Replication dependent instability at (CTG)•(CAG) repeat hairpins in human cells

Guoqi Liu, Xiaomi Chen, John J. Bissler, Richard R. Sinden, and Michael Leffak

Supplementary Results.

Supplementary Figure 1. Targeted integration of $(CTG)_n$•$(CAG)_n$ TNRs. (a) The HeLa cell acceptor subline HeLa/406 contains a single copy of the Flp recombinase target plasmid pHyg.FRT.TK. Hyg, hygromycin resistance gene; TK, HSV-TK gene; unfilled rectangles, vector. Donor plasmids (shown linearized) contain a G418 resistance gene (Neo) with its promoter replaced by the FRT, and the 2.4 kb Hind III/Xho I fragment of the c-myc replicator (pFRT.myc) alongside $(CTG)_{12}$•$(CAG)_{12}$ or $(CTG)_{102}$•$(CAG)_{102}$ plus 19 bp of 5' genomic flanking DNA and 16 bp of 3' genomic flanking DNA (denoted $(CTG)_n$•$(CAG)_n$). pOG44 is a Flp recombinase expression plasmid. Horizontal arrowheads), primers for PCR diagnostic for the unoccupied (primers 1 and 2) or the occupied (primers 1 and 3) acceptor site. Restriction sites (E, Eco RI) and probes (Hyg, Neo) relevant to Southern analyses are shown. (b) PCR using primers 1 and 2, or 1 and 3, with DNA from HeLa/406 acceptor cells (unoccupied FRT), and DNA from cells containing the wild type c-myc origin fragment or the mutant (ΔDUE) c-myc origin fragment. (c) Hybridization with the Hyg or Neo probe to Eco RI digested DNA from HeLa/406 acceptor cells, or from cell lines containing the wild type c-myc origin fragment [$(CTG)_{12}$, $(CAG)_{12}$, $(CTG)_{102}$, $(CAG)_{102}$] or the mutant c-myc origin fragment [ΔDUE-$(CTG)_{102}$, ΔDUE-$(CAG)_{102}$] at the FRT.

Supplementary Figure 2. Slipped strand products formed during PCR. Linear PCR products from (a) $(CTG)_{102}$•$(CAG)_{102}$ cells, (b) $(CTG)_{45}$•$(CAG)_{45}$ cells, or (c) $(CTG)_{12}$•$(CAG)_{12}$ cells were gel purified and reamplified (lanes 2, 4, 6, 8, 10, 12) and compared to PCR products of genomic DNA (Lanes 1, 3, 5, 7, 9, 11). G, genomic DNA PCR; R, reamplified DNA PCR.

Supplementary Figure 3. Aphidicolin enhances $(CTG)_{102}$•$(CAG)_{102}$ instability. (a) $(CTG)_{102}$•$(CAG)_{102}$ cells cultured for 25 population doublings were exposed to five cycles of aphidicolin treatment (Methods) (lanes 1, 2) or five cycles of mock treatment (lanes 3, 4) before standard PCR. (b) The variability between cell populations growing under identical conditions was assessed by spPCR of DNA from cells drug treated (lanes 1-16) or mock treated (lanes 17-20) in (a). A short extension time (30 seconds) was used for spPCR (Methods).

Supplementary Figure 4. Emetine enhances $(CTG)_{102}$•$(CAG)_{102}$ instability. (a) $(CTG)_{102}$•$(CAG)_{102}$ cells cultured for 25 population doublings were exposed to five cycles of emetine treatment (Methods) (lanes 1, 2) or five cycles of mock treatment (lanes 3, 4) before PCR. DNAs from bands labeled 1, 2, 3, and 4 were cloned and sequenced. These bands contained uninterrupted, pure (CTG)•(CAG) sequences of $n > 172$, and $n = 150$, 33, and 20 repeats, respectively. (b) spPCR results of DNA from cells drug treated (lanes 1-16) or mock treated (lanes 17-24) in (a).

Supplementary Figure 5. Effects of emetine or Fen1 siRNA treatment. (a) Flow cytometry analysis of $(CTG)_{102}$ and $(CAG)_{102}$ cells. Cells were grown in DMEM medium with 1 μM emetine for 18 hr, and allowed to recover in drug free medium for two population doublings; this cycle was repeated 5 times. Alternatively, 30% confluent cells were cultured with Fen1 siRNA or control siRNA for 48 hr and allowed to recover for 48 hr in normal medium; this cycle was repeated 3 times. (b) Expression of Fen1 in $(CTG)_{102}$ or $(CAG)_{102}$ cells treated with emetine. (-) mock treatment; (+) emetine treatment. (c) Expression of Fen1 in $(CTG)_{102}$ or $(CAG)_{102}$ cells treated with Fen1 siRNA. (-) control siRNA treatment; (+) Fen1 siRNA treatment. Mcm7 loading control.

Supplementary Figure 6. Fen1 siRNA enhances $(CTG)_{102}$•$(CAG)_{102}$ instability. (a) $(CTG)_{102}$•$(CAG)_{102}$ cells cultured for 25 population doublings were exposed to Fen1 siRNA (Methods) (lanes 1, 2) or control siRNA (lanes 3, 4) before PCR. (b) spPCR of DNA from cells drug treated (lanes 1-16) or control siRNA treated (lanes 17-24) in (a).

Supplementary Figure 7. ZFN, ZFP expression. (a) Whole cell extracts were prepared from vector transfected (mock) or ZFN expression plasmid transfected $(CTG)_{102}$ cells cultured in 10% or 0.5% newborn calf serum. (b) Whole cell extracts were prepared from vector transfected (mock) or ZFP expression plasmid transfected $(CTG)_{102}$ cells. Extracts were probed with anti-actin and anti-FLAG (ZFN, ZFP) antibody.

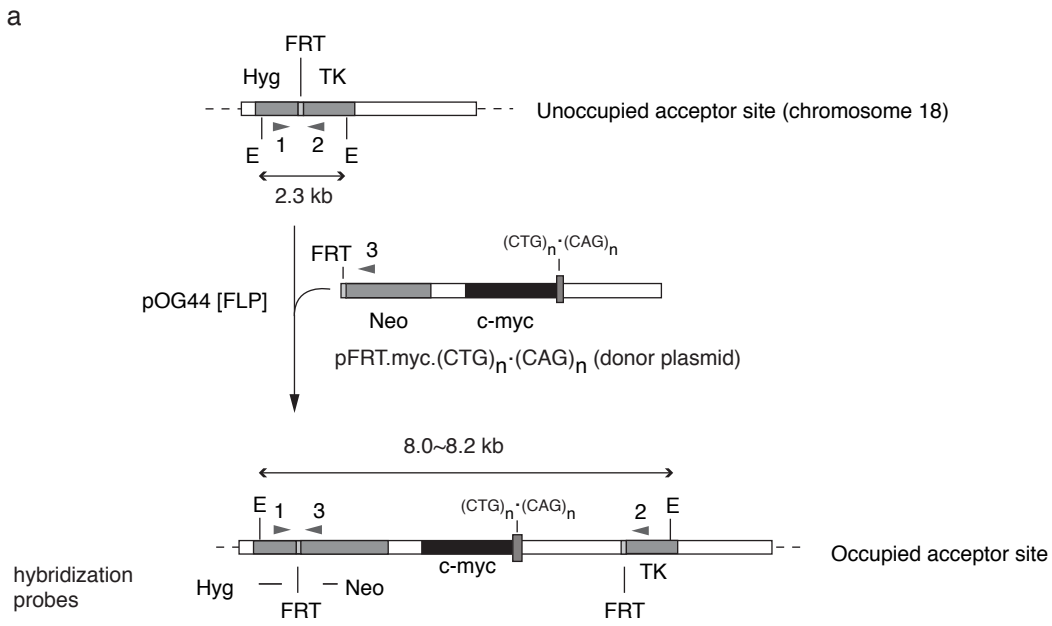
Supplementary Figure 8. Time courses of ZFN, ZFP digestion of $(CTG)_{102}$•$(CAG)_{102}$ DNA. The linear $(CTG)_{102}$•$(CAG)_{102}$ PCR product was gel purified and reamplified. Time course of cleavage of the reamplified $(CTG)_{102}$•$(CAG)_{102}$ PCR products with immunoprecipitated (a) ZFN_{CTG}, (b) ZFP_{CTG}, (c) ZFN_{CAG}, (d) ZFP_{CAG}, (e) ZFN_{CTG} + ZFN_{CAG}, (f) ZFP_{CTG} + ZFP_{CAG}. Individual immunoprecipitate digestions, 10% of immunoprecipitate; combined immunoprecipitate digestions, 5% of each immunoprecipitate.

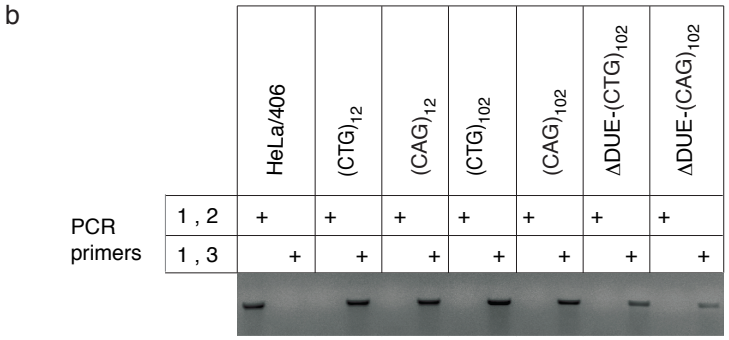
Supplementary Figure 9. Time courses of ZFN, ZFP digestion of $(CNG)_{46}$ hairpin DNA. The indicated $(CNG)_{46}$ hairpins (Methods) were digested with immunoprecipitated (a) ZFN_{CTG}, (b) ZFN_{CAG}, (c) ZFN_{CAG}, (d) ZFN_{CTG}, (e, f) ZFP_{CTG} + ZFP_{CAG}. Individual immunoprecipitate digestions, 10% of immunoprecipitate; combined immunoprecipitate digestions, 5% of each immunoprecipitate.

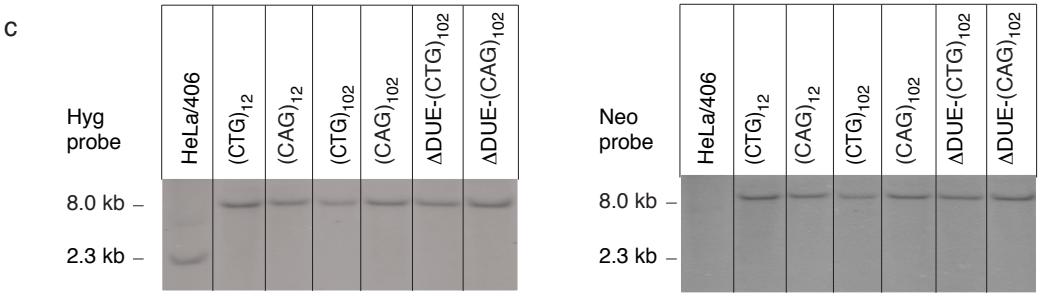
Supplementary Figure 10. ZFN hairpin cleavage in $(CTG)_{45}$•$(CAG)_{45}$ cells. $(CTG)_{45}$•$(CAG)_{45}$ cells were cultured for 25 population doublings. (a) spPCR of DNA from untransfected $(CTG)_{45}$ cells (lanes 1-8) or $(CAG)_{45}$ cells (lanes 9-12). (b) spPCR of DNA from $(CTG)_{45}$ cells (lanes 1-4) or $(CAG)_{45}$ cells (lanes 5-8) transfected with the ZFN_{CTG} expression plasmid. (c) spPCR of DNA from $(CTG)_{45}$ cells (lanes 1-5) or $(CAG)_{45}$ cells (lanes 5-8) transfected with the ZFN_{CAG} expression plasmid. (d) spPCR of DNA from $(CTG)_{45}$ cells or $(CAG)_{45}$ cells transfected with ZFP_{CTG} (lanes 1-8) or ZFP_{CAG} (lanes 9-16) expression plasmids. Lanes 1-4 and 5-8 were merged from nonadjacent

lanes of the same gel (see Supplementary Figure 11 for full gel images.) (e) spPCR of DNA from $(CTG)_{45}$ cells (lanes 1-8) or $(CAG)_{45}$ cells (lanes 9-16) transfected with a 0.5:0.5 mixture of ZFN_{CTG} and ZFN_{CAG} expression plasmids.

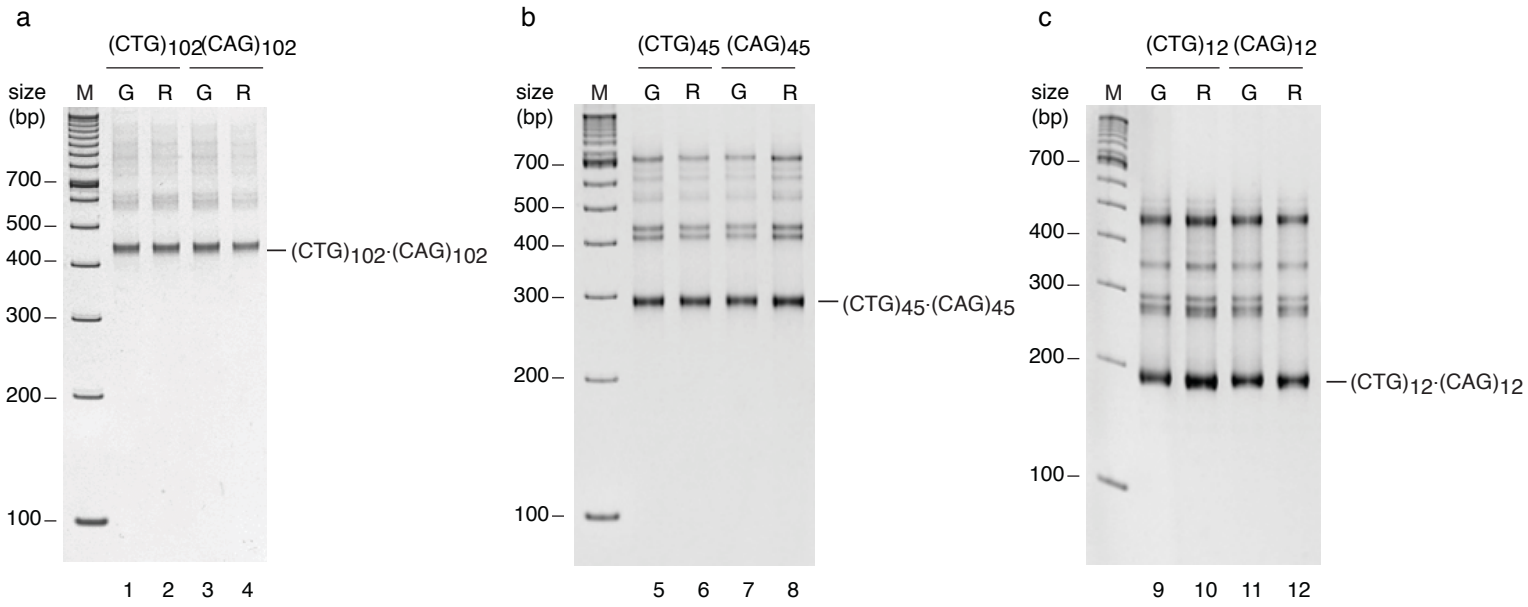
Supplementary Figure 11. Full gel photographs. (a) Gels of Figure 4d. (b) Gels of Supplementary Figure 10d.

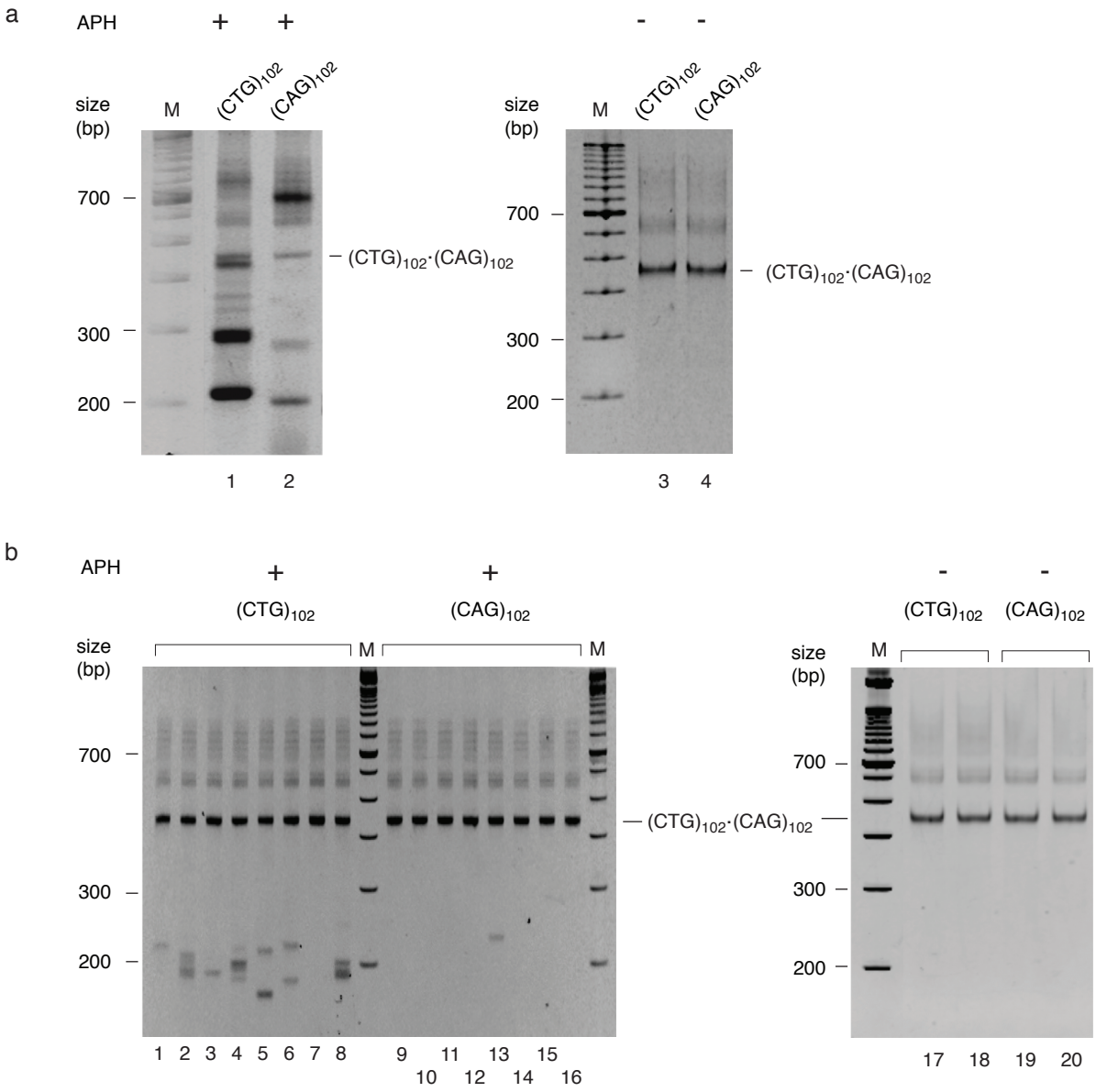

a
FRT
Hyg
TK
Unoccupied acceptor site (chromosome 18)
E
1
2
E
2.3 kb
FRT
3
(CTG)n·(CAG)n
pOG44 [FLP]
Neo
c-myc
pFRT.myc.(CTG)n·(CAG)n (donor plasmid)
8.0~8.2 kb
E
1
3
(CTG)n·(CAG)n
2
E
c-myc
TK
Occupied acceptor site
hybridization probes
Hyg
Neo
FRT
FRT


b
HeLa/406
(CTG)12
(CAG)12
(CTG)102
(CAG)102
ΔDUE-(CTG)102
ΔDUE-(CAG)102
PCR primers
1 , 2
1 , 3

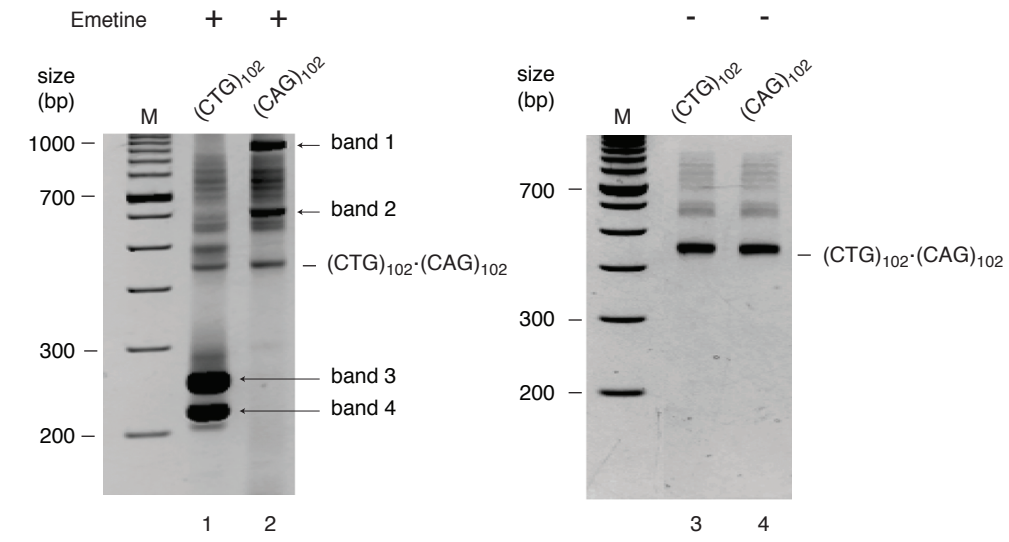

c
Hyg probe
HeLa/406
(CTG)12
(CAG)12
(CTG)102
(CAG)102
ΔDUE-(CTG)102
ΔDUE-(CAG)102
8.0 kb
2.3 kb
Neo probe
HeLa/406
(CTG)12
(CAG)12
(CTG)102
(CAG)102
ΔDUE-(CTG)102
ΔDUE-(CAG)102
8.0 kb
2.3 kb

Supplementary Figure 2

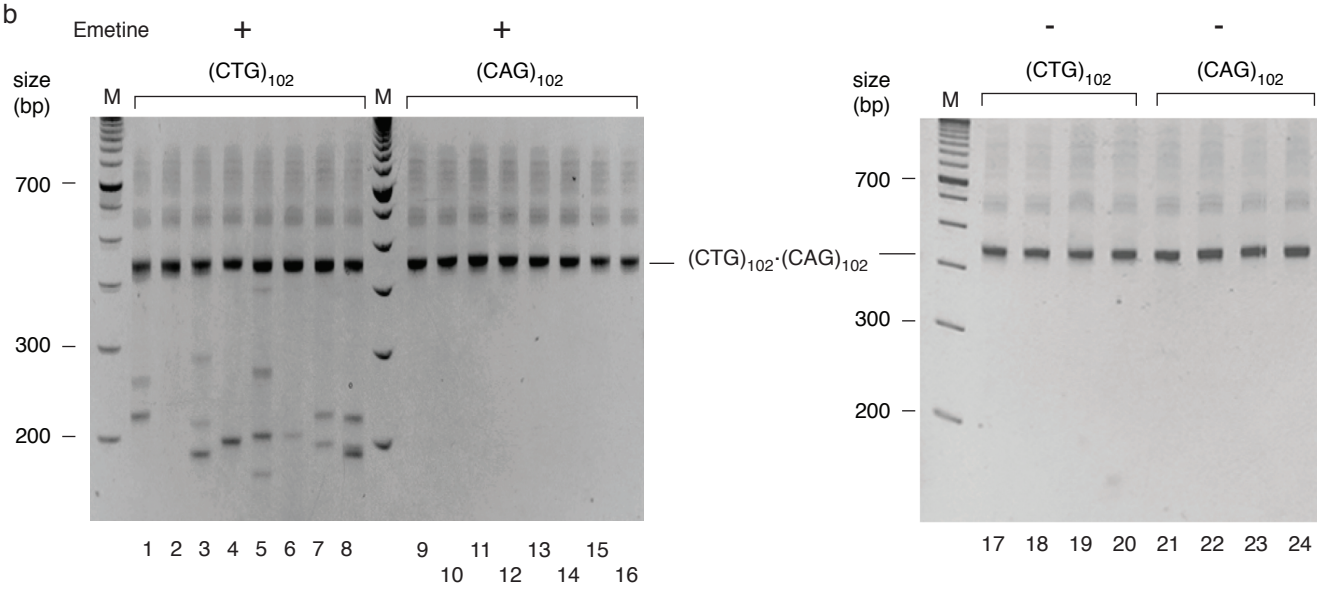


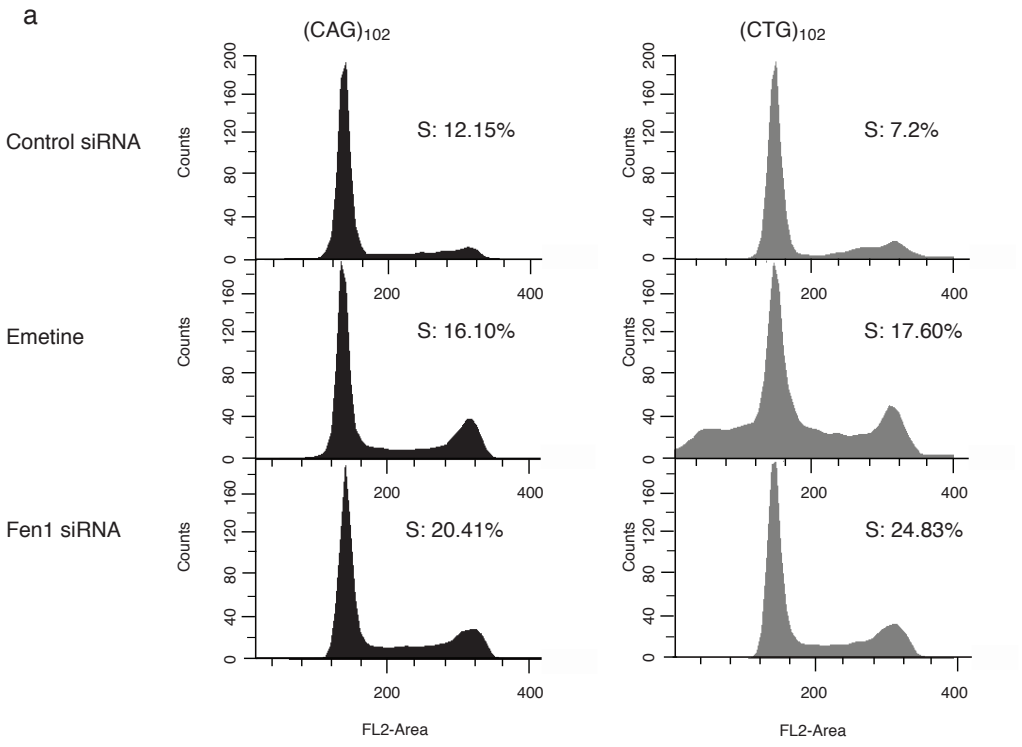

a
APH
+
+
-
-
size
(bp)
M
(CTG)102
(CAG)102
700
300
200
(CTG)102·(CAG)102
1
2
3
4
b
APH
+
+
-
-
(CTG)102
(CAG)102
size
(bp)
M
700
300
200
(CTG)102·(CAG)102
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
17 18 19 20

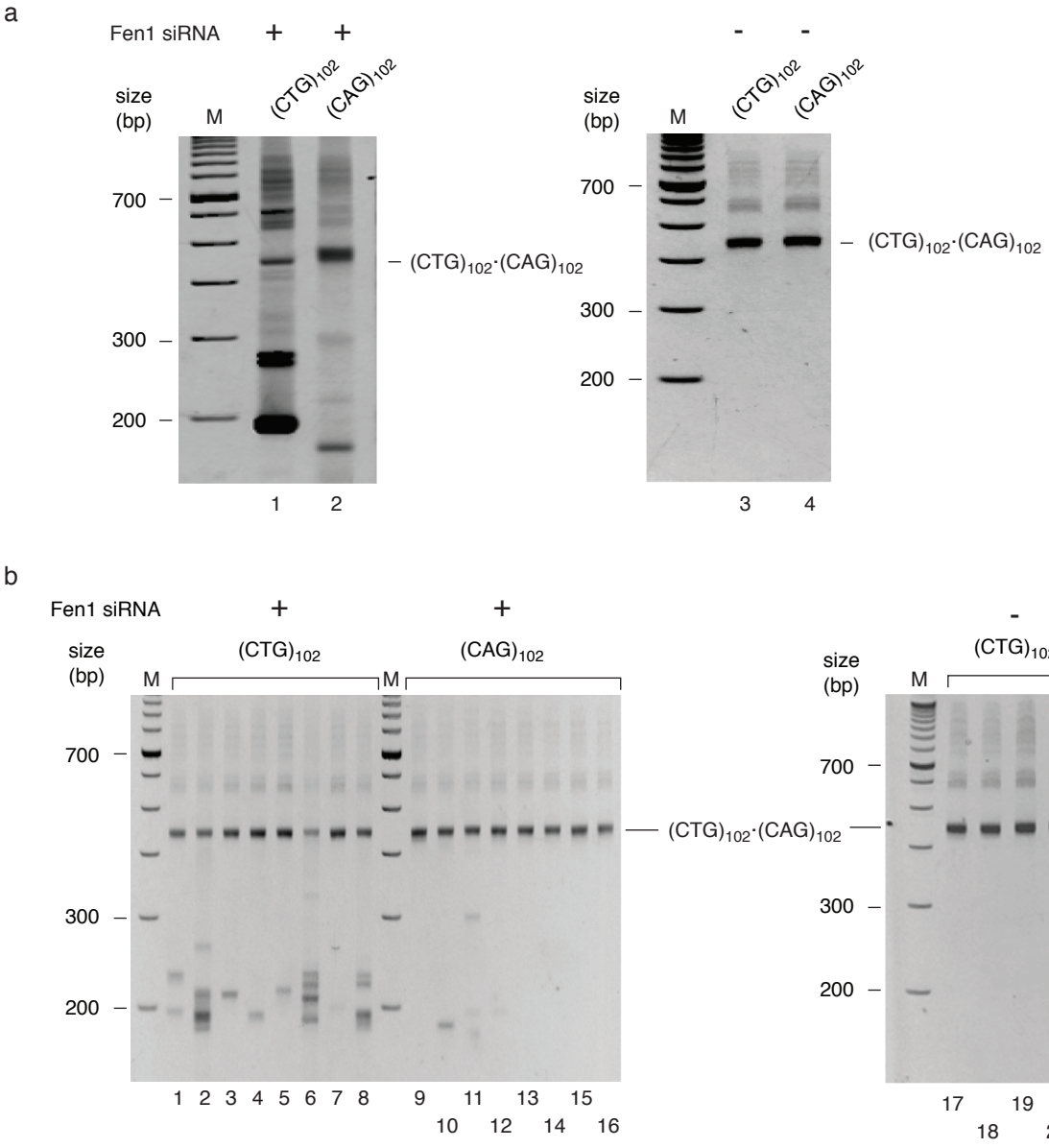
a


Emetine
+
+
-
-
size (bp)
M
(CTG)102
(CAG)102
1000
700
300
200
band 1
band 2
(CTG)102·(CAG)102
band 3
band 4
1
2
3
4

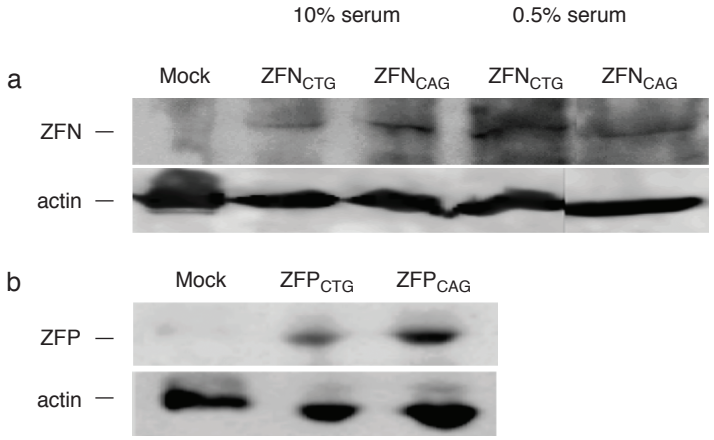
b

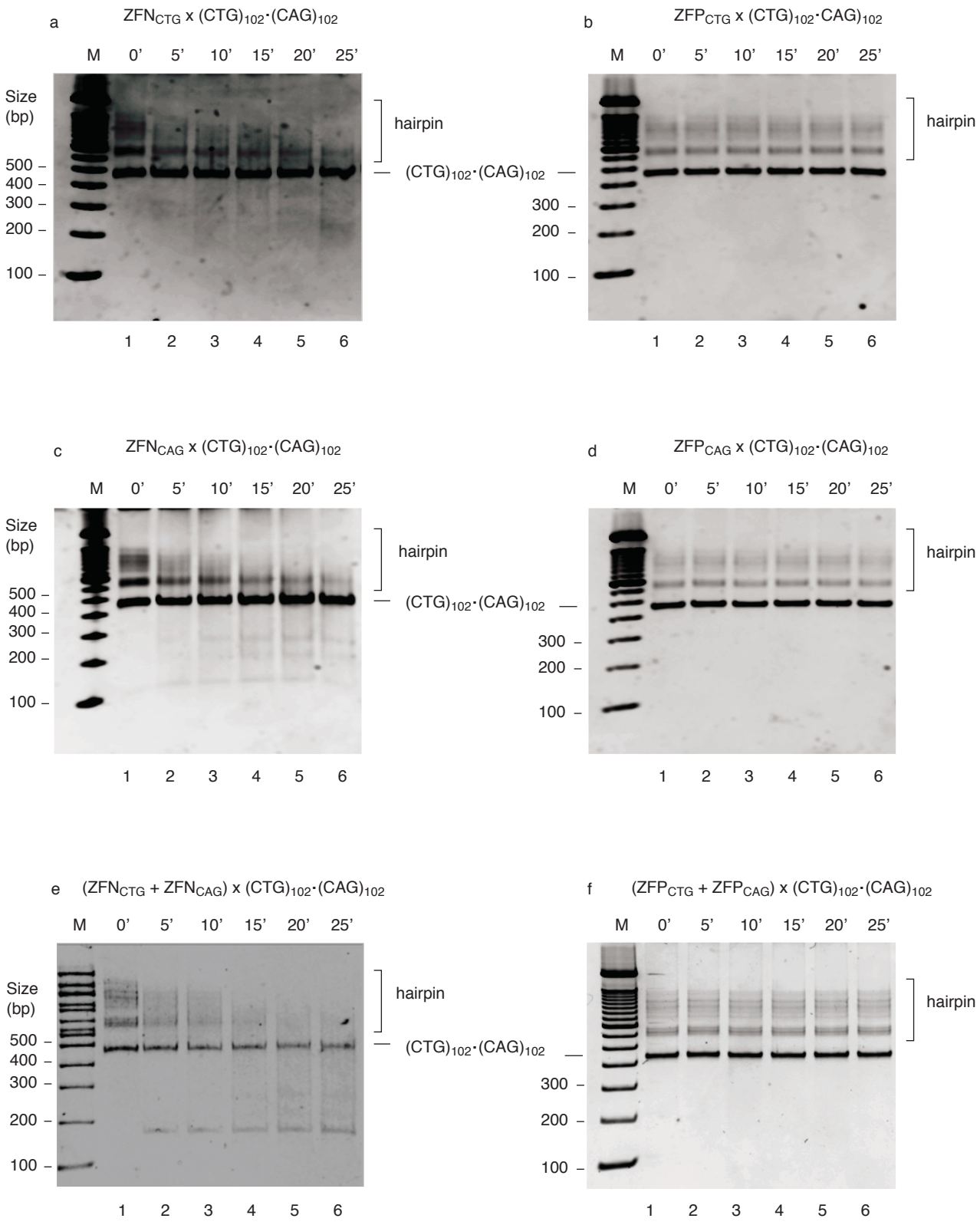

Emetine
+
+
-
-
size (bp)
M
(CTG)102
(CAG)102
700
300
200
(CTG)102·(CAG)102
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24

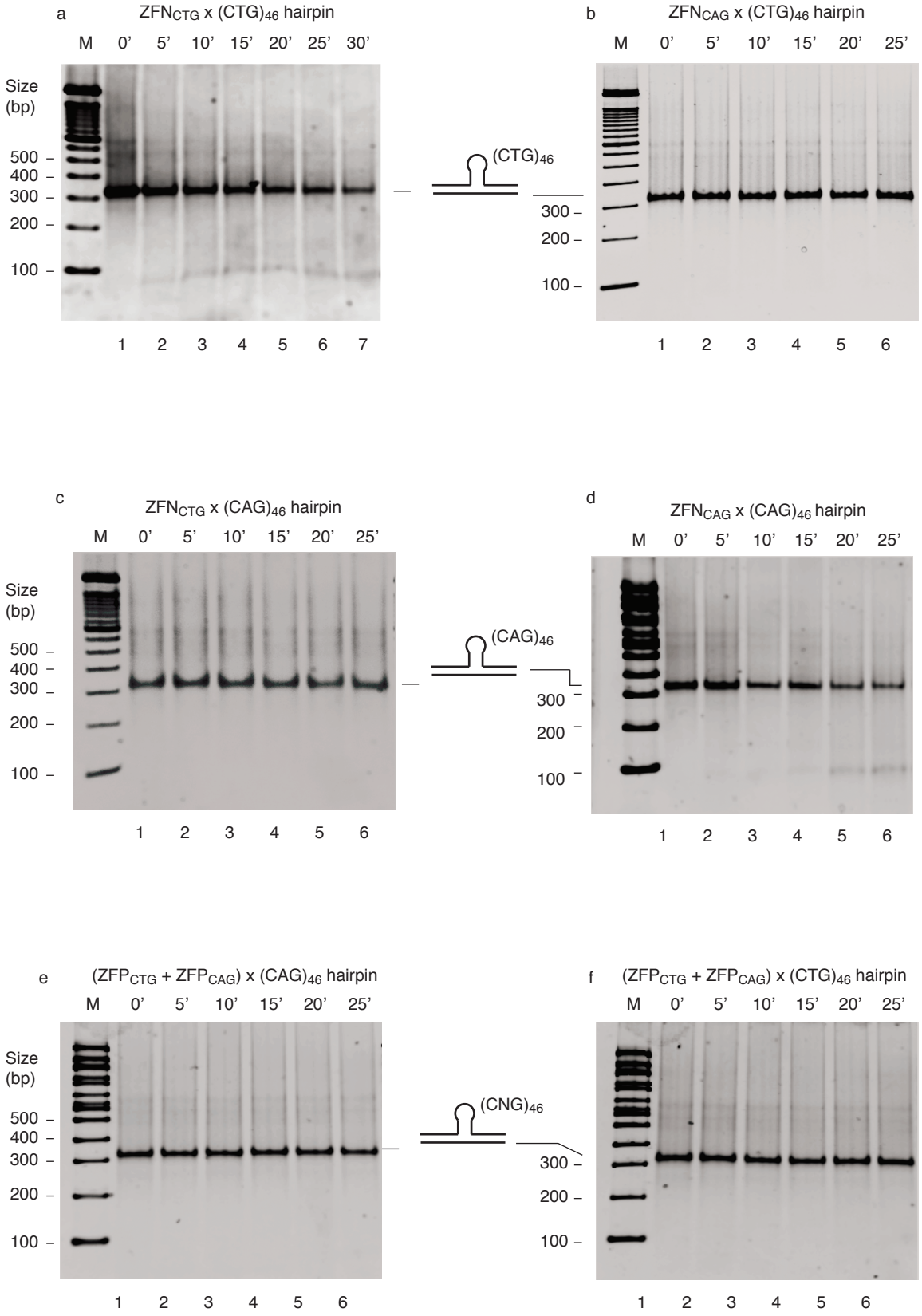

a
(CAG)102
(CTG)102
Control siRNA
Emetine
Fen1 siRNA
Counts
S: 12.15%
S: 7.2%
S: 16.10%
S: 17.60%
S: 20.41%
S: 24.83%
FL2-Area
b
Emetine - + - +
Fen1
Mcm7
c
Fen1 siRNA - + - +
Fen1
Mcm7


a
Fen1 siRNA + +
size (bp)
M
(CTG)102
(CAG)102
700
300
200
(CTG)102·(CAG)102
1 2
- -
3 4
b
(CTG)102
(CAG)102
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24

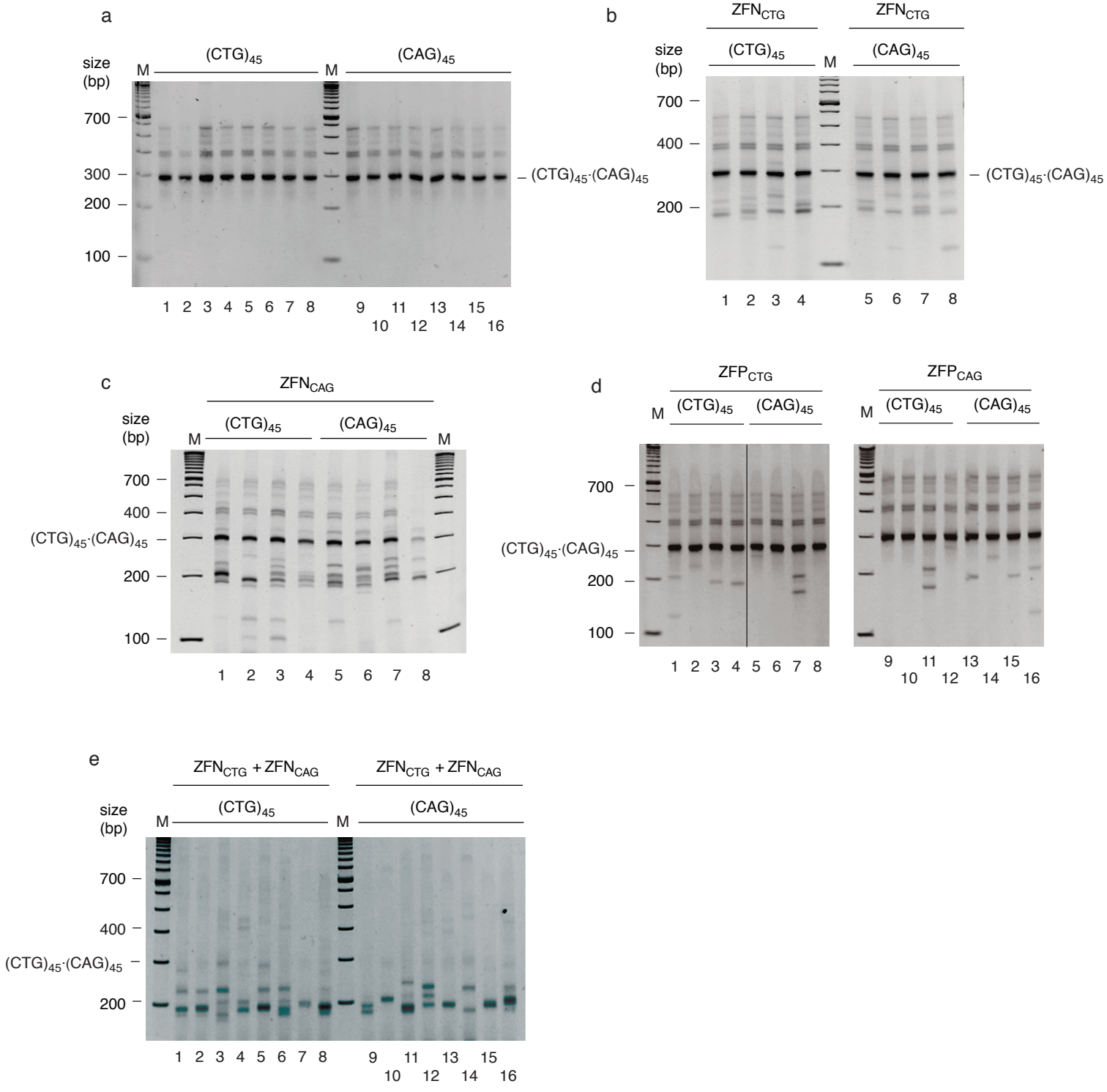
Supplementary Figure 7

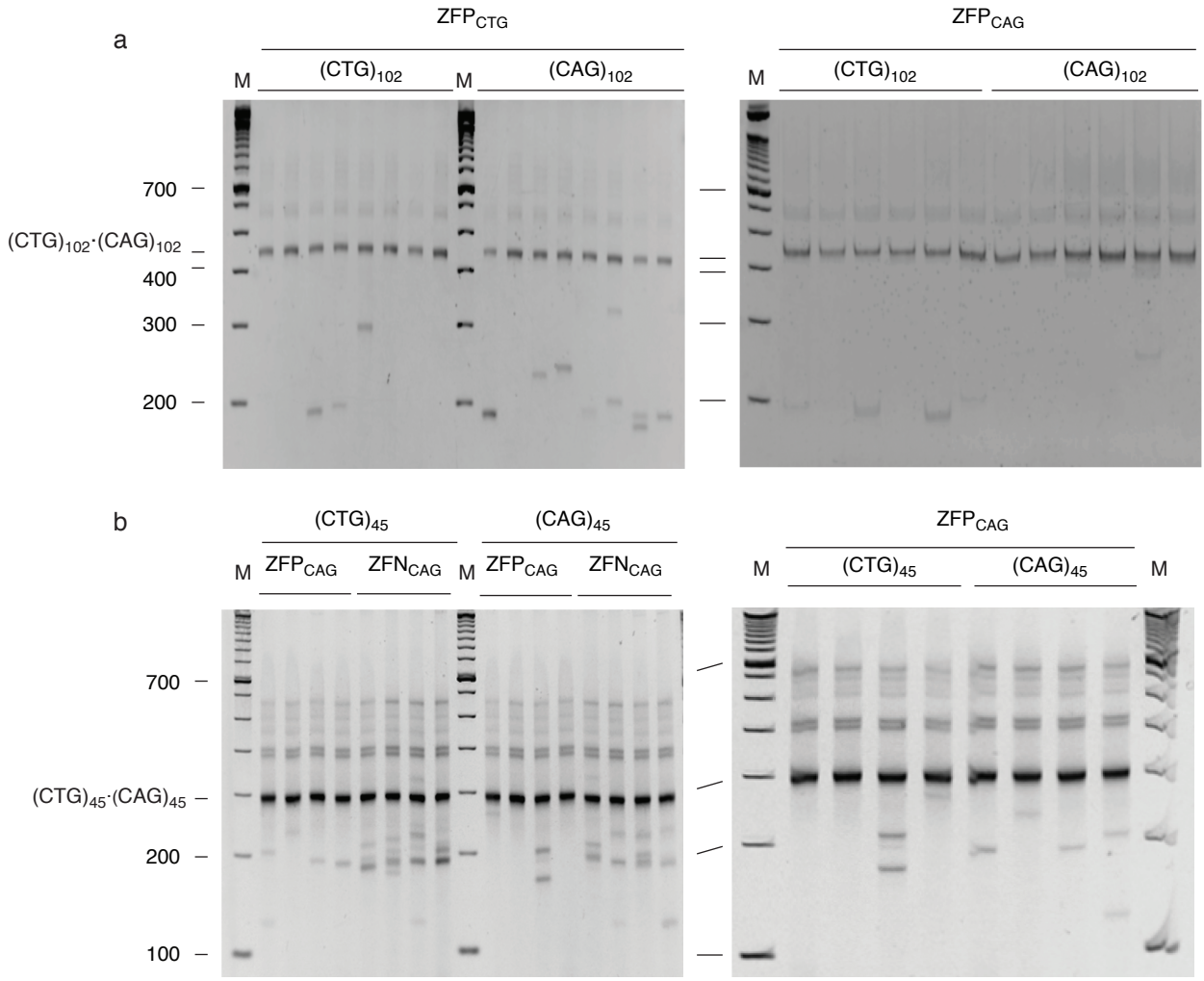



a
ZFN$_{CTG}$ x (CTG)$_{102}$·(CAG)$_{102}$
M 0' 5' 10' 15' 20' 25'
Size (bp)
500
400
300
200
100
hairpin
(CTG)$_{102}$·(CAG)$_{102}$
1 2 3 4 5 6
b
ZFP$_{CTG}$ x (CTG)$_{102}$·CAG)$_{102}$
M 0' 5' 10' 15' 20' 25'
300
200
100
hairpin
1 2 3 4 5 6
c
ZFN$_{CAG}$ x (CTG)$_{102}$·(CAG)$_{102}$
M 0' 5' 10' 15' 20' 25'
Size (bp)
500
400
300
200
100
hairpin
(CTG)$_{102}$·(CAG)$_{102}$
1 2 3 4 5 6
d
ZFP$_{CAG}$ x (CTG)$_{102}$·(CAG)$_{102}$
M 0' 5' 10' 15' 20' 25'
300
200
100
hairpin
1 2 3 4 5 6
e
(ZFN$_{CTG}$ + ZFN$_{CAG}$) x (CTG)$_{102}$·(CAG)$_{102}$
M 0' 5' 10' 15' 20' 25'
Size (bp)
500
400
300
200
100
hairpin
(CTG)$_{102}$·(CAG)$_{102}$
1 2 3 4 5 6
f
(ZFP$_{CTG}$ + ZFP$_{CAG}$) x (CTG)$_{102}$·(CAG)$_{102}$
M 0' 5' 10' 15' 20' 25'
300
200
100
hairpin
1 2 3 4 5 6


a
ZFN$_{CTG}$ x (CTG)$_{46}$ hairpin
M 0' 5' 10' 15' 20' 25' 30'
Size (bp)
500
400
300
200
100
1 2 3 4 5 6 7
(CTG)$_{46}$
300
200
100
b
ZFN$_{CAG}$ x (CTG)$_{46}$ hairpin
M 0' 5' 10' 15' 20' 25'
1 2 3 4 5 6
c
ZFN$_{CTG}$ x (CAG)$_{46}$ hairpin
M 0' 5' 10' 15' 20' 25'
Size (bp)
500
400
300
200
100
1 2 3 4 5 6
(CAG)$_{46}$
300
200
100
d
ZFN$_{CAG}$ x (CAG)$_{46}$ hairpin
M 0' 5' 10' 15' 20' 25'
1 2 3 4 5 6
e
(ZFP$_{CTG}$ + ZFP$_{CAG}$) x (CAG)$_{46}$ hairpin
M 0' 5' 10' 15' 20' 25'
Size (bp)
500
400
300
200
100
1 2 3 4 5 6
(CNG)$_{46}$
300
200
100
f
(ZFP$_{CTG}$ + ZFP$_{CAG}$) x (CTG)$_{46}$ hairpin
M 0' 5' 10' 15' 20' 25'
1 2 3 4 5 6

Supplementary Figure 10




a
ZFP$_{CTG}$
ZFP$_{CAG}$
M
(CTG)$_{102}$
(CAG)$_{102}$
M
(CTG)$_{102}$
(CAG)$_{102}$
700
(CTG)$_{102}$·(CAG)$_{102}$
400
300
200
b
(CTG)$_{45}$
(CAG)$_{45}$
ZFP$_{CAG}$
M
ZFP$_{CAG}$
ZFN$_{CAG}$
M
ZFP$_{CAG}$
ZFN$_{CAG}$
M
(CTG)$_{45}$
(CAG)$_{45}$
M
700
(CTG)$_{45}$·(CAG)$_{45}$
200
100