Supplementary information

Adaptor Aly and Co-Adaptor Thoc5 Function in the Tap-p15-Mediated Nuclear Export of *HSP70* mRNA

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Supplemental materials and methods

Plasmid construction

Full-length human Tap cDNA was subcloned in the yeast two-hybrid bait plasmid pGBKT7 (Clontech). The resulting plasmid was designated pGBKT7-Tap. N- and C-terminal deletion mutants were constructed by restriction digestion of pGBKT7-Tap with appropriate enzymes, followed by T4 DNA polymerase treatment and self-ligation. cDNA fragments encoding aa 1-619, 372-551, and 551-619 of human Tap were amplified by PCR and subcloned to the pGEX6P vector (GE Healthcare) as BamHI-SalI fragments. Full-length Thoc5 cDNA was amplified by PCR using a human liver cDNA library as template. The amplified cDNA was inserted into a modified pET-NH_6 vector (Takano et al., 2007), which harbors a C-terminal FLAG peptide tag sequence in addition to an N-terminal 6x histidine tag. To remove the C-terminal FLAG peptide tag, the vector was cut open by Bsu36I and NruI, and annealed oligonucleotides (5'-TCAGGGATTCTTCAGCCATCGCTGATAGTCG-3' and 5'-CGACTATCAGCGATGGCTGAAGAATCCC-3') were inserted. The resulting vector for expression of 6x histidine-tagged Thoc5 without the C-terminal FLAG tag

was used for antibody production. The fragments of Thoc5 cDNA (encoding aa 1-251 and 251-683) were also amplified by PCR and cloned in the same manner. Full-length hHpr1 cDNA was amplified by PCR and subcloned into the pGEX6P vector. Full-length Thoc5 and hHpr1 cDNAs were also subcloned to pEBO-GFP to obtain expression vectors for C-terminally GFP-tagged Thoc5 and hHpr1 in mammalian cells (Zhou et al., 2000). cDNAs encoding full length human Thoc7 and a C-terminal portion of human Thoc2 (aa 1331-1478) were amplified by PCR and subcloned into the pGEX6P vector, and the resulting expression plasmids were used for antibody production. cDNA fragments of mouse *HSP70* (*HSPA1B*, GenBank accession NM_010478; nt 578-1474) and cytoplasmic *β-actin* (*ACTB*, GenBank accession NM_007393; nt 78-1892) genes were amplified by PCR using a mouse embryo cDNA library as template and subcloned to the pBluescript vector (Stratagene).

Anti-sense oligonucleotide probe sequences

Sequences of oligonucleotide probes for *in situ HSP70* mRNA detection were as follows:

5’-TTGTTCAGG*CGCGCCCG*TGAAGAAG*CCTGCAGCAGC*TCTGCACC*T-3’

5’-GAGTAGG*GGTGAAGATC*GCGTCTGCT*GGTGGGGA*GGTGGAGT*GCG-3’

5’-CGATC*CCTCCTTGC*CAGGCGGCCC*TGTCGTTGG*GATGGTGA*CTTG-3’.

Sequences of oligonucleotide probes for *in situ β-actin* mRNA detection were as follows (Zhang et al., 1994):

5'-CA*AGGAATCC*TCTGACCCA*GCCCACCA*CACGCCCTGGTGCC*GGGGC-3'

5'-GG*GTTGAAGGTC*CAAACATGA*CTGGGTCATC*TCTCGCGGTTGGCC*T-3'

5'-G*TGAAGGTAG*TTCGTGGA*GCCACAGGAC*CCATGCCCAGGAAGGAAGGC*G-3'

5'-C*CAGGAGGAGCAA*GATCTTGATCT*CATTGTGCTGGG*GCCAGGGCAG*G-3'

FITC-labeled phosphoramidite (fluorescein-dT) was incorporated at the indicated positions (indicated by asterisks) during synthesis. The specificity of the oligonucleotide probes was determined by Northern blot (Shibata *et al.*, 2006).

SiRNA sequences

Sequences of siRNAs used to target Thoc5, Aly and Tap were as follows:

SiThoc5-1: 5'-CCAAAUGUUUGGAGUUUAAdTdT-3'

SiThoc5-2: 5'-GAAAGUAGAUGCCUAUCAUdTdT-3'

SiALY-1: 5'-GGAACUCUUUGCUGAAUUUdTdT-3'

SiALY-2: 5'-GCACGAUCUUUUCGACAGUdTdT-3'

SiTap-1: 5'-GGAUAUCUAUCAUCAUCAAdTdT-3'

SiTap-2: 5'-ACAUUGACGUUGUCCUGAAdTdT-3'

SiThoc6-1: 5'-GGAUGGAGCUGUUCGACUUdTdT-3'

SiThoc6-2: 5'-AGAAGCCGGUGGUGACUUUdTdT-3'

The sequence of siRNA against DsRed has been described in the literature (Shibata et al., 2006).

RNA co-immunoprecipitation

Subconfluent HeLa cell cultures (4 x 10 cm dishes) were temperature shifted at 43 ^{o}C for 1 hr. The cells were cross-linked by 1 % formaldehyde, washed, and resuspended in 2 ml of RSBN buffer (10 mM Tris-HCl (pH 7.4)/ 100 mM NaCl/ 2.5 mM $MgCl_2$/ 0.1 % NP-40) (Taniguchi and Ohno, 2008) supplemented with 400 U/ml of RNasin (Promega). The cells were disrupted by brief sonication, and cell debris was removed by

centrifugation. An aliquot of the supernatant (100 μl, input fraction) was sampled, and it was adjusted to a final of 10 mM EDTA and 1 % SDS and reverse cross-linked during over night incubation at 65 ^{o}C. The remaining supernatant (600 μl each) was subjected to immunoprecipitation with Protein A Sepharose beads pre-adsorbed to rabbit anti-mouse IgG (for negative control) or anti-Thoc5 antibodies. For Aly immunoprecipitation, rabbit anti-mouse IgG was used to bridge the interaction between the mouse anti-Aly/REF antibody and Protein A Sepharose beads (Taniguchi *et al.*, 2008). After extensive washing with RSBN buffer followed by RSBN buffer supplemented with 0.5 % Na-deoxycholate, the immune-complexes were dissociated by being incubated in 50 mM Tris-HCl (pH 8.0)/ 10 mM EDTA/ 1 % SDS at 65 ^{o}C for 30 min, and the released mRNPs were reverse cross-linked during over night incubation at 65 ^{o}C. For re-immunoprecipitation experiments, the immune-complexes were released from the anti-Thoc5 bound beads by incubating in 15 mM DTT for 30 min at 37 ^{o}C and further immunoprecipitated with Protein A Sepharose beads pre-adsorbed to rabbit anti-Tap, anti-Thoc2 or anti-Thoc7 antibodies. The beads pre-adsorbed to rabbit anti-mouse IgG was used for negative control. Washing and reverse cross-linking were performed as above. Total RNAs in the input fraction and immune-complexes were extracted by TRIzol reagent and contaminating DNA was removed using the TURBO DNA-free kit (Ambion) in 50 μl reactions. Of these DNase I treated samples, 5 μl each was subjected to a 20 μl of first-strand cDNA synthesis reaction by the RETROscript kit using a random decamer (Ambion). Then 2 μl each of the first strand of cDNA was used for a 25 μl PCR reaction using an Expand High Fidelity PCR system (Roche) and the primers listed below. To monitor contaminating chromosomal DNA, control reactions, from which reverse transcriptase was omitted, were run in parallel. Thirty PCR cycles

were performed, with each cycle consisting of a 30 sec denaturation at 95 ^{o}C, a 30 sec annealing at 60 ^{o}C, and a 30 sec extension at 68 ^{o}C. For re-immunoprecipitation experiments 40 PCR cycles were performed.

PCR primer sequences

Sequences of primers for detection of human *HSP70* mRNA by RT-PCR were as follows:

HSPRTS1: 5'-GATCGGCATCGACCTGGGCACCACCTACTC-3'

HSPRTS2: 5'-TTGACAACAGGCTGGTGAACCACTTCGTGG-3'

HSPRTA1: 5'-TCGCGCCCGTTGAAGAAGTCCTGCAGCAGC-3'

HSPRTA2: 5'-GCCTCGGCGATCTCCTTCATCTTGGTCAGC-3'

Combinations of HSPRTS1 and HSPRTA2 (5' primer pair) or HSPRTS2 and HSPRTA1 (3' primer pair) amplify the 5'- and 3'-portions of *HSPA1B* cDNA with 381 and 376 bps in length, respectively.

Sequences of PCR primers for detection of human *β-actin* (Kendirgi et al., 2005) were as follows:

HSACTRT3FW: 5'-AGAAGGATTCCTATGTGGGCGACGAG-3'

HSACTRT3RV: 5'-GTCGCCATCTCTTGCTCGAAGTCCAG-3'

Miscellaneous

Isolation of total RNAs from HeLa cells was done using TRIzol reagent (Invitrogen). Cell fractionation was performed as reported (Kendirgi *et al.*, 2005). Northern blot analysis was performed as described (Sasaki et al., 2005) using the cDNA fragments of mouse *HSP70* and human *β-actin* (*ACTB*) genes as probe templates. GST pull-down

assays were done as reported previously (Kunzler and Hurt, 1998;Sasaki *et al.*, 2005). RNA band shift assays were performed as described (Katahira et al., 1999;Shibata *et al.*, 2006). Immunofluorescence and heterokaryon assays were carried out as described (Katahira *et al.*, 1999;Zhou *et al.*, 2000). The structural coordinate of human Tap-p15 and FG repeat (accession number; 1JN5) was taken from the PDB database and displayed using the GRASP2 software (Petrey and Honig, 2003). To model a structure of the N-terminal domain of Thoc5, proteins showing structural similarity were identified using the PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/) (Jones, 1999;McGuffin and Jones, 2003). Alignment of the domains identified (aa 51-252 of Thoc5 versus repeat 16 and 17 of chicken brain α-spectrin, accession number; 1cunA) was repeated by the ClustalW server (http://www.ebi.ac.uk/Tools/clustalw2/). The resulting sequence alignment was used to predict the secondary structure and to model the structure of the N-terminal region of Thoc5 by SWISS-MODEL Workspace (http://swissmodel.expasy.org/workspace/) (Arnold et al., 2006). The predicted structure was displayed using the GRASP2 software.

Legends for the supplemental figures

Figure S1.

A yeast two-hybrid strain AH109 was transformed with indicated combinations of bait and prey plasmids. FG-repeat containing domain of human FG-nucleoporin hCG1 (Katahira *et al.*, 1999) was used as the positive control. Growth on his-, ade- selection plates and expression of *MEL1* gene activity were scored. -; negative, +; positive, ±;

weakly positive

Figure S2. The N-terminal region of Thoc5 could exhibit α-solenoid structure.

(A) Amino acid sequence alignment of Thoc5 (aa 51-252) and repeats 16 and 17 of chicken brain α-spectrin (1cunA). The secondary structures (h: α-helical region) of Thoc5 and α-spectrin are indicated.

(B) The predicted structural model of the N-terminal region of Thoc5.

Figure S3.

(A) HeLa total cell extract was subjected to Western blot using the anti-Thoc2, anti-Thoc5 and anti-Thoc7 antibodies. Alkaline phosphatase-conjugated anti-rabbit IgG was used as secondary antibody.

(B) Formaldehyde fixed and Triton X-100 permeabilized HeLa cells were subjected to immunofluorescence assays using indicated antibodies. The cells were doubly stained with anti-Aly/REF (upper left) or anti-hHpr1 (upper right). Alexa488-labeled anti-rabbit and Alexa568-labeled anti-mouse antibodies were used as secondary antibodies. Nuclei were stained with Hoechst 33342 dye. The cells were observed using confocal microscopy.

(C) HeLa cell extract was subjected to immunoprecipitation using the antibodies indicated on the top of each lane. Immune-pellets were subjected to Western blot using the indicated antibodies. Note that under the condition used, Thoc2 was not efficiently extracted and thus it was detectable only after immunoprecipitation (indicated by an asterisk). Input (5 % of total) was run on lane 1.

Figure S4.

(A) Specificity of the oligonucleotide probes. HeLa cells were left untreated (indicated by 37) or temperature shifted at 43 ^{o}C for 1 hr (indicated by 43). Total RNAs (10 μg/lane) prepared from each culture were subjected to Northern blot analysis using mixtures of the *HSP70* (left) and *β-actin* (right) oligonucleotide probes end-labeled with γ-[^{32}P]-ATP. 28 S rRNA detected by ethidium bromide staining served as loading control.

(B) HeLa cells grown on glass bottom dishes were left untreated (37 ^{o}C, upper panels) or temperature shifted at 43 ^{o}C for 1 hr (lower panels). The cells were then fixed and the localization of *HSP70* transcripts were examined by *in situ* hybridization using a mixture of FITC-labeled oligonucleotide probes. Nuclei were counter-stained with Hoechst 33342 dye. Arrowheads indicate *HSP70* mRNA-containing nuclear foci.

(C) HeLa cells treated with the indicated siRNAs were subjected to *in situ* hybridization as in (B) without heat shock. These control experiments confirmed the nuclear foci are not caused by siRNA treatment.

(D) HeLa cells were left untreated (upper panels) or temperature shifted at 43 ^{o}C for 1hr (lower panels). The cells were fixed, permeabilized and incubated at 37 ^{o}C for 15 min in the absence (left panels) or presence (right panels) of RNase A. Localization of *β-actin* transcripts was detected by *in situ* hybridization using a mixture of FITC-labeled oligonucleotide probes.

(E) HeLa cells were treated with the indicated siRNAs. The cells were temperature shifted at 43 ^{o}C for 1 hr and subjected to *in situ* hybridization as in (D). Note that *β-actin* transcripts did not show nuclear dot-like accumulation upon siRNA treatment.

Figure S5.

A series of Z-sections of the pictures (consecutive 1 μm sections) displayed in Fig. 4A.

Figure S6.

A series of Z-sections of the pictures (consecutive 1 μm sections) displayed in Fig. 7C.

Supplementary References

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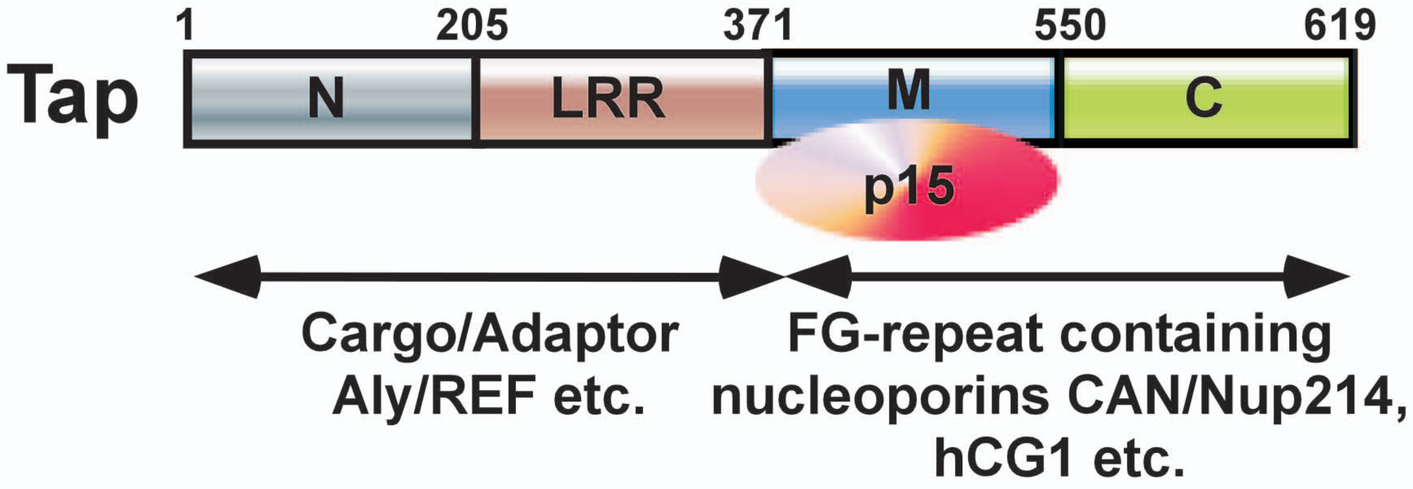
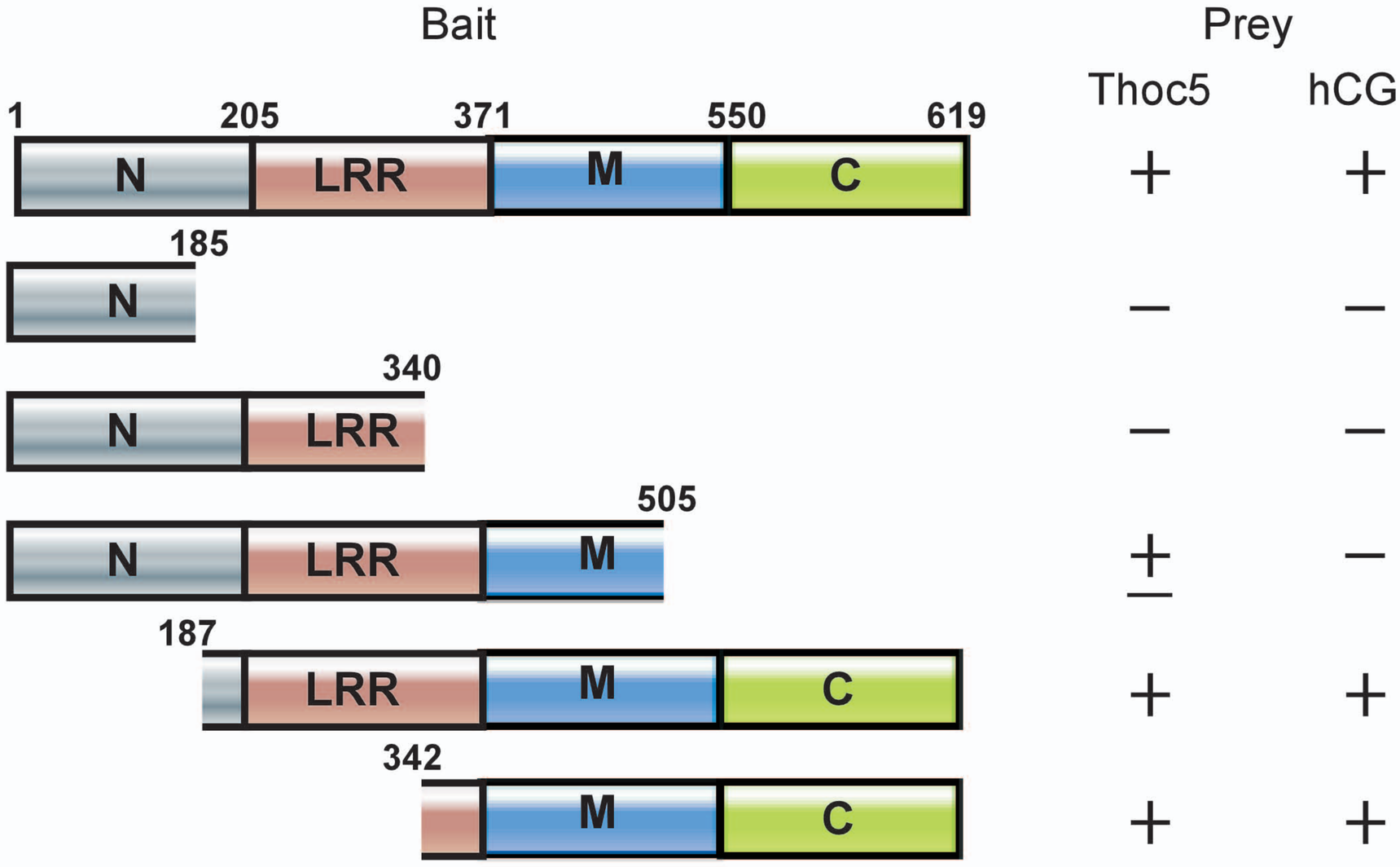

Tap
1
205
371
550
619
N
LRR
M
C
p15
Cargo/Adaptor
Aly/REF etc.
FG-repeat containing
nucleoporins CAN/Nup214,
hCG1 etc.


Bait
Prey
Thoc5
hCG1
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205
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550
619
N
LRR
M
C
+
+
185
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−
340
N
LRR
−
−
505
N
LRR
M
+
−
−
187
LRR
M
C
+
+
342
M
C
+
+

A

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Thoc5(51-252)  1        DYELYKY TCQELQRLMA EIQDLKSRG- GKDV----AI EIEERRIQSC
1cunA          7     m--vhqffrd mddeeswike kkllvssedy grdltgvqnl rkkhkrleae

                        hhhhhh hhhhhhhhhh hhhhh               hhhhhhhhhh
                       hhhhhhh hhhhhhhhhh hhhhh         hhhhhhh hhhhhhhhhh

               43    VHFMTLKKLN RLAHIRL--- --KKGRDQTH EAKQKVDAYH LQLQNLLYEV
               55    laahepaiqs vldtgkklsd dntigkeeiq qrlaqfvdhw kelkqlaaar

                     hhhhhhhhhh hhhhhhh          hhhh hhhhhhhhhh hhhhhhhhhh
                     hhhhhhhhhh hhhhhhhhhh      hhhhh hhhhhhhhhh hhhhhhhhhh

               88    MHLQKEITKC LEFKSKHEEI DLVSLEEFYK EAPPDISKAE VTMGDPHQQT
               105   gqrleesley qqfvanveee eawinekmtl vasedygdtl aaiqgllkkh

                     hhhhhhhhhh hhhhhhhhhh hhhhhhhhhh h        h hhhhhhhhhh
                     hhhhhhhhhh hhhhhhhhhh hhhhhhhhhh h        h hhhhhhhhhh

               138   LARLDWELEQ RKRLAEKYRE CLSNKEKILK EIEVKKEYLS SLQPRLNSIM
               155   eafetdftvh kdrvndvcan gedlikknnh hvenitakmk glkgkvsdle

                     hhhhhhhhhh hhhhhhhhhh hhhhhh      hhhhhhhhhh hhhhhhhhhh
                     hhhhhhhhhh hhhhhhhhhh hhhhhh      hhhhhhhhhh hhhhhhhhhh

               188   QASLPVQEYL FMPFD
               205   kaaaqrkakl densa-

                     hhhhhhhhhh hhhh
                     hhhhhhhhhh hhhh
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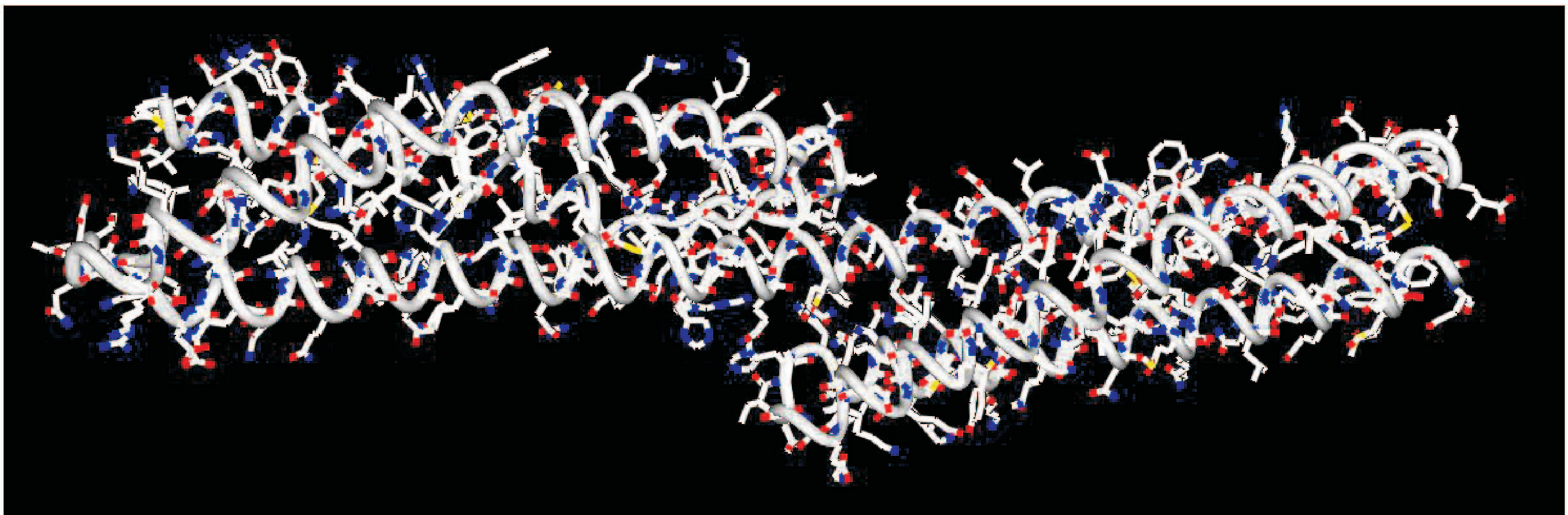
B



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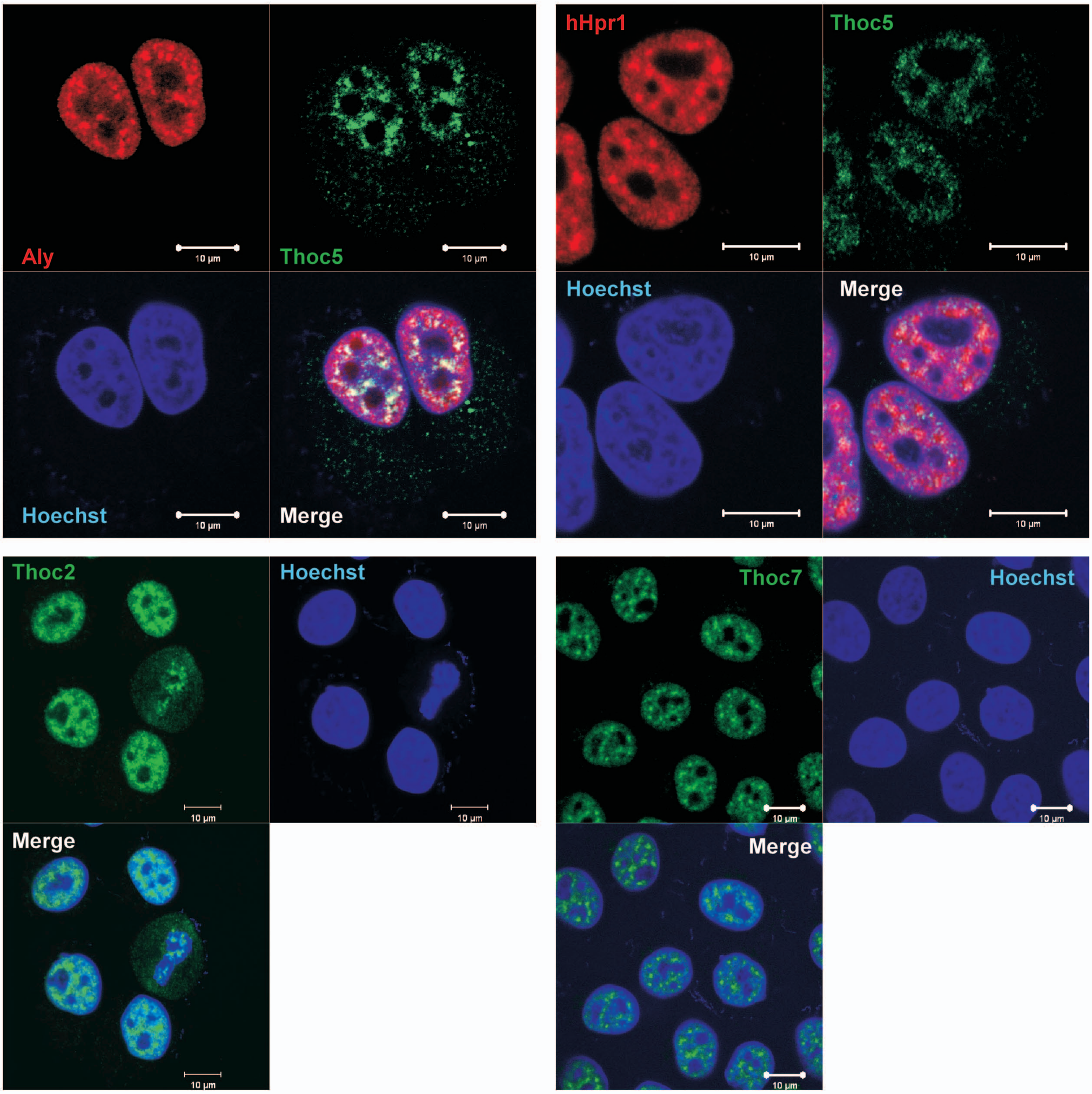
Aly
10 μm
Thoc5
10 μm
Hoechst
10 μm
Merge
10 μm
hHpr1
10 μm
Thoc5
10 μm
Hoechst
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Merge
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Thoc2
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Merge
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Thoc7
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Hoechst
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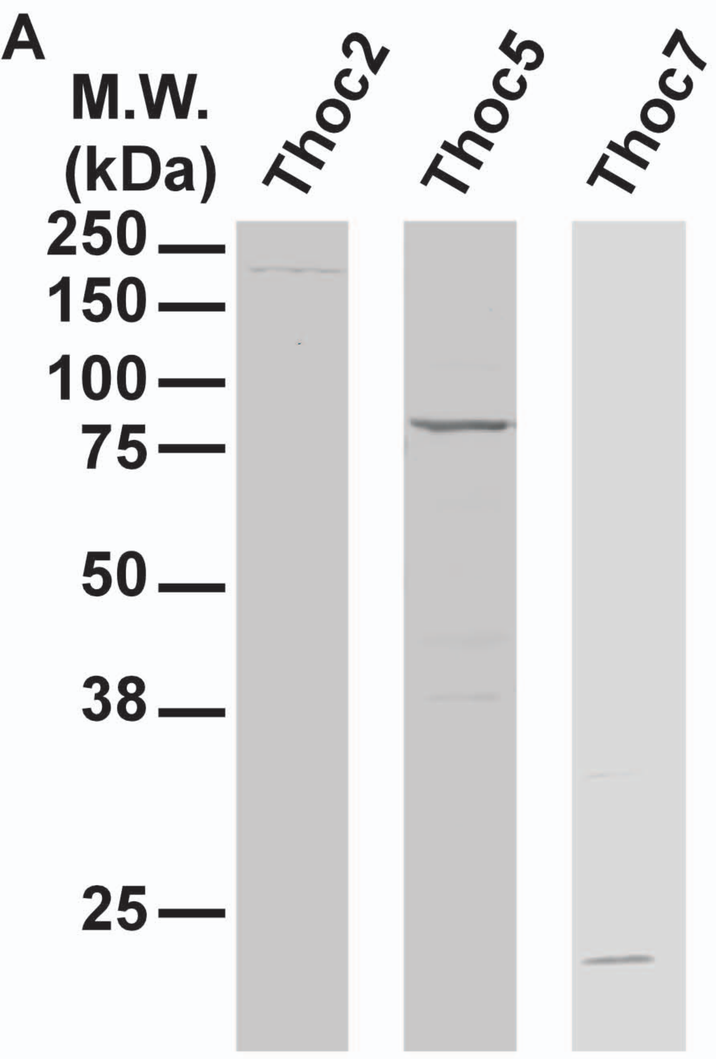
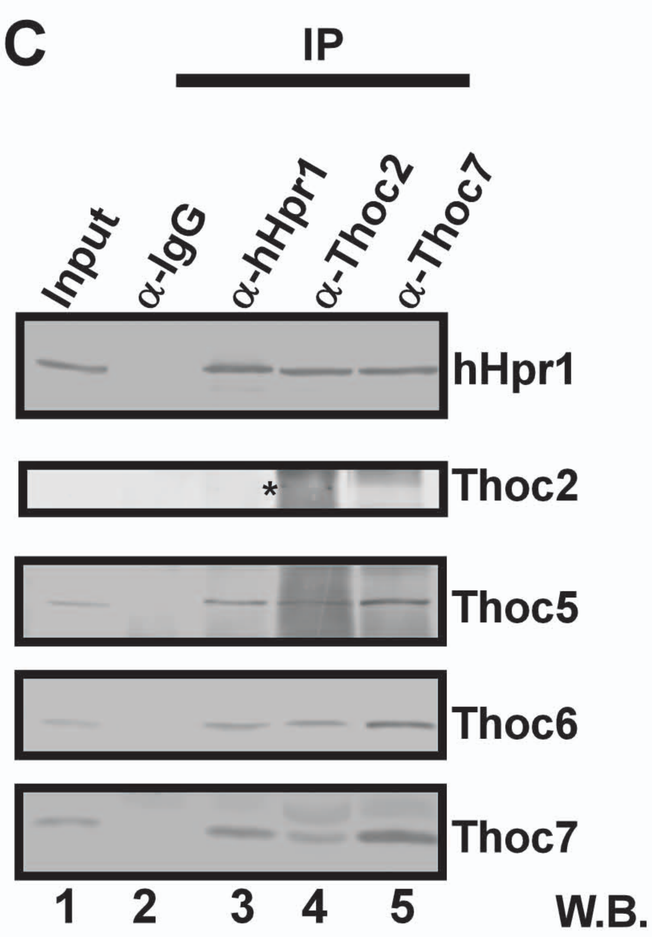
Merge
10 μm

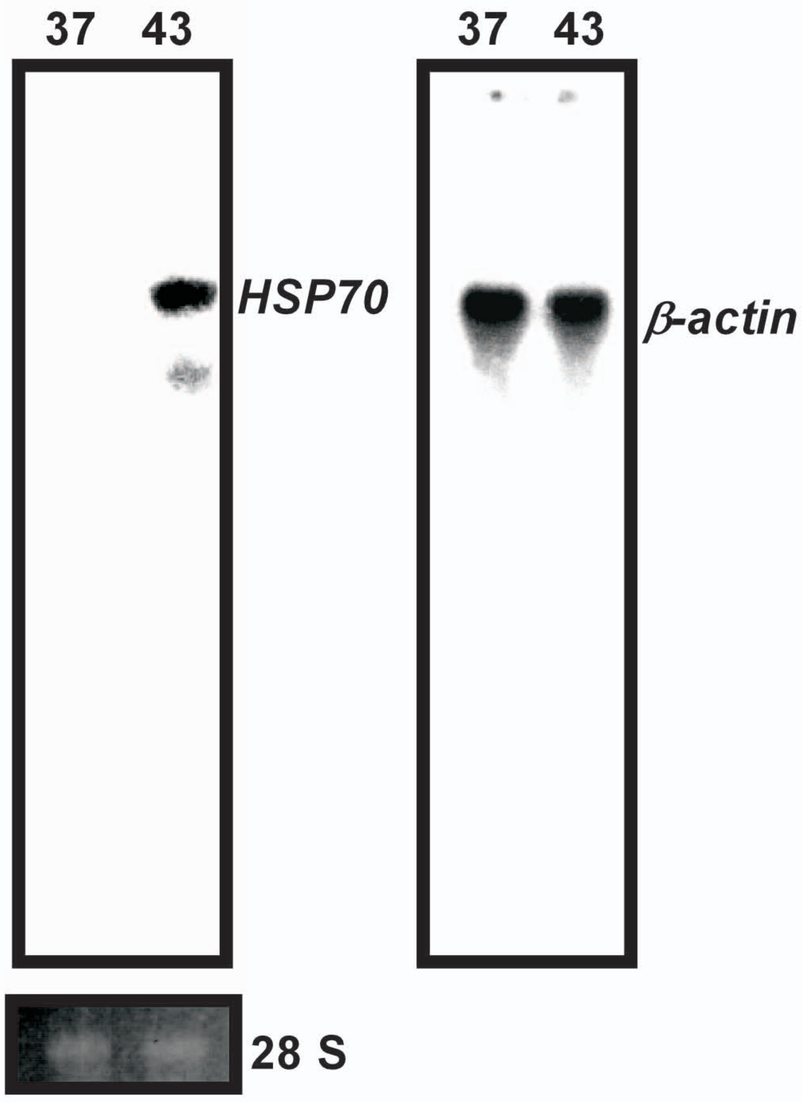
A


M.W.
(kDa)
Thoc2
Thoc5
Thoc7
250
150
100
75
50
38
25

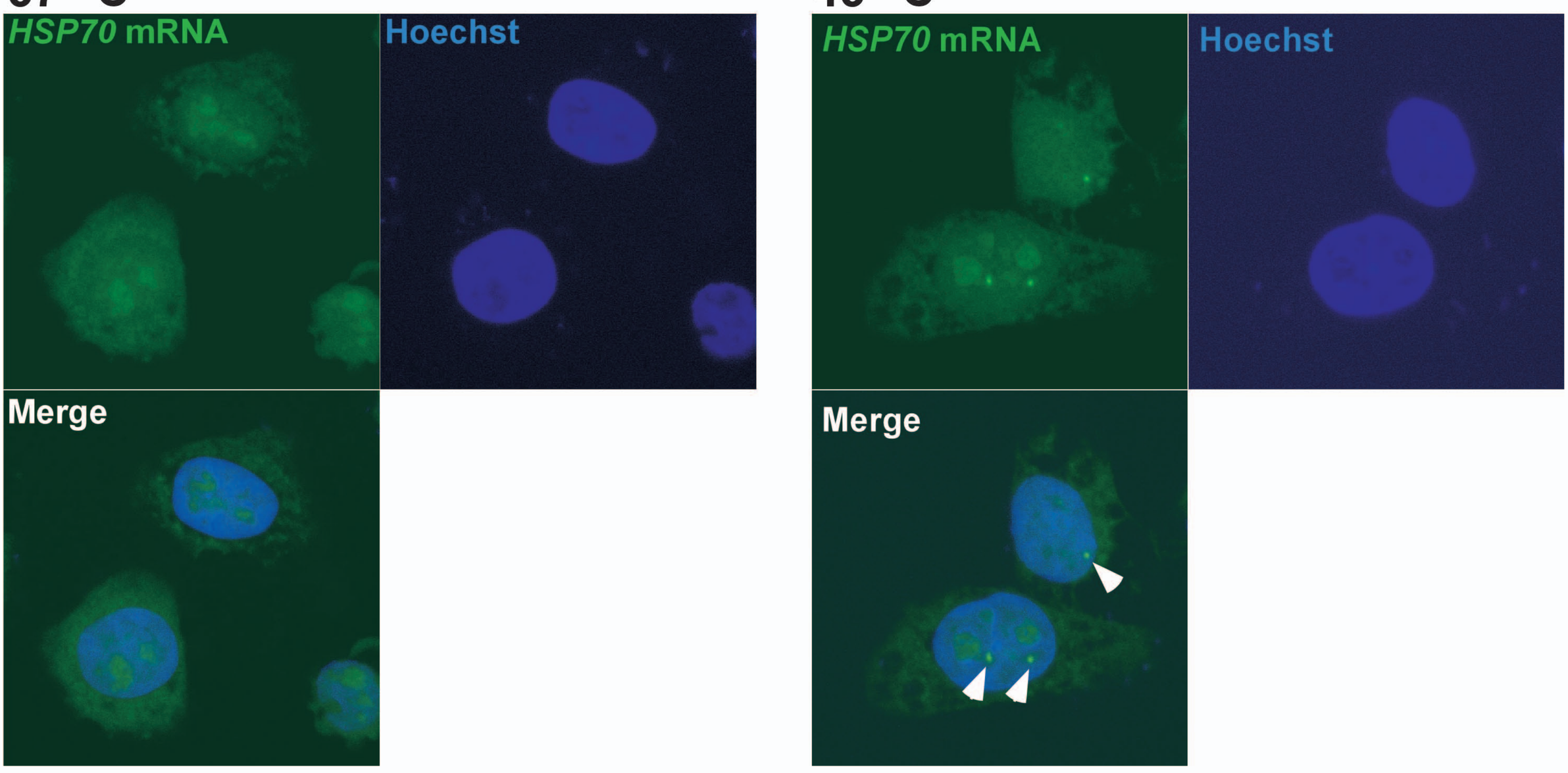
C


IP
Input
α-IgG
α-hHpr1
α-Thoc2
α-Thoc7
hHpr1
*
Thoc2
Thoc5
Thoc6
Thoc7
1
2
3
4
5
W.B.

A


37
43
HSP70
37
43
β-actin
28 S

B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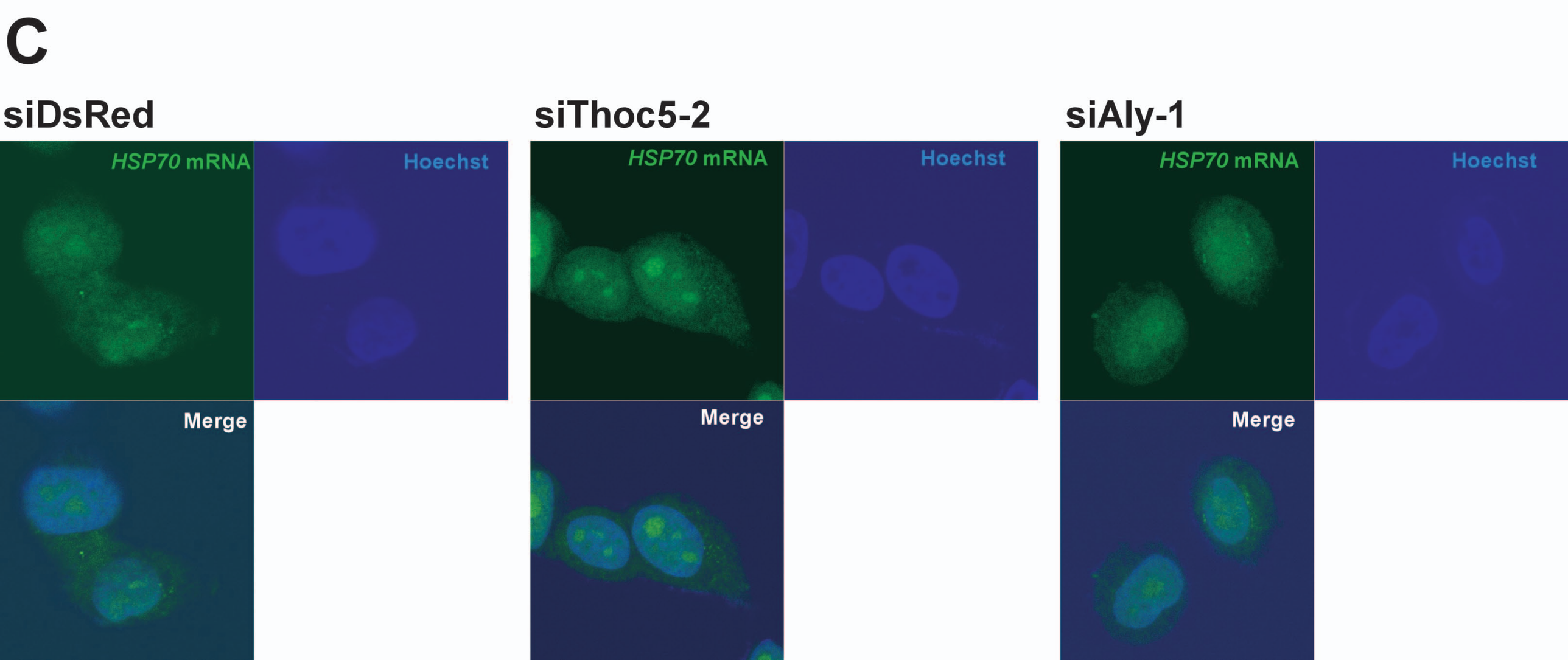
HSP70 mRNA
Hoechst
Merge
43 ºC
HSP70 mRNA
Hoechst
Merge

C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HSP70 mRNA
Hoechst
Merge
siThoc5-2
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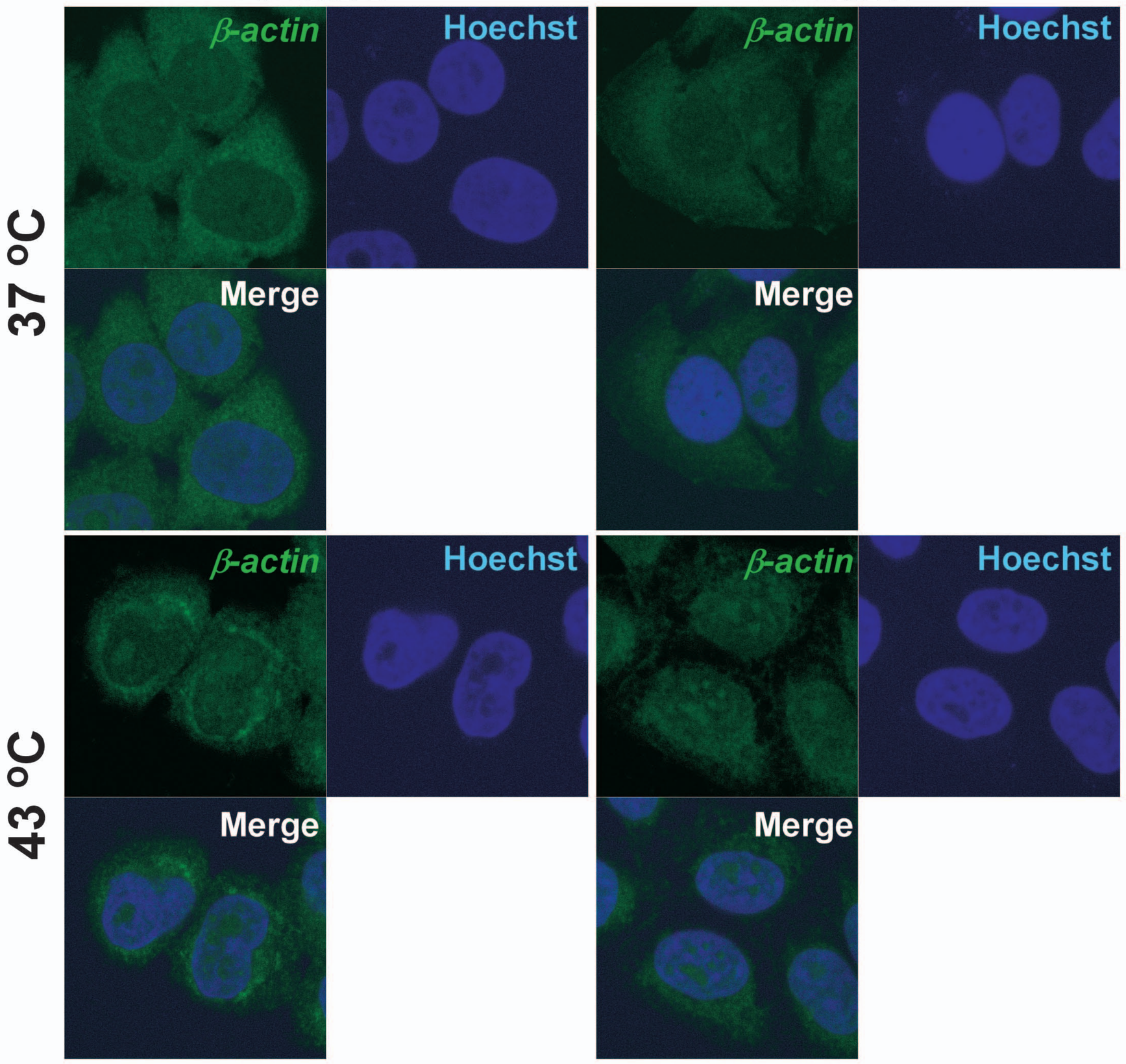
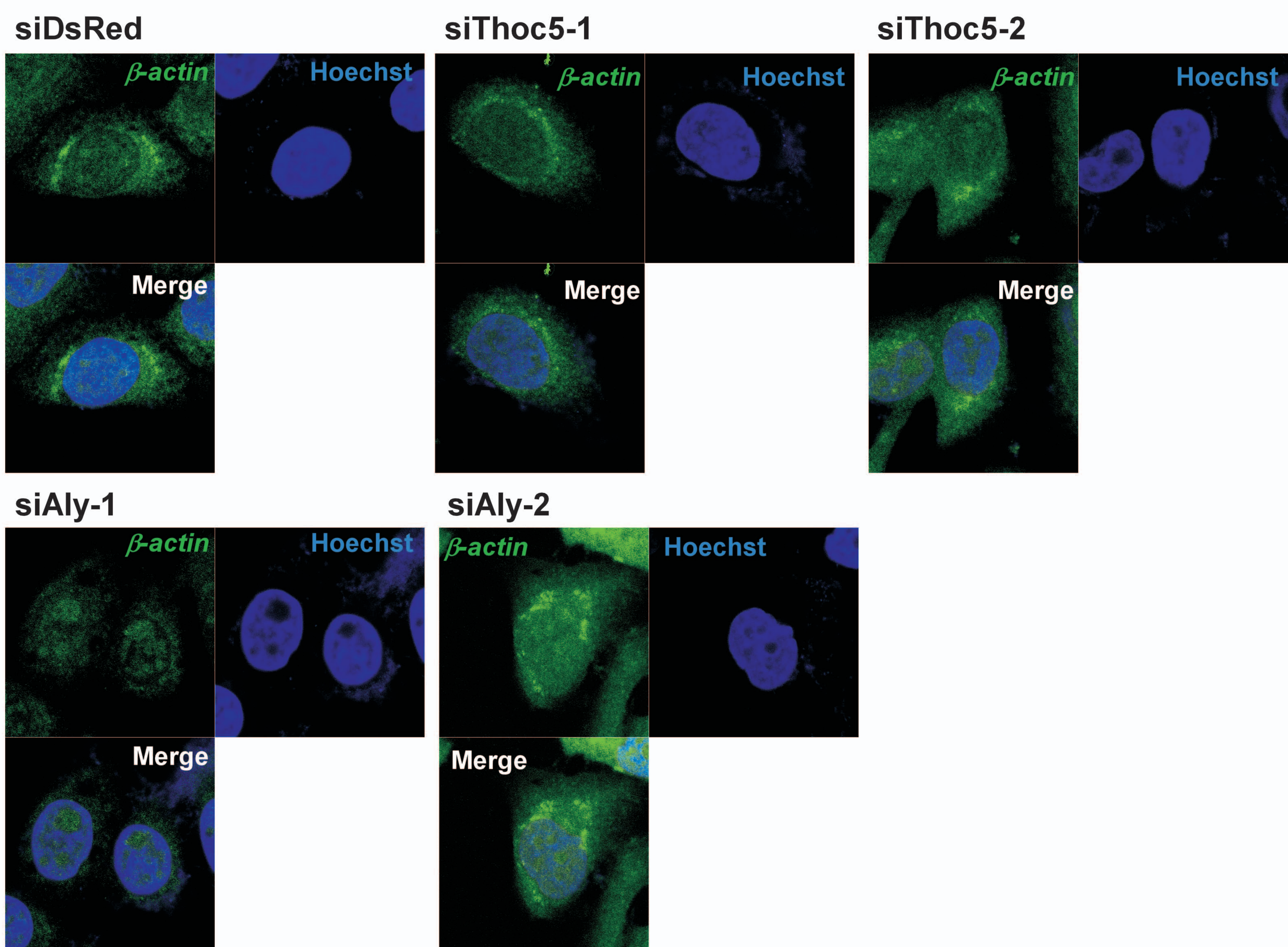
Hoechst
Merge
siAly-1
HSP70 mRNA
Hoechst
Merge

D


RNase -
RNase +
37 °C
43 °C
β-actin
Hoechst
Merge

E


siDsRed
siThoc5-1
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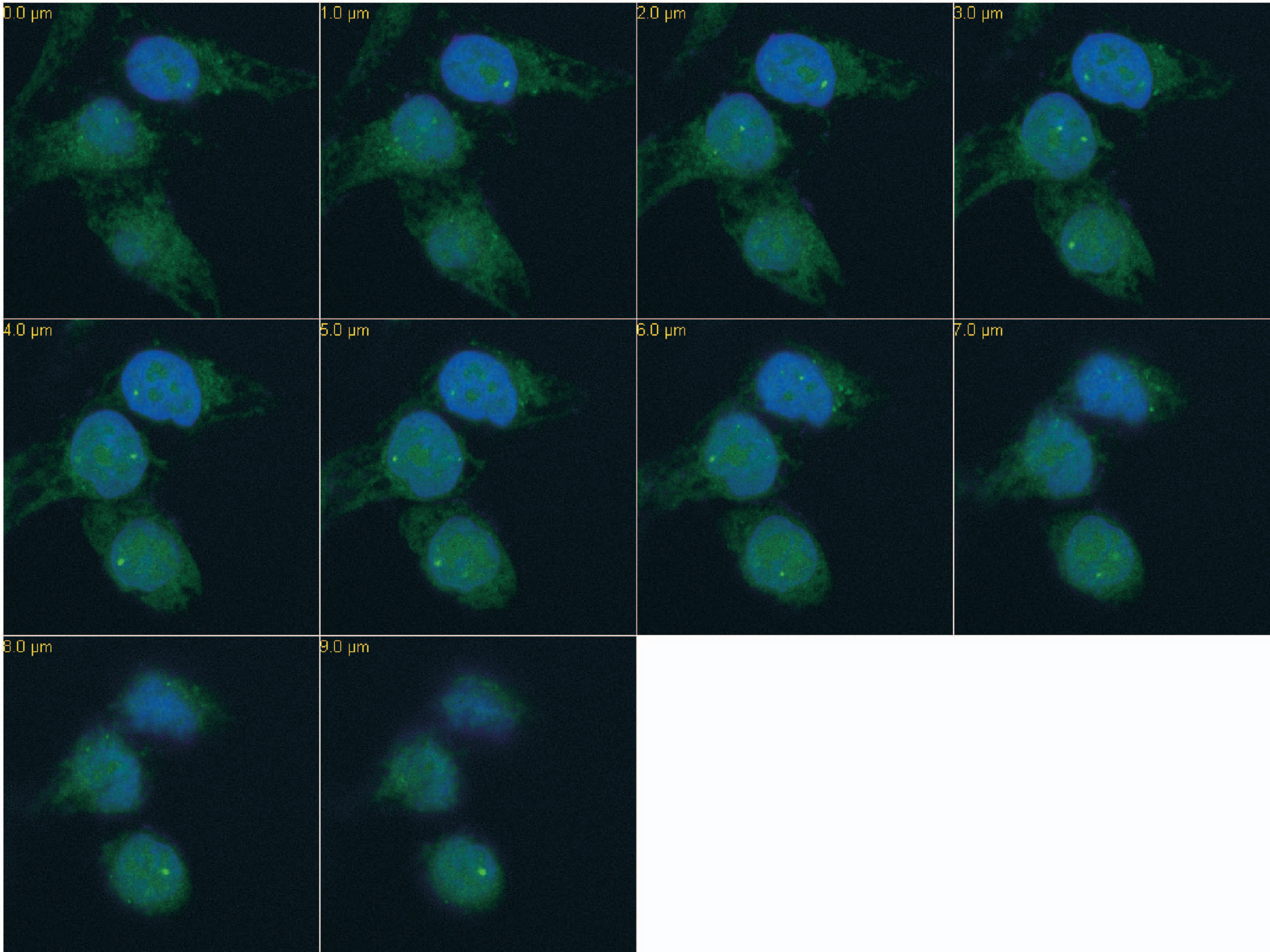
siAly-1
siAly-2
β-actin
Hoechst
Merge

Katahira_supplementary
Fig.S5

siDsRed



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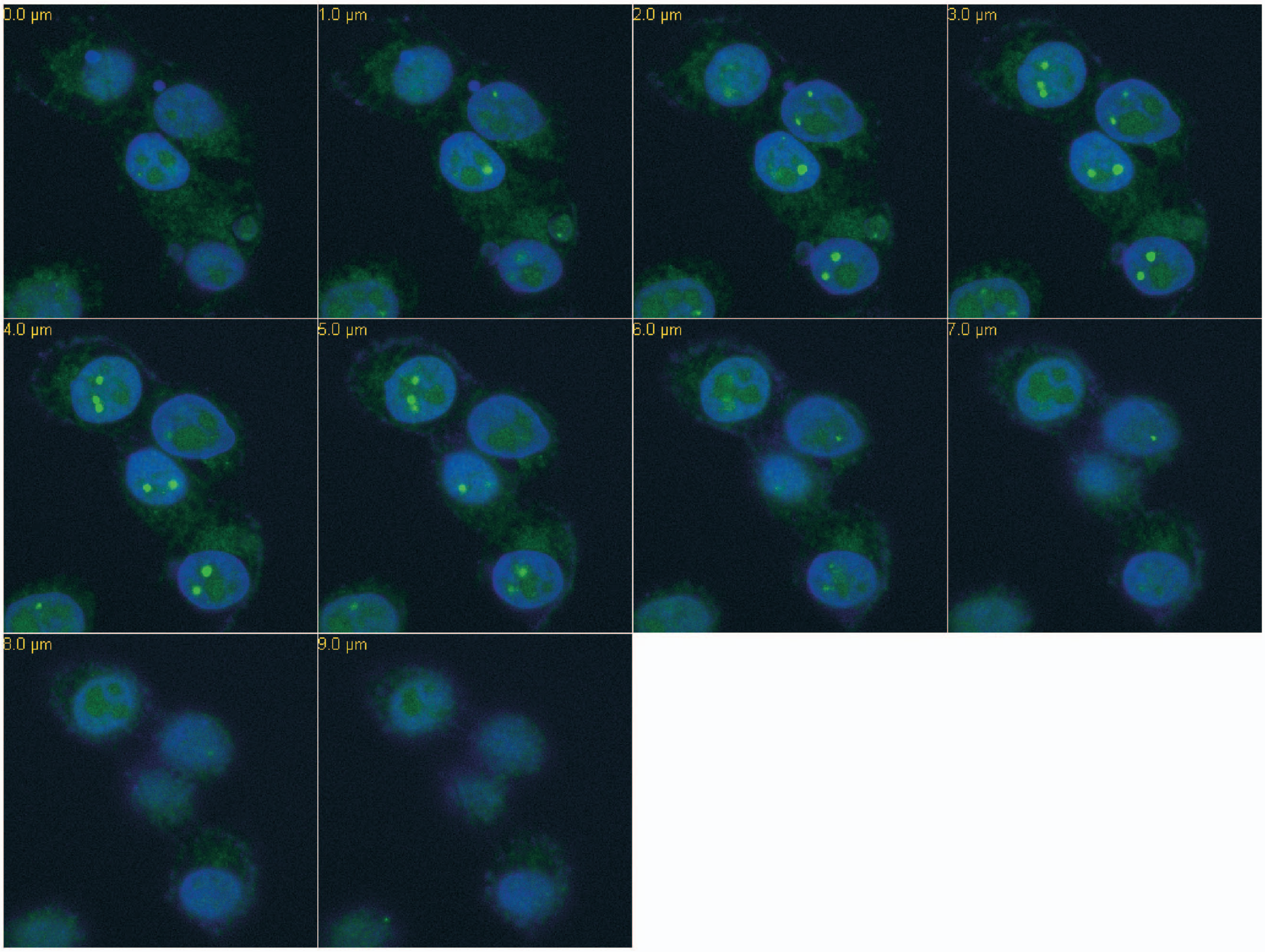
siAly-1



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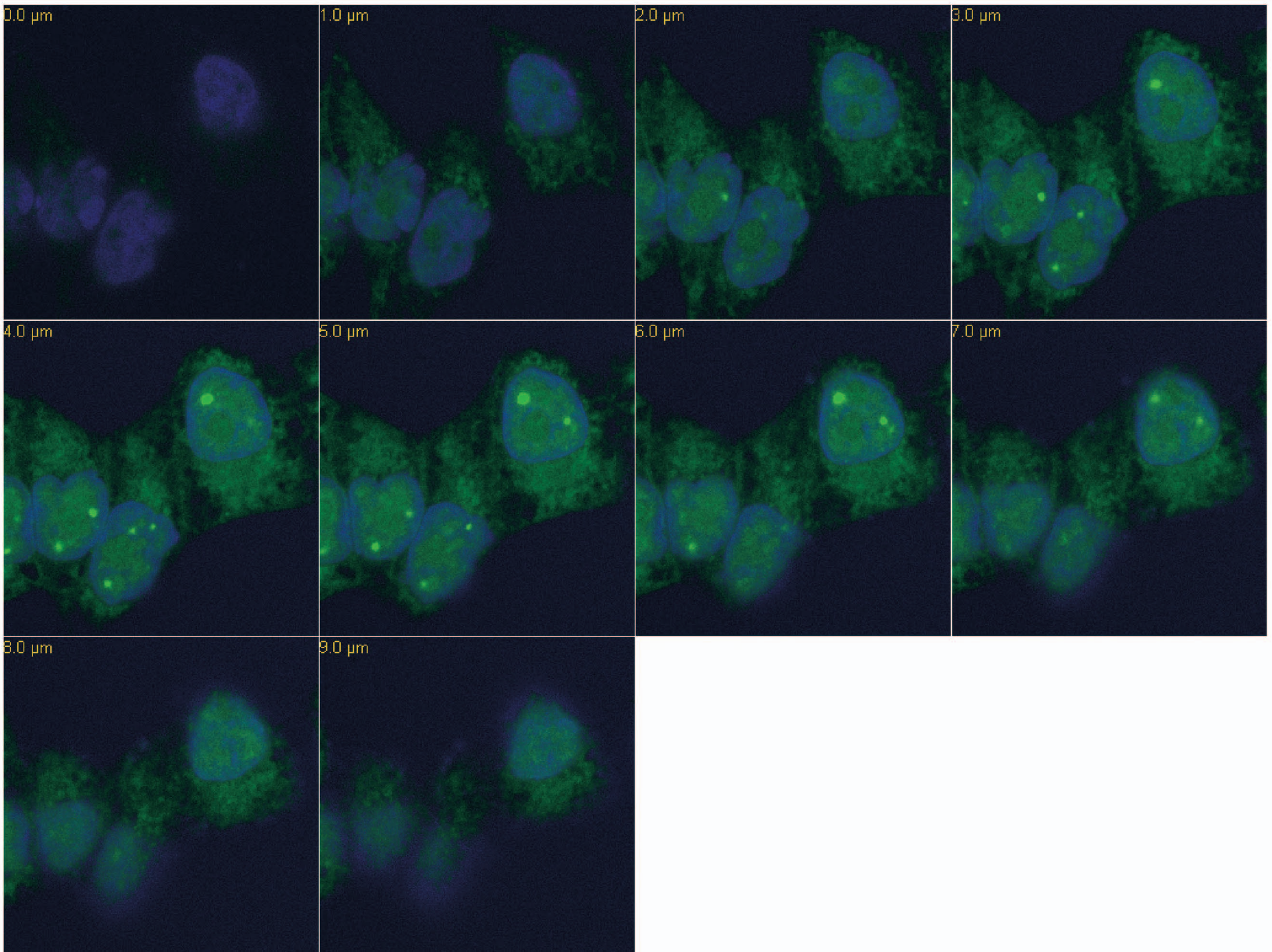

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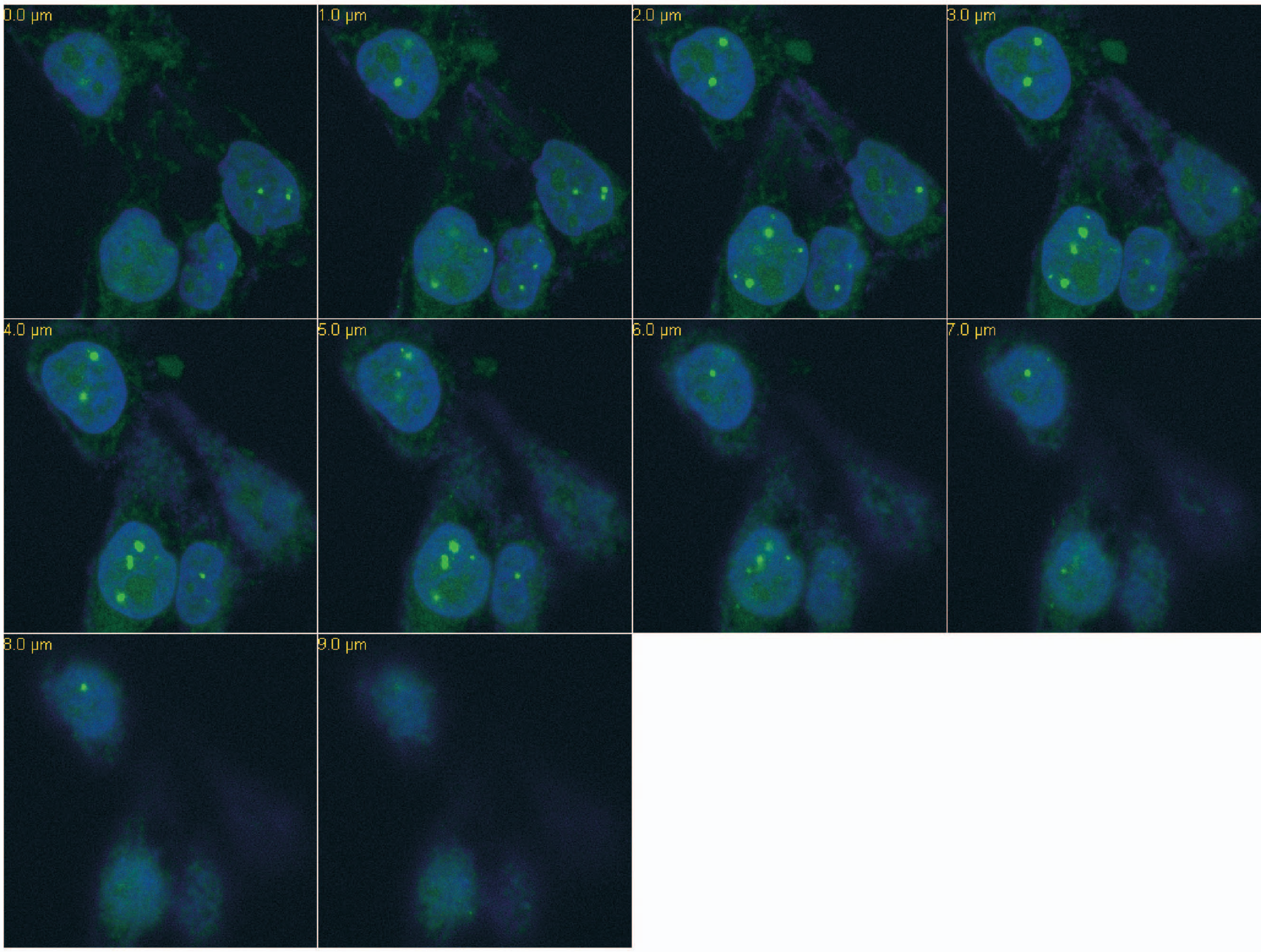

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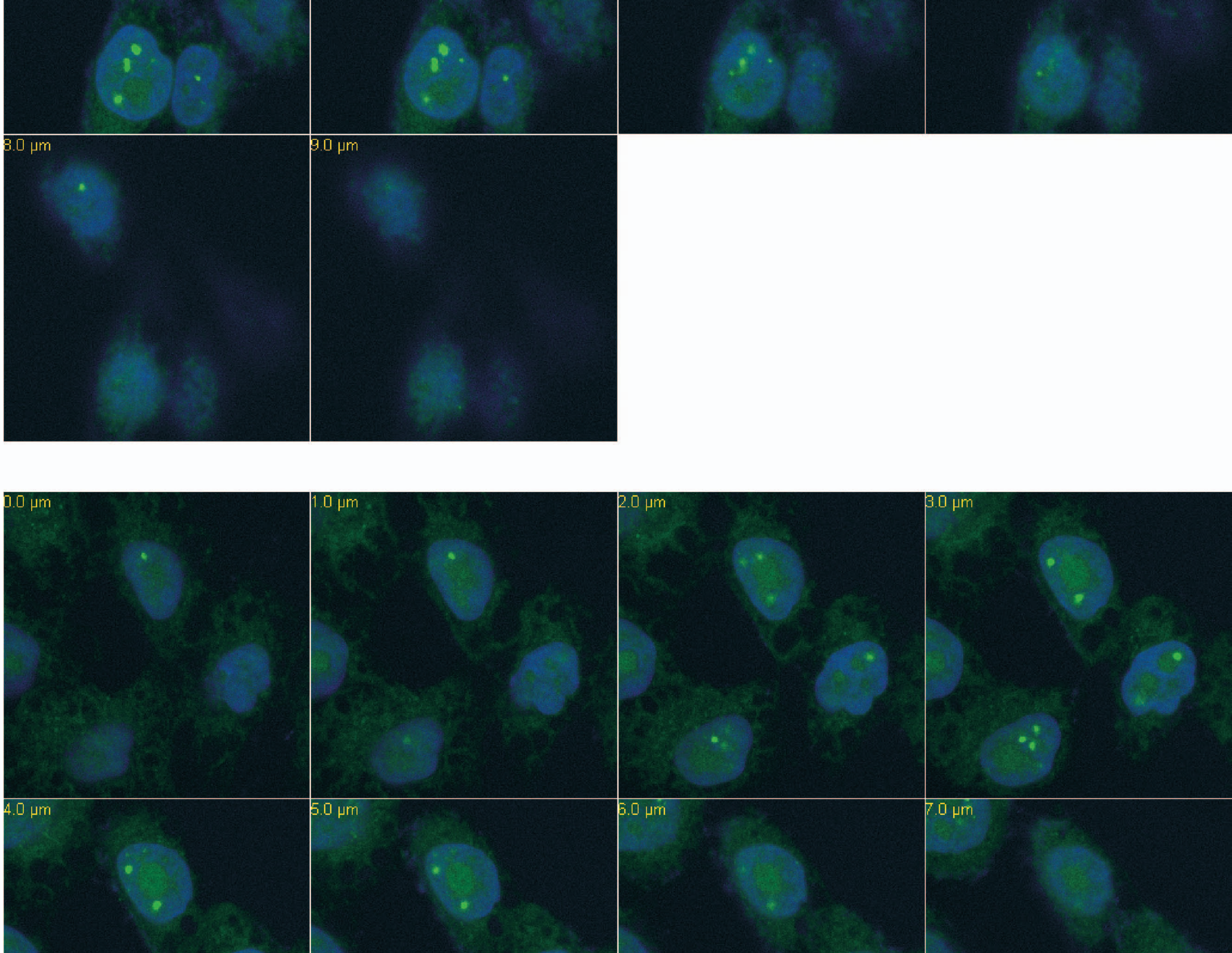

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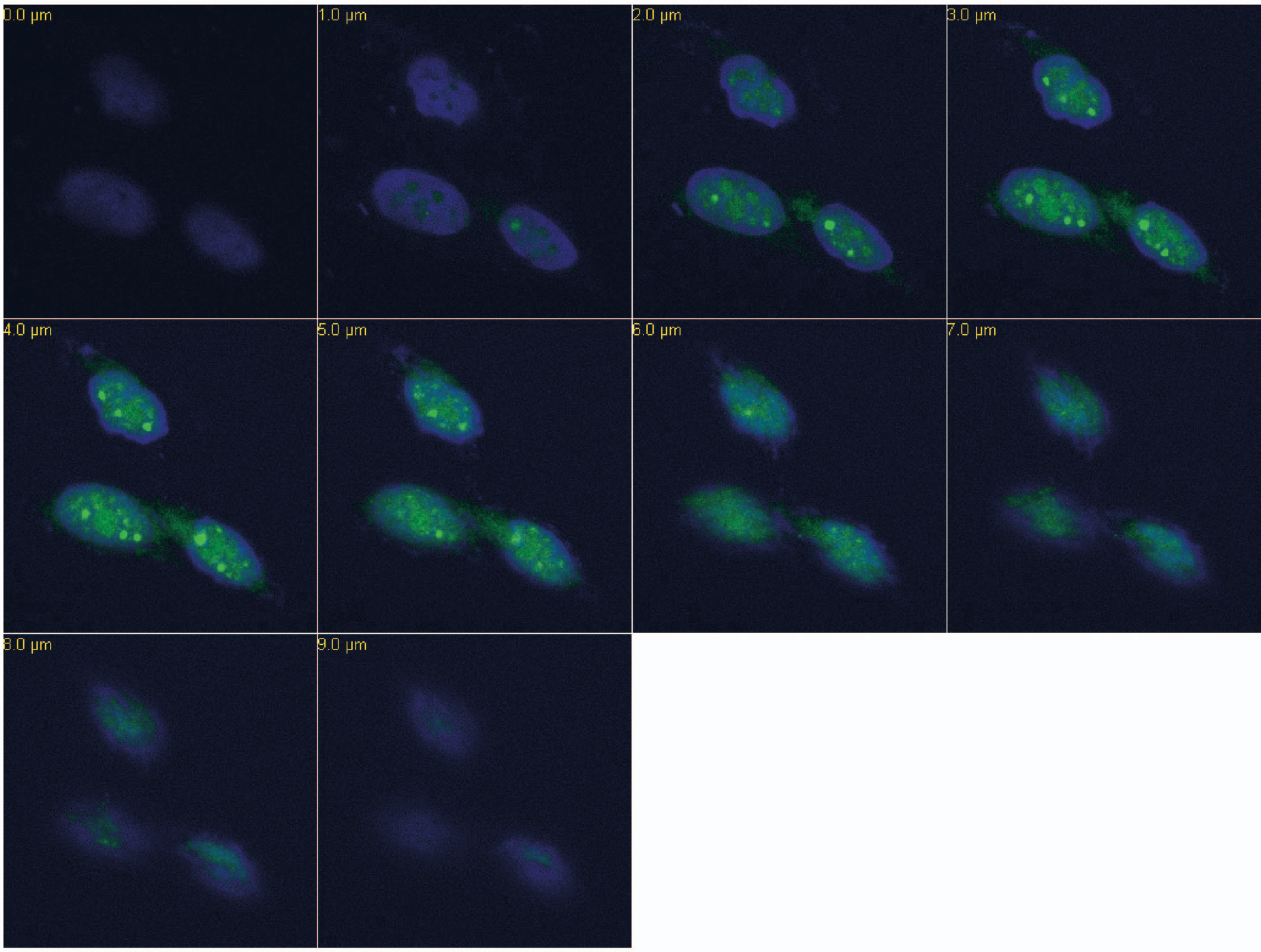
Fig.S5

siTap-1



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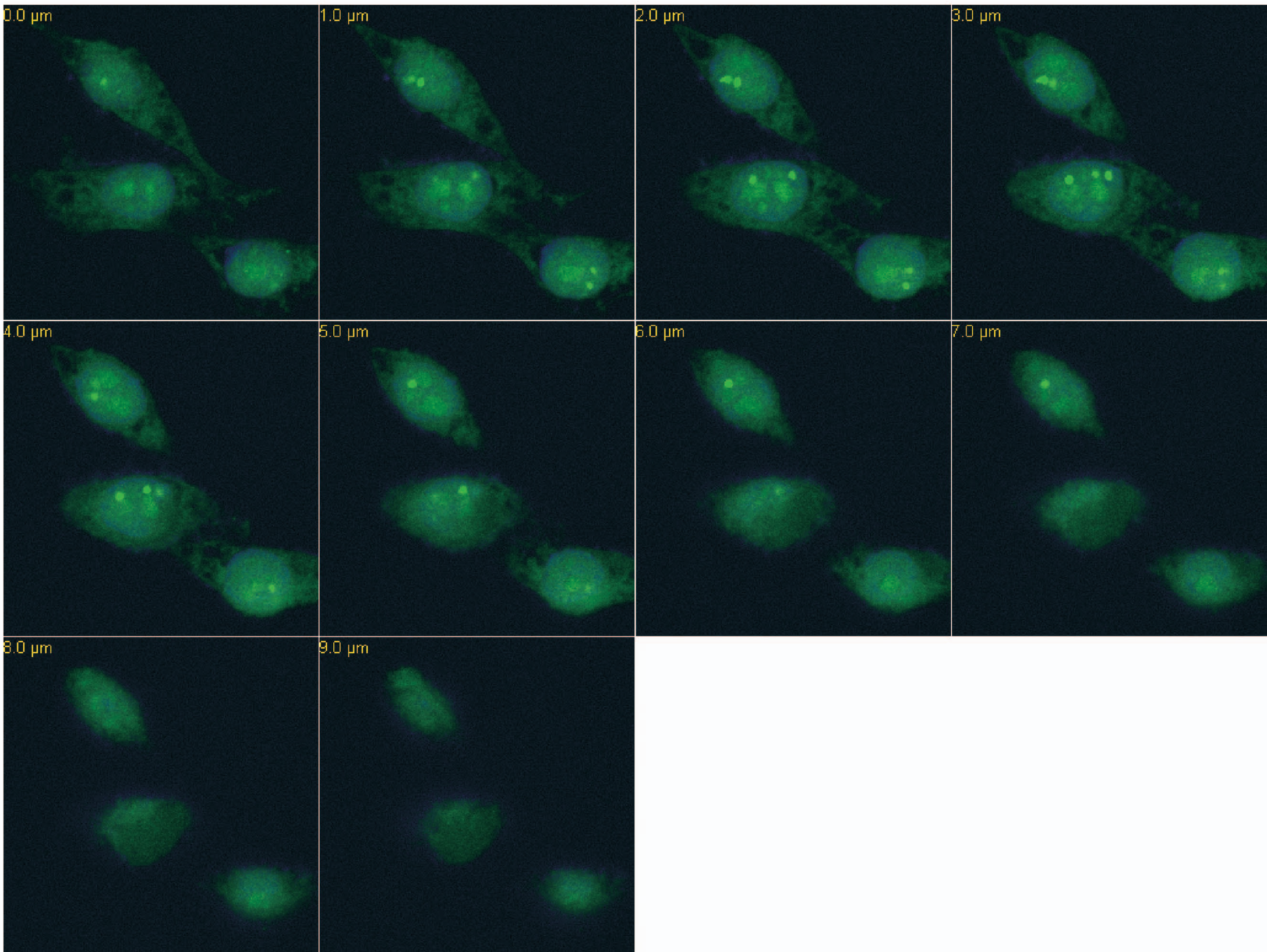

Katahira_supplementary Fig.S6

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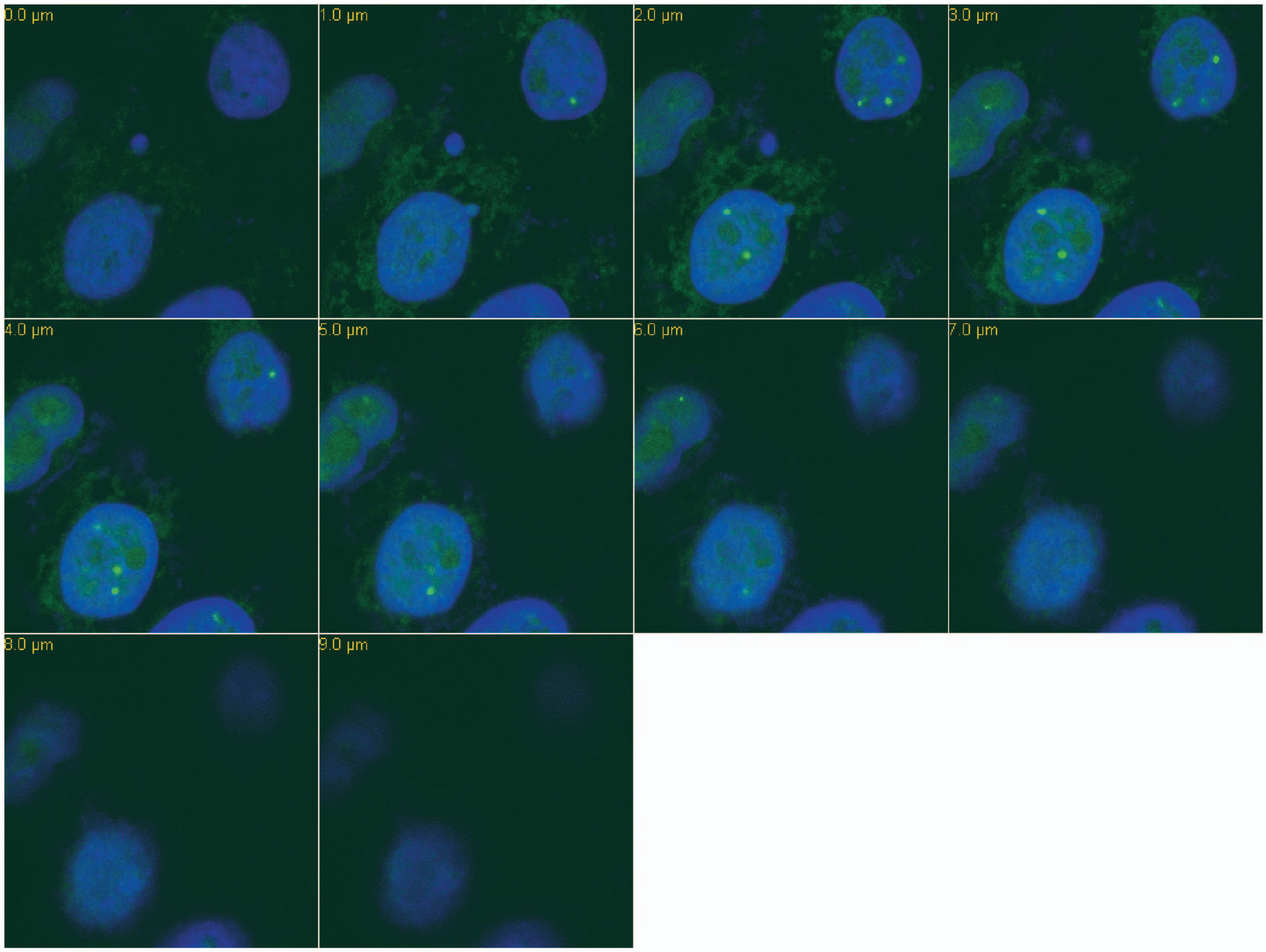

siThoc6-2