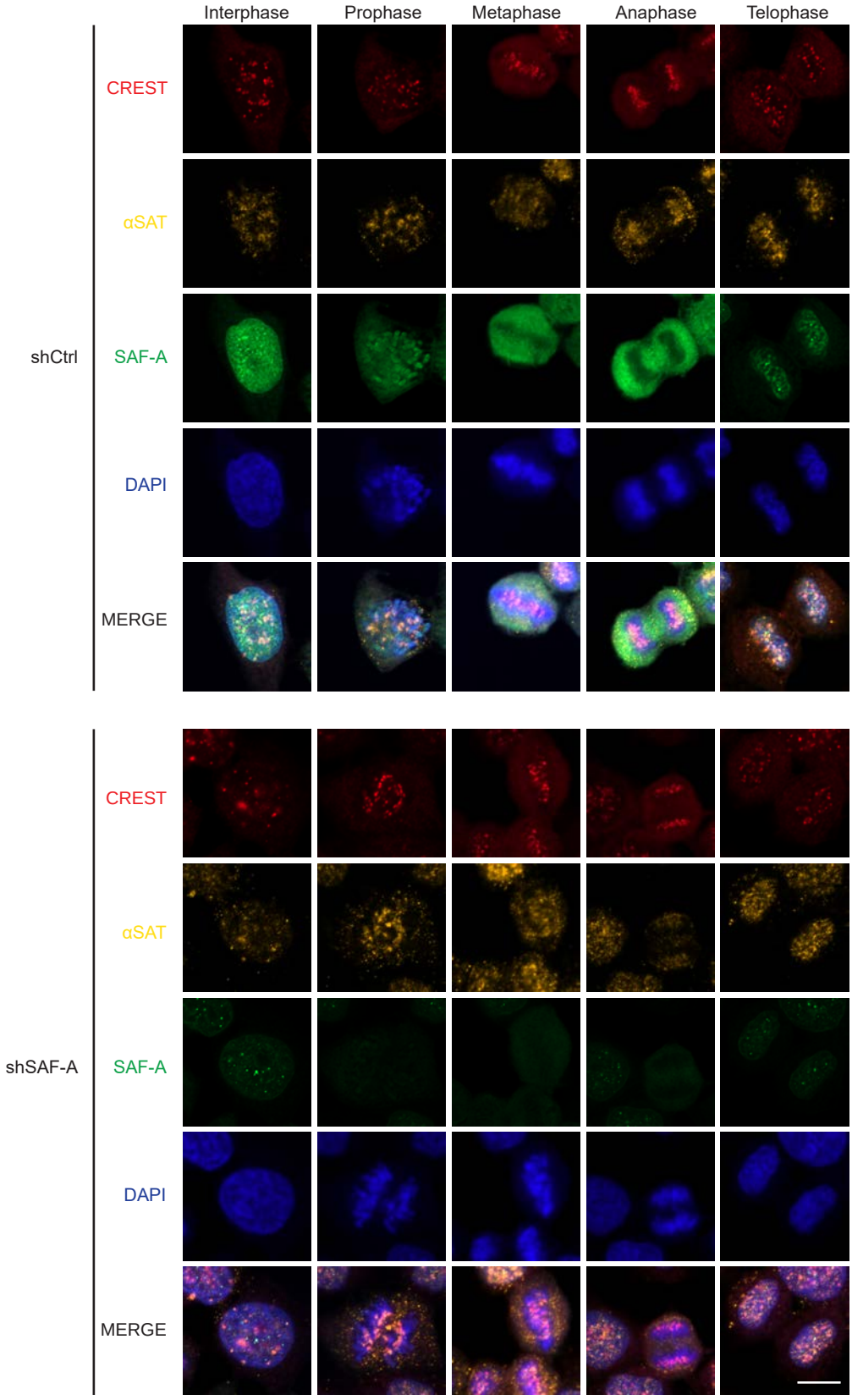

Figure S10 SAF-A knockdown does not influence the localization of α-satellite RNA. Representative images of α-satellite RNA smFISH, SAF-A IF and CREST staining at different cell cycle stages after SAF-A knockdown with shRNA constracts. shCtrl, non-specific control shRNA construct. Scale bar, 10 μm.

Figure S11

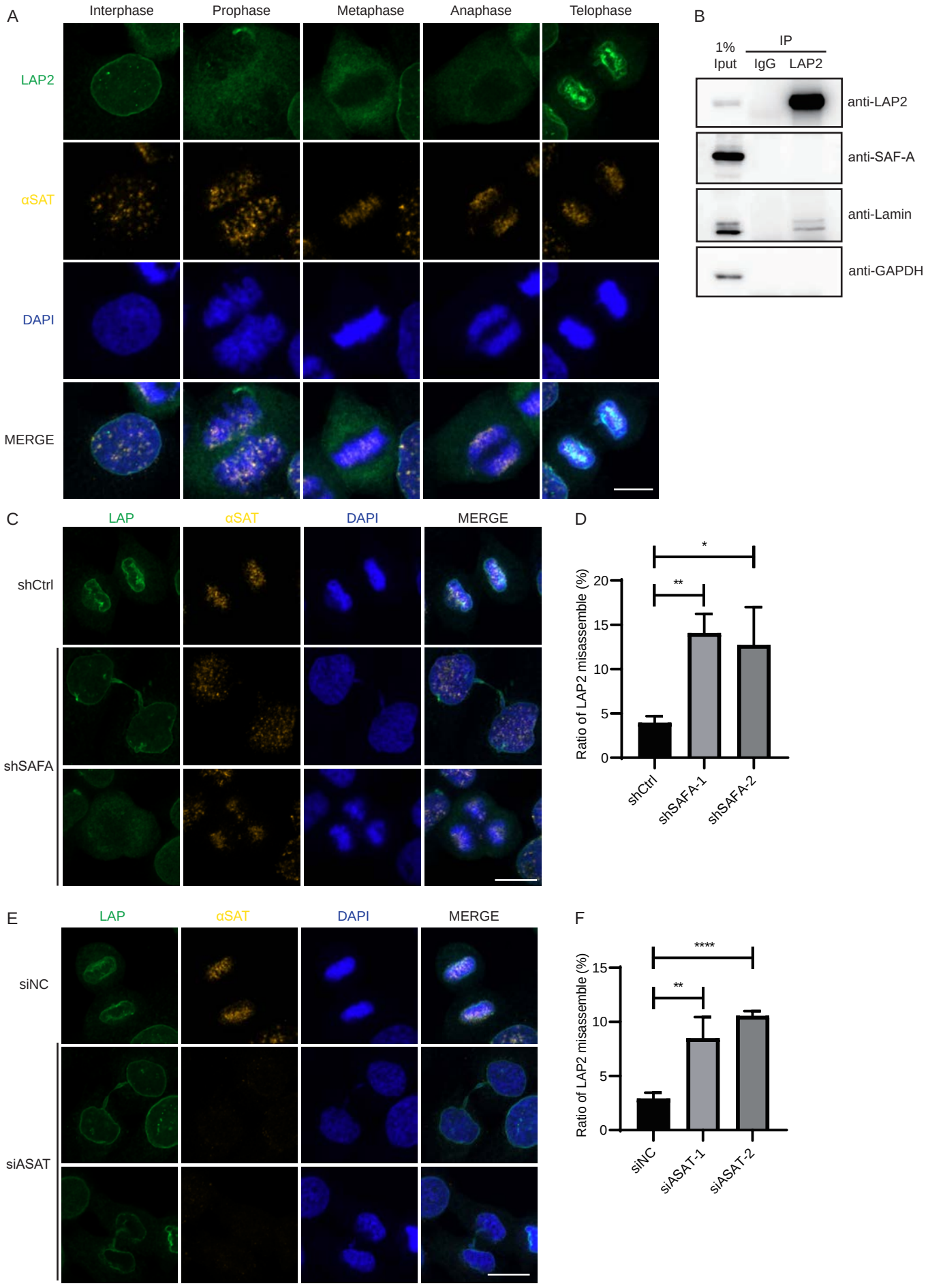

Figure S11 α-Satellite RNA regulates the reassembly of LAP2. (A) Representative images of α-satellite RNA smFISH and LAP2 IF at different cell cycle stages. (B) IP with LAP2 antibody with co-IP of SAF-A. Lamin, positive control; GAPDH, negative control. (C) Representative α-satellite RNA smFISH and LAP IF images show the misassembly of LAP2 upon SAF-A knockdown. (D) Quantification of mitotic cells with misassmbled LAP in (C). (E) Representative α-satellite RNA smFISH and LAP2 IF images show the misassembly of LAP2 upon siASAT knockdown. (F) Quantification of mitotic cells with misassmbled LAP2 in (E). Scale bar, 10 μm. The error bars represent SD, three independent experiments are conducted, n > 200 mitotic cells in each experiment. Statistical significance was calculated using unpaired t-tests and is reported as $P < 0.05$ *, $P < 0.01$ **, $P < 0.0001$ ****.