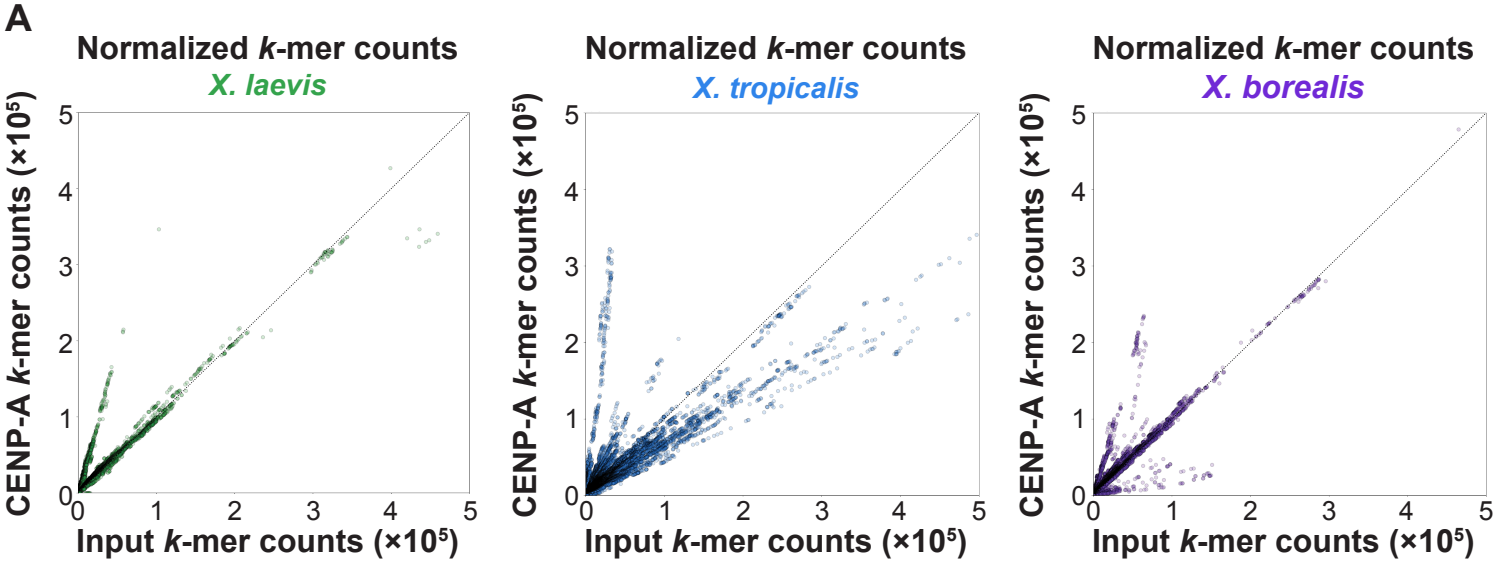
A


Normalized k-mer counts
X. laevis
Normalized k-mer counts
X. tropicalis
Normalized k-mer counts
X. borealis
CENP-A k-mer counts (×10⁵)
Input k-mer counts (×10⁵)
0
1
2
3
4
5

B

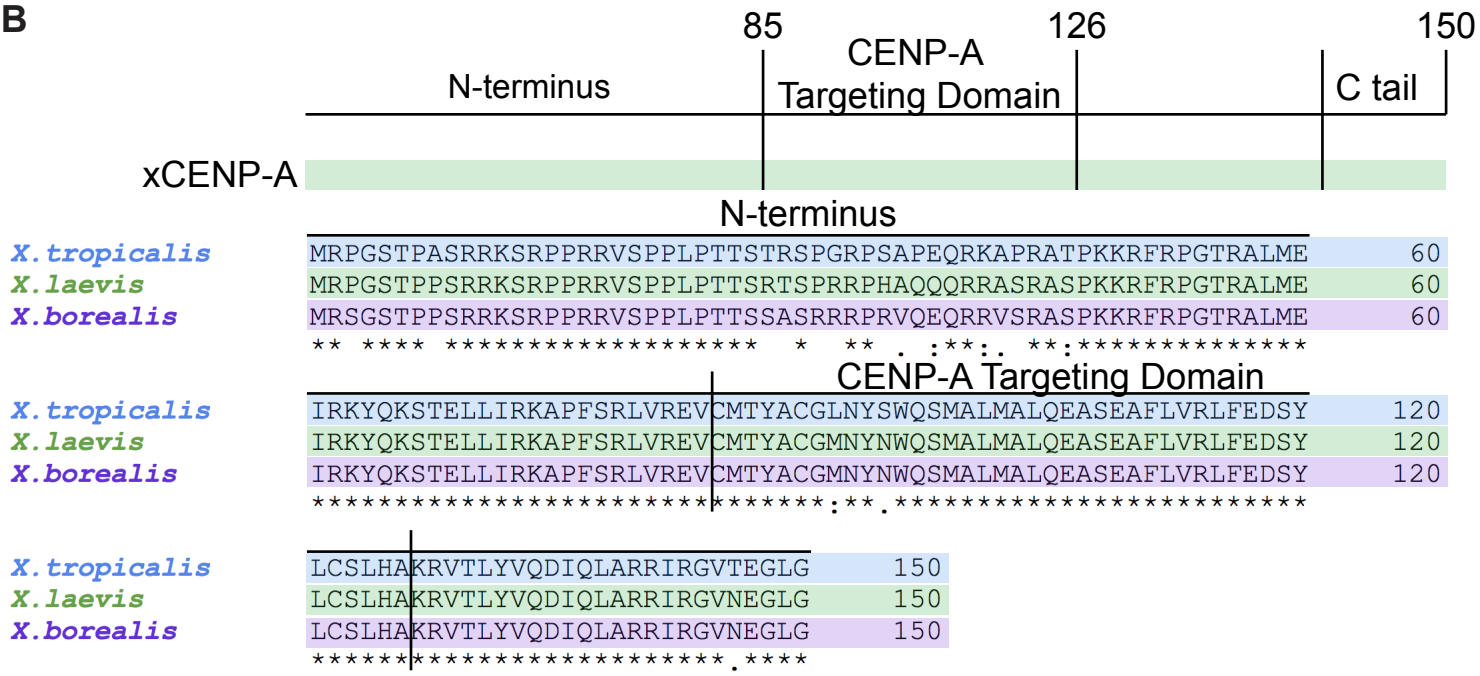

85
126
150
N-terminus
CENP-A Targeting Domain
C tail
xCENP-A
N-terminus
X.tropicalis MRPGSTPASRRKSRPPRRVSPPLPTTSTRSPGRPSAPEQRKAPRATPKKRFRPGTRALME 60
X.laevis MRPGSTPPSRRKSRPPRRVSPPLPTTSRTSPRRPHAQQQRRASRASPKKRFRPGTRALME 60
X.borealis MRSGSTPPSRRKSRPPRRVSPPLPTTSSASRRRPRVQEQRRVSRASPKKRFRPGTRALME 60
CENP-A Targeting Domain
X.tropicalis IRKYQKSTELLIRKAPFSRLVREVCMTYACGLNYSWQSMALMALQEASEAFLVRLFEDSY 120
X.laevis IRKYQKSTELLIRKAPFSRLVREVCMTYACGMNYNWQSMALMALQEASEAFLVRLFEDSY 120
X.borealis IRKYQKSTELLIRKAPFSRLVREVCMTYACGMNYNWQSMALMALQEASEAFLVRLFEDSY 120
X.tropicalis LCSLHAKRVTLYVQDIQLARRIRGVTEGLG 150
X.laevis LCSLHAKRVTLYVQDIQLARRIRGVNEGLG 150
X.borealis LCSLHAKRVTLYVQDIQLARRIRGVNEGLG 150

Figure S1: Comparison of centromere DNA and CENP-A protein sequences, Related to Figure 1

(**A**) Scatter plots of normalized *k*-mer counts from Input and CENP-A ChIP-seq sequencing libraries from *X. laevis*, *X. tropicalis*, and *X. borealis*. The dotted line (x=y) indicates *k*-mers that are equally abundant in both libraries. *k*-mer counts reveal distinct patterns in the three species.

(**B**) Protein sequence alignment comparing CENP-A across the three *Xenopus* species. Differences are observed in the N-terminal region and the CENP-A targeting domain (CATD).

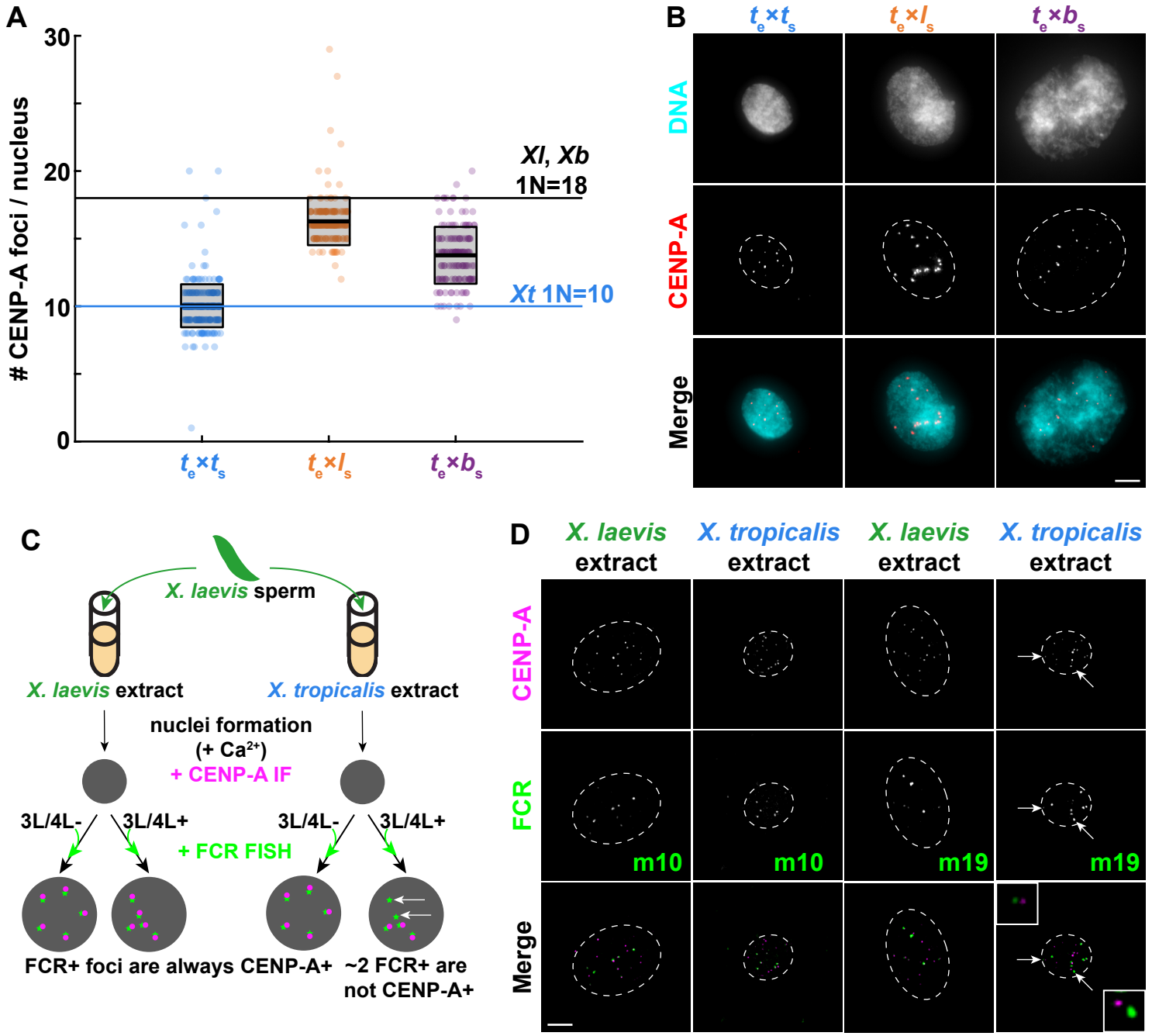

A
CENP-A foci / nucleus
30
20
10
0
Xl, Xb
1N=18
Xt 1N=10
te×ts
te×ls
te×bs
B
te×ts
te×ls
te×bs
DNA
CENP-A
Merge
C
X. laevis sperm
X. laevis extract
X. tropicalis extract
nuclei formation
(+ Ca2+)
+ CENP-A IF
3L/4L-
3L/4L+
3L/4L-
3L/4L+
+ FCR FISH
FCR+ foci are always CENP-A+
~2 FCR+ are
not CENP-A+
D
X. laevis
extract
X. tropicalis
extract
X. laevis
extract
X. tropicalis
extract
CENP-A
FCR
m10
m10
m19
m19
Merge
E
foci / X. laevis nucleus
30
20
10
0
ns
ns
ns
ns
ns
***
ns
***
Xl ext.
Xt ext.
Xl ext.
Xt ext.
Xl ext.
Xt ext.
Xl ext.
Xt ext.
FCR m4
FCR m10
FCR m16
FCR m19
Chr. 3L/4L-
Chr. 3L/4L+

Figure S2: CENP-A is lost from *X. laevis* chromosomes 3L and 4L, Related to Figure 2

(**A**) Quantification of the number of CENP-A foci in interphase nuclei assembled in *X. tropicalis* egg extract. Whereas *X. tropicalis* nuclei on average possess 10 foci corresponding to the 10 sperm chromosomes, *X. laevis* and *X. borealis* interphase nuclei possess an average of 16 and 14 CENP-A foci, respectively, which does not correspond to the 18 sperm chromosomes of these two species. Note that nuclei assembled around sperm chromosomes in egg extract do not always contain a single set of chromosomes, even in control reactions, so averages must be calculated. Quantification with N = 3 extracts, N > 64 nuclei per extract.

(**B**) Representative images of *X. tropicalis*, *X. laevis* and *X. borealis* nuclei formed in *X. tropicalis* extract. DNA in cyan, CENP-A in red. Scale bar is 5 μm.

(**C**) Experimental schematic for specific centromere quantification. *X. laevis* sperm nuclei were cycled into interphase in either *X. laevis* or *X. tropicalis* egg extract. All centromeres were detected by CENP-A immunofluorescence and a subset of centromeres were identified by FCR (frog centromeric repeat) FISH (Smith et al., 2021). Probes prepared from two sequences not present in centromeres of chromosomes 3L or 4L (3L/4L- = m4, m10), were compared with probes made using two sequences present in centromeres of chromosomes 3L and 4L (3L/4L+ = m16, m19). m16 and m19 recognize ~60% of *X. laevis* chromosomes, and are not specific solely to 3L and 4L. In *X. laevis* extract, all FCR+ foci should co-localize with CENP-A, as 18/18 centromeres are maintained. If centromeric CENP-A staining is lost specifically from chromosomes 3L and 4L in *X. tropicalis* extract, 2 3L/4L+ FCR+ foci should not colocalize with CENP-A (panel A). This experiment was

performed with interphase nuclei to infer chromosome identity, since mitotic chromosomes formed in egg extracts cannot withstand the FISH protocol.

(**D**) Images of *X. laevis* sperm nuclei formed in *X. laevis* or *X. tropicalis* extract probed by FISH for FCR monomer m10 or m19 (green) and by immunofluorescence of CENP-A (magenta). Insets show the 2 m19 FCR+ foci not co-localized with CENP-A, while all other FCR+ foci co-localize with CENP-A. DNA periphery is marked by the dashed white lines. Scale bar is 5 μm.

(**E**) Quantification of CENP-A foci that co-localize with FCR+ foci in *X. laevis* vs. *X. tropicalis* extract. In *X. laevis* extract, all FCR+ foci co-localize with CENP-A. However, in *X. tropicalis* extract, ~2 m16 or m19 FCR+ foci do not co-localize with CENP-A, corresponding to the loss of CENP-A localization on chromosomes 3L and 4L. Quantification with N = 2 extracts, N > 50 nuclei and > 800 centromeres per probe per extract. p-values by two-tailed two-sample unequal variance t-tests (left to right): 0. 3562, 0.0916, 0.3708, 0.0499, 0.2426, 2.797e-19, 0.5485, 7.972e-13; ns, not significant. Open circles are FCR+ foci, filled circles represent foci that are both FCR+ and CENP-A+.

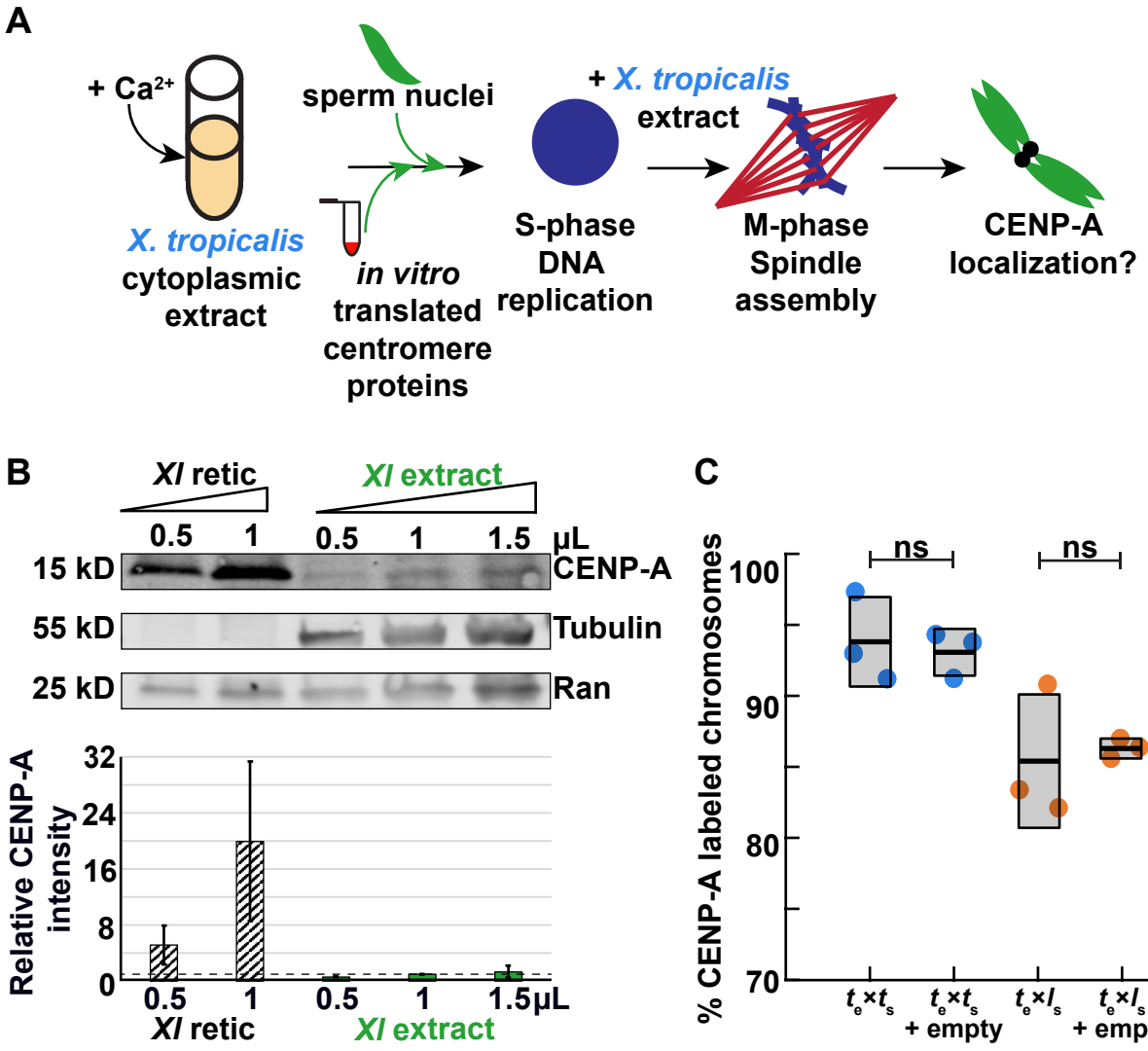

A
+ Ca2+
X. tropicalis
cytoplasmic
extract
sperm nuclei
in vitro
translated
centromere
proteins
S-phase
DNA
replication
+ X. tropicalis
extract
M-phase
Spindle
assembly
CENP-A
localization?
B
Xl retic
Xl extract
0.5
1
0.5
1
1.5
µL
15 kD
CENP-A
55 kD
Tubulin
25 kD
Ran
Relative CENP-A
intensity
32
24
16
8
0
0.5
1
0.5
1
1.5µL
Xl retic
Xl extract
C
% CENP-A labeled chromosomes
100
90
80
70
ns
ns
te×ts
te×ts
+ empty
te×ls
te×ls
+ empty

Figure S3: Driving CENP-A assembly with proteins expressed in reticulocyte lysate, Related to Figure 3

(**A**) Experimental schematic of extract reactions in which reticulocyte lysate is added at the onset of interphase to mimic the timing of CENP-A deposition in G1.

(**B**) Representative Western blot of *X. laevis* CENP-A protein expressed in reticulocyte lysate and quantification of three blots showing band intensity normalized to CENP-A levels in 1 µL of *X. laevis* egg extract (dotted line). CENP-A is approximately twenty times more concentrated in reticulocyte lysate compared to *X. laevis* extract, with amounts added to chromosome/nuclear assembly reactions corresponding to 8 or 80 times endogenous CENP-A levels.

(**C**) Percentage of replicated *X. laevis* or *X. tropicalis* chromosomes with CENP-A staining in *X. tropicalis* extract supplemented with unprogrammed reticulocyte lysate. Lysates containing empty expression vectors have no effect on centromere staining. Quantification from N = 3 extracts, N > 298 chromosomes per extract. p-values (left to right) by two-tailed two-sample unequal variance t-tests: 0.7433, 0.7755; ns, not significant.

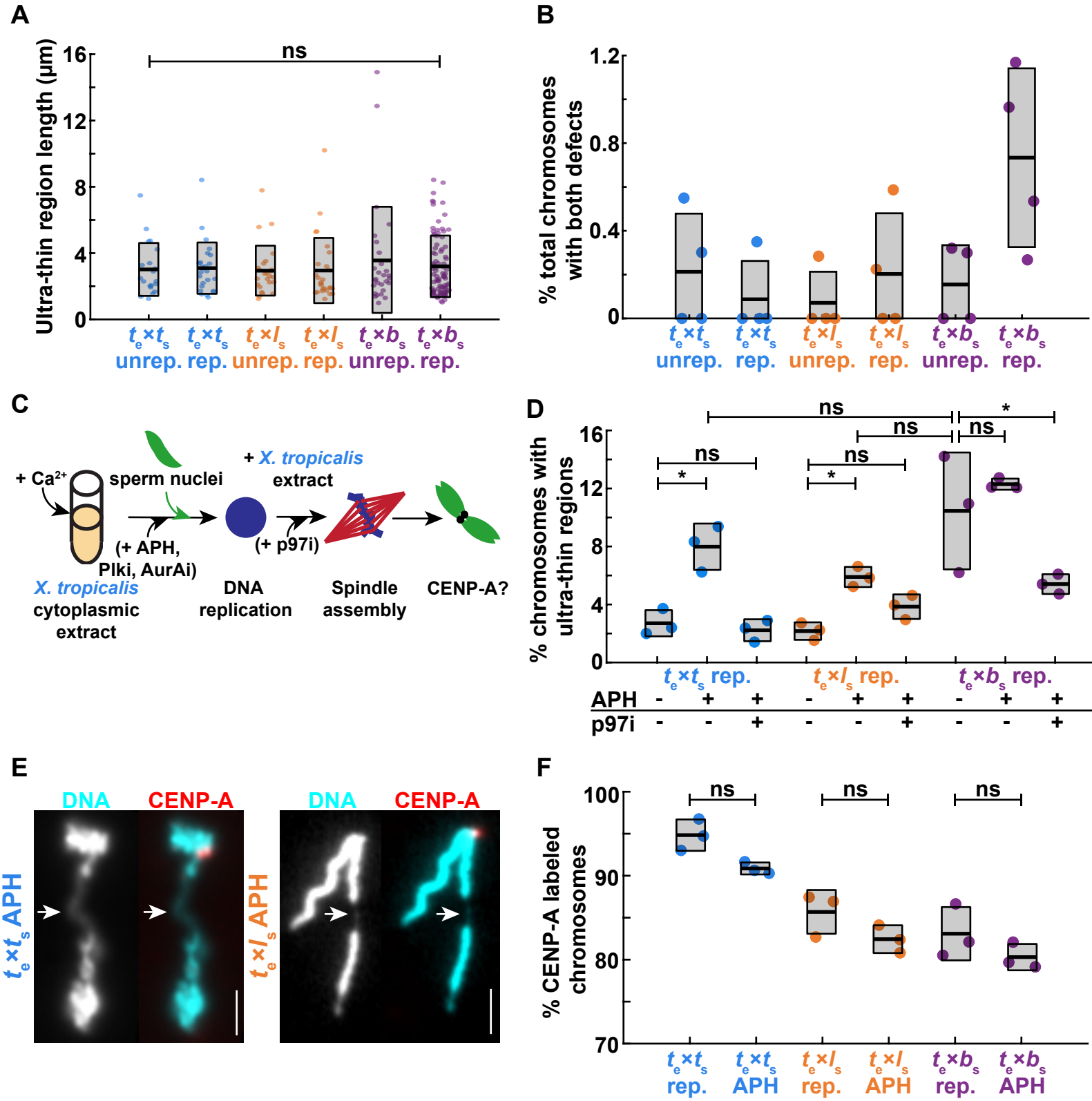

A
Ultra-thin region length (µm)
16
12
8
4
0
ns
$t_e \times t_s$ unrep.
$t_e \times t_s$ rep.
$t_e \times l_s$ unrep.
$t_e \times l_s$ rep.
$t_e \times b_s$ unrep.
$t_e \times b_s$ rep.
B
% total chromosomes with both defects
1.2
0.8
0.4
0
C
+ Ca^{2+}
sperm nuclei
+ X. tropicalis extract
(+ APH, Plki, AurAi)
(+ p97i)
X. tropicalis cytoplasmic extract
DNA replication
Spindle assembly
CENP-A?
D
% chromosomes with ultra-thin regions
16
12
8
4
0
ns
*
$t_e \times t_s$ rep.
$t_e \times l_s$ rep.
$t_e \times b_s$ rep.
APH
p97i
E
DNA
CENP-A
$t_e \times t_s$ APH
$t_e \times l_s$ APH
F
% CENP-A labeled chromosomes
100
90
80
70
$t_e \times t_s$ rep.
$t_e \times t_s$ APH
$t_e \times l_s$ rep.
$t_e \times l_s$ APH
$t_e \times b_s$ rep.
$t_e \times b_s$ APH

Figure S4: Characterization of chromosome morphology defects that can be induced by aphidicolin and rescued by p97 inhibition, Related to Figure 4

(**A**) Quantification of ultra-thin region lengths, which average ~2-3 μm on mitotic chromosomes of all three *Xenopus* species. p-value by one-way ANOVA = 0.8712.

(**B**) Percentage of *X. tropicalis*, *X. laevis*, and *X. borealis* chromosomes with ultra-thin regions that have also lost CENP-A staining. Only a small fraction of chromosomes with ultra-thin regions also show centromere loss. Across all species, only ~0.2-0.6% of all chromosomes exhibit both morphological and centromere defects, corresponding to 1-4 chromosomes out of ~350 total chromosomes per extract.

(**C**) Experimental schematic illustrating when inhibitors are added to *X. tropicalis* extract reactions.

(**D**) Percentage of mitotic chromosomes with ultrathin regions in *X. tropicalis* extracts treated with solvent control or 10 μg/mL aphidicolin (APH) to inhibit DNA replication, and with or without 10 μM p97 ATPase inhibitor NMS-873 (p97i) to prevent removal of stalled replication forks. Aphidicolin increased the prevalence of ultra-thin chromosome regions on *X. tropicalis* and *X. laevis* chromosomes, but did not significantly exacerbate these regions on *X. borealis* chromosomes. Inhibition of p97 rescued the chromosome morphology defects. Quantification from N = 3 extracts, N > 138 chromosomes per extract. p-values (top to bottom, then left to right) by two-tailed two-sample unequal variance t-tests: 0.6106, 0.0217, 0.9986, 0.8708, 0.9999, 0.9159, 0.0151, 0.0023.

(**E**) Representative images of *X. tropicalis* and *X. laevis* mitotic chromosomes following aphidicolin treatment. DNA in cyan, CENP-A in red. Scale bar is 5 μm.

(**F**) Percentage of replicated chromosomes with centromeric CENP-A staining in *X. tropicalis* extracts treated with solvent control or 10 µg/mL aphidicolin. Inhibition of DNA replication does not affect centromere formation on any species' chromosomes. p-values (left to right) by two-tailed two-sample unequal variance t-tests: 0.0523, 0.1554, 0.2679.

A, B: N = 3 extracts, N > 20 chromosomes per condition.

D, F: N = 3 extracts, N > 150 chromosomes per extract.

A, D, F: ns, not significant.

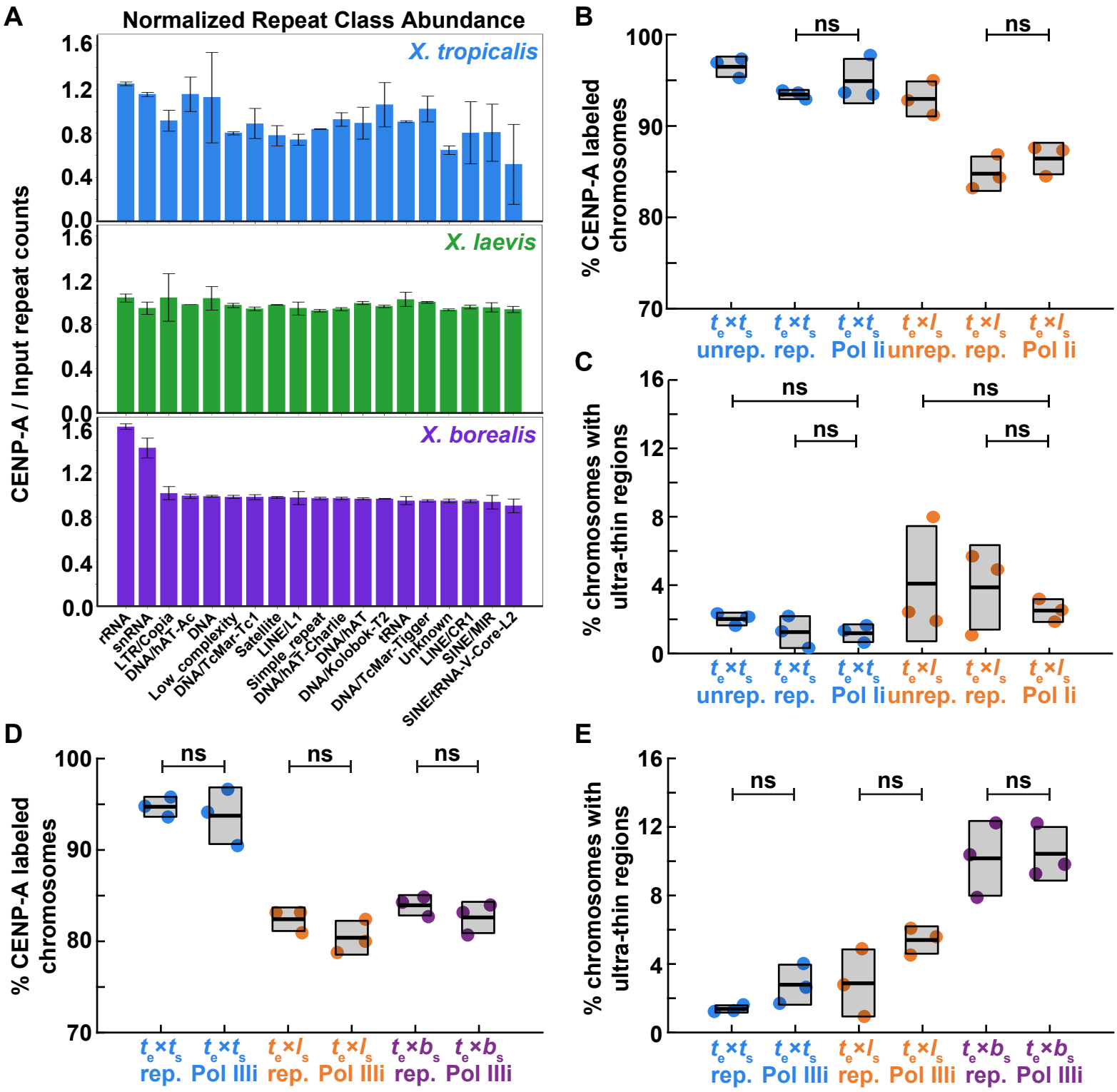

A
Normalized Repeat Class Abundance
X. tropicalis
X. laevis
X. borealis
CENP-A / Input repeat counts
rRNA
snRNA
LTR/Copia
DNA/hAT-Ac
DNA
Low_complexity
DNA/TcMar-Tc1
Satellite
LINE/L1
Simple_repeat
DNA/hAT-Charlie
DNA/hAT
DNA/Kolobok-T2
tRNA
DNA/TcMar-Tigger
Unknown
LINE/CR1
SINE/MIR
SINE/tRNA-V-Core-L2
B
% CENP-A labeled chromosomes
ns
unrep.
rep.
Pol Ii
C
% chromosomes with ultra-thin regions
D
% CENP-A labeled chromosomes
Pol IIIi
E
% chromosomes with ultra-thin regions

Figure S5: Pol I transcription inhibition does not affect *X. tropicalis* or *X. laevis* chromosomes, while Pol III inhibition had no effect on any species, Related to Figure 5

(**A**) Bar plots showing the makeup of repeat classes in the core centromere (CENP-A-associated) sequences normalized to the input sequences of each species. Mean and standard deviation of CENP-A/Input counts from 3 one million read subsets are shown. Only repeat classes found more than 25 times per one million reads in the CENP-A dataset are plotted.

(**B**, **C**) The percentage of *X. tropicalis* or *X. laevis* mitotic chromosomes formed in *X. tropicalis* egg extract with centromeric CENP-A staining (B) or ultrathin regions (C) is unchanged upon treatment with 1 μM BMH-21 to inhibit RNA Pol I (Pol Ii). Quantification with N = 3 extracts, N > 113 chromosomes per extract. p-values by one-way ANOVA with Tukey post-hoc analysis: (B, left to right) 0.9702, 0.9413, (C, left to right, then top to bottom) 0.9995, 0.9711, 1, 0.9882.

(**D**, **E**) The percentage of *X. tropicalis*, *X. laevis*, or *X. borealis* mitotic chromosomes formed in *X. tropicalis* egg extract with centromeric CENP-A staining (D) or ultrathin regions (E) is unchanged upon treatment with 20 μM ML-69218 to inhibit RNA Pol III (Pol IIIi). Quantification with N = 3 extracts, N > 179 chromosomes per extract. p-values by one-way ANOVA with Tukey post-hoc analysis: (D, left to right) 0.9389, 0.7506, 0.9416, (E, left to right) 0.8431, 0.3540, 0.9999.

B-E: ns, not significant.

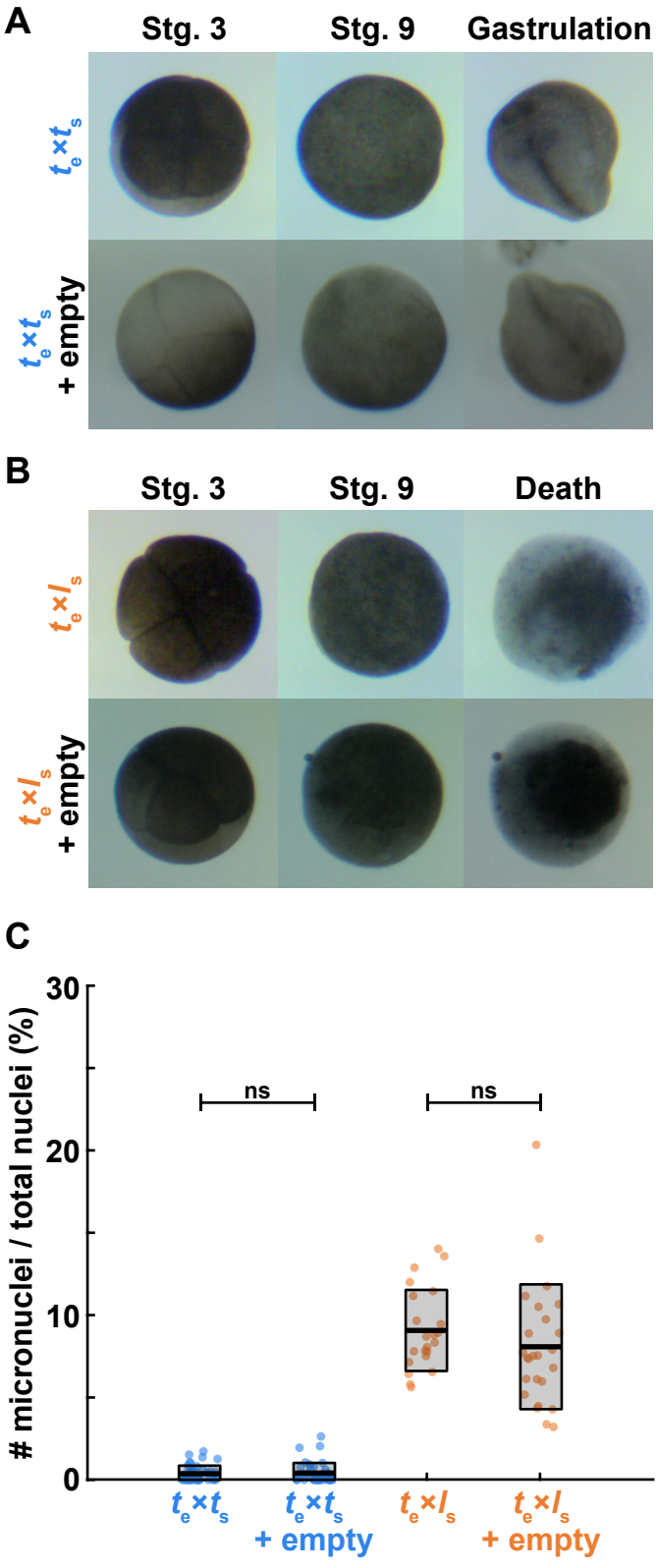

A
Stg. 3
Stg. 9
Gastrulation
$t_e \times t_s$
$t_e \times t_s$ + empty
B
Stg. 3
Stg. 9
Death
$t_e \times l_s$
$t_e \times l_s$ + empty
C
micronuclei / total nuclei (%)
30
20
10
0
ns
ns
$t_e \times t_s$
$t_e \times t_s$ + empty
$t_e \times l_s$
$t_e \times l_s$ + empty

Figure S6: Microinjection with reticulocyte lysate does not affect embryo development or chromosome segregation, Related to Figure 6

(**A**) Video frames of untreated *X. tropicalis* embryos and embryos microinjected at the two-cell stage with empty reticulocyte lysate into both blastomeres show that embryonic development is not affected by the procedure. N = 15 embryos across 3 clutches. Scale is 200 μm.

(**B**) Video frames of untreated *X. tropicalis/X. laevis* hybrid embryos and hybrid embryos microinjected at the two-cell stage with empty reticulocyte lysate into both blastomeres show that the embryonic death phenotype is not affected by the procedure. N = 12 embryos across 2 clutches. Scale is 200 μm.

(**C**) Quantification of the number of micronuclei compared to total nuclei in stage 9 *X. tropicalis* embryos or *X. tropicalis*/*X. laevis* hybrid embryos microinjected with empty reticulocyte lysate. The prevalence of micronuclei is unaffected by the procedure. p-values (left to right) by two-tailed two-sample unequal variance t-tests: 0.749, 0.288; ns, not significant.