Supplementary Information:

Cdk1 regulates the temporal recruitment of telomerase and CST complex for telomere replication

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*Corresponding author

Supplemental Data:

Table S1: **List of yeast strains used in this study.**

Figure S1: Putative Cdk1 phosphorylation sites in Stn1. A. Four putative Cdk1 phosphorylation sites (containing the minimal Cdk1 consensus motif S/T*-P, as highlighted in red) can be identified in Stn1 amino acid sequence. **B.** Schematic representation of the functional domains in Stn1 and the relative locations of the four putative Cdk1 phosphorylation sites on the protein. **C.** Fungal sequence alignment results showing that threonine at position 223 and

serine at position 250 are well conserved. Putative Cdk1 phosphorylation sites are highlighted in red. Two additional putative Cdk1 phosphorylation sites in the Stn1 homologues are also underlined and highlighted in blue.

Figure S2: The Stn1 S250 phospho-peptide specific antibody detects stn1 recombinant protein that is phosphorylated by Cdk1/cyclin complexes *in vitro*. Top panel, the blot is probed with Stn1 S250 phospho-peptide specific antibody. Bottom panel, the same blot is probed with anti-His antibody to detect the recombinant 6xHis-Stn1(M) proteins that are used in the in vitro kinase assay.

Figure S3: Telomere length measurement. A. Introduction of 13xMyc to the C terminal of the endogenous *STN1* results in a slightly telomere shortening. **B.** Progressive telomere lengthening in STN1 phosphorylation site mutants. The telomere length of each individual yeast colony derived from the dissected spores was measured after ~25 (S1) and ~50 (S2) cell divisions on YPD plate at 30°C. Progressive telomere lengthening can be readily observed in *stn1-T223A,S250A* mutant yeast strain.

Figure S4: Mutations in Stn1 phosphorylation sites result in telomere elongation *in vivo*. Diploid yeast strains heterozygous for wild type and Cdk1

phosphorylation site mutants in *STN1* were sporulated and dissected. The telomere length of each individual yeast colony derived from the dissected spores was measured after ~100 cell divisions on an YPD plate at 30°C.

Figure S5: Cdk1-dependent Stn1 phosphorylation affects telomerase-dependent telomere elongation. Telomere length was analyzed in the various yeast strains obtained by dissecting spores of diploid yeast strains harboring *STN1* threonine 223 and serine 250 to alanine mutations in combination with deletion of known factors involved in telomerase-dependent telomere length regulation pathways (Lanes 1-4 y*ku70Δ*; lanes 5-8 y*ku80Δ*; lanes 9-12 *est2Δ*; lanes 13-16 *tel1Δ*; and lanes 17-20 *mec1Δ/sml1Δ*). The telomere length of each individual yeast colony derived from the dissected spores was measured ~100 cell divisions after dissection, except for *est2Δ* strains that was measured ~50 cell divisions after dissection.

Figure S6: Single mutations in *STN1* T223 and S250 also result in a cell cycle-dependent increase in single-stranded telomere G-rich overhangs. A. In gel-hybridization at non-denaturing condition showed that increased signal from the single-stranded telomere G-rich overhangs can be detected in yeast strain harboring the Stn1 phosphorylation site mutations (*stn1-T223A and stn1-S250A*). **B.** The same gel from A that is probed with a telomere-specific probe under denaturing condition. FACS analysis shows the DNA content of *stn1-T223A* (**C.**)*, stn1-S250A* (**D.**) and *STN1* (**E.**) yeast strains from alpha-factor

synchronization release that were used for the in-gel hybridization. **F.** Quantification of the relative intensity of cell-cycle-dependent generation of single-stranded telomere DNA in *stn1-T223A*, *stn1-S250A* and *STN1*, yeast strains. The data is derived from three independent experiments.

Figure S7: The cell cycle-dependent increase in single-stranded telomere G overhangs in *STN1* phosphorylation mutant is not significantly affected by *est2Δ*. A. In gel-hybridization at non-denaturing condition showed that the increased signal from single-stranded telomere G-rich overhang was detected in the *est2Δ/stn1-T223A,S250A* yeast strain compared to the *est2Δ* yeast strain. **B.** The same gel from A that was probed with telomere-specific probe under denaturing condition. **C.** and **D.** FACS analysis showing the DNA content of *est2Δ* and *est2Δ/stn1-T223A,S250A* yeast strains from alpha-factor synchronization release that were used for the in-gel hybridization. **E.** Quantification of the relative intensity of cell-cycle-dependent generation of single-stranded telomere DNA in *STN1/est2Δ* and *stn1-T223A,S250A/est2Δ* mutant yeast strains. The data is derived from three independent experiments.

Figure S8: Increased single-stranded telomere G-rich overhangs in *STN1* phosphorylation mutant do not activate DNA damage checkpoint. In yeast harbor stn1-T223A,S250A mutations, there is no obvious increase in phosphorylation of Rad53-3xHA. In contrast, phosphorylation of Rad53-3xHA is readily observed in yeast treated with Zeocin for 4hr. The expression of

phosphoglycerate kinase (PGK) is used as loading control.

Figure S9: The association between Stn1 and Ten1 is not affected by the Cdk1-dependent phosphorylation of Stn1. A yeast strain co-expressing Ten1-3xFlag and Stn1-13xMyc from endogenous promoters was used for the co-immunoprecipitation assay. **A.** Immunoprecipitation of Ten1-3xFlag protein results in the efficient co-immunoprecipitation of wild-type Stn1-13xMyc protein as well as Stn1(T223A,S250A)-13xMyc mutant proteins (compare lane 3 and 4). **B.** In the reciprocal experiment, immunoprecipitation of Stn1-13xMyc protein or Stn1(T223A,S250A)-13xMyc protein also results in the efficiently co-immunoprecipitation of Ten1 protein (compare lane 2 and 3).

Figure S10: DNA content of yeast cultures from alpha-factor synchronization that were used for ChIP experiment. A. *CDC13-13xMyc* tagged strain with wild-type *STN1*; **B.** *CDC13-13xMyc* tagged strain with *stn1-T223A,S250A*; **C.** *STN1-13xMyc* tagged strain; **D.** *stn1 (T223A,S250A)-13xMyc* tagged strain; **E.** *TEN1-13xMyc* tagged strain with wild-type *STN1*; **F.** *TEN1-13xMyc* tagged strain with *stn1-T223A,S250A*; **G.** *EST1-13xMyc* tagged strain with wild-type *STN1* and **H.** *EST1-13xMyc* tagged strain with *stn1-T223A,S250A* were synchronized in late G1 using alpha-factor (0 minute), and further samples were taken at 15 minute intervals after alpha-factor removal for FACS analysis.

Figure S11: The cell cycle-dependent recruitment of Ten1 to telomere is dramatically reduced in stn1-T223A,S250A mutant. ChIP assays were done in synchronous yeast cultures, and the immunoprecipitated chromatin DNA was then probed on the dot blot using ^{32}P–labeled telomere specific oligonucleotides or ARO1 cDNA probe. The enrichment of telomeric DNA in ChIP samples was quantified as the percentage of total input. The data is derived from two independent experiments.

Figure S12: Single mutations in *STN1* T223 and S250 affect the recruitment of CST complex as well as telomerase complex to telomeres. A. ChIP assays in synchronous yeast cultures show that the cell cycle-dependent recruitment of Cdc13 to telomere is not affected in *stn1-T223A* or *stn1-S250A* mutant yeast strain. **B.** ChIP assays in synchronous yeast cultures show that the cell cycle-dependent recruitment of Ten1 to telomere is dramatically reduced in *stn1-T223A* and *stn1-S250A* mutant yeast strains. **C.** ChIP assays in synchronous yeast cultures show that the cell cycle-dependent recruitment of Stn1 to telomere is dramatically reduced in *stn1-T223A* and *stn1-S250A* mutant yeast strains. **D.** In contrast, ChIP assays in synchronous yeast cultures show the prolonged association of Est1 and telomere in *stn1-T223A* as well as *stn1-S250A* mutant yeast strains.

Figure S13: DNA content of yeast cultures from alpha-factor synchronization that were used for ChIP experiment. A. *CDC13-13xMyc*

tagged strain with wild-type *STN1*; **B.** *CDC13-13xMyc* tagged strain with *stn1-T223A*; **C.** *CDC13-13xMyc* tagged strain with *stn1-S250A*; **D.** *STN1-13xMyc* tagged strain; **E.** *stn1 (T223A)-13xMyc* tagged strain; **F.** *stn1 (S250A)-13xMyc* tagged strain; **G.** *TEN1-13xMyc* tagged strain with wild-type *STN1*; **H.** *TEN1-13xMyc* tagged strain with *stn1-T223A*; **I.** *TEN1-13xMyc* tagged strain with *stn1-S250A*; **J.** *EST1-13xMyc* tagged strain with wild-type *STN1*; **K.** *EST1-13xMyc* tagged strain with *stn1-T223A* and **L.** *EST1-13xMyc* tagged strain with *stn1-S250A* were synchronized in late G1 using alpha-factor (0 minute), and further samples were taken at 15 minute intervals after alpha-factor removal for FACS analysis.

Supplemental material and methods

Plasmid Constructions:

pRS406-STN1-13xMyc was engineered by PCR amplification of *STN1* cDNA with 500bp 5' and 3' flanking genomic DNA using PCR primers with 5' BamHI site and 3' XhoI site, then subsequently cloned into BamHI/XhoI sites in pRS406 vector. Site-directed mutagenesis reactions were done subsequently to introduce phosphorylation sites mutants. These constructs were then used in pop-in and pop-out procedure to introduce these phosphorylation mutations into the endogenous *STN1* locus in a diploid yeast strain.

pET28a-Stn1(M), expressing 6xHis tagged recombinant Stn1(M) proteins, was engineered by PCR amplification of *STN1* amino acids 161-287 using primers with 5' NdeI site and 3' XhoI site, then subsequently cloned into NdeI/XhoI sites in the pET28a vector. Site-directed mutagenesis reactions were done subsequently to introduce phosphorylation sites mutants.

pBTM116-Pol12(N), expressing Pol12 N terminal 381 amino acids fused to LexA DNA binding domain, was engineered by PCR amplification of *POL12* amino acid 1-381 using PCR primers with 5' SmaI site and 3' SalI site, then subsequently cloned into SmaI/SalI sites in the pBTM116 vector.

pGADT7-STN1, expressing Stn1 fused to Gal4 activation domain, was engineered by PCR amplification of *STN1* full-length cDNA using PCR primers with 5' SmaI site and 3' XhoI site, then subsequently cloned into SmaI/XhoI sites

in the pGADT7 vector. Site-directed mutagenesis reactions were done subsequently to introduce phosphorylation sites mutants.

pGBKT7-CDC13, expressing Cdc13 with DNA-binding domain deletion fused to Gal4 DNA binding domain, was engineered by PCR amplification of *CDC13* full-length cDNA using PCR primers with 5' SmaI site and 3' SalI site, then subsequently cloned into SmaI/SalI sites in the pGBKT7 vector. The BstBI fragment containing the DBD domain of *CDC13* was subsequently eliminated by digestion with BstBI restriction enzyme followed by self-ligation.

pGBKT7-TEN1, expressing Ten1 fused to Gal4 DNA binding domain, was engineered by PCR amplification of *TEN1* full-length cDNA using PCR primers with 5' SmaI site and 3' SalI site, then subsequently cloned into SmaI/SalI sites in the pGBKT7 vector.

Primers used for qPCR and qRT-PCR:

TEL: Forward: 5'-TCATGTACGTCTCCTCCAAGCCC-3'

Reverse: 5'-GCAGTAGCGAGAGACAAGTGGGAAA-3'

ARO1: Forward: 5'-TGACTGGTACTACCGTAACGGTTC-3'

Reverse: 5'- GAATACCATCTGGTAATTCTGTAGTTTTGAC-3'

TLC1: Forward: 5'- CATCGAACGATGTGACAGAGAA-3'

Reverse: 5'-TAATGCGACAAAAATACCGTATTGATCATCAA-3'

Actin: Forward: 5'-ATGGATTCTGAGGTTGCTGCTTTGGTTA-3'

Reverse: 5'-TGTTCTTCTGGGGCAACTCTCAATT-3'

Strain Name	Genotype
SL10284	*MATa bar1::natMX ura3-52 trp1-289 leu2-3,112 STN1-13myc-TRP1*
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SL10428	*MATa bar1::natMX ura3-52 trp1-289 leu2-3,112 STN1-13myc-TRP1 sml1Δ::TRP1 tel1Δ::kanMX6 mec1Δ::kanMX6*
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SL10435	*MATa bar1::natMX ura3-52 trp1-289 leu2-3,112 stn1(S250A)-13myc-TRP1*
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All the strains are S. cerevisiae A364a background.

Figure S1

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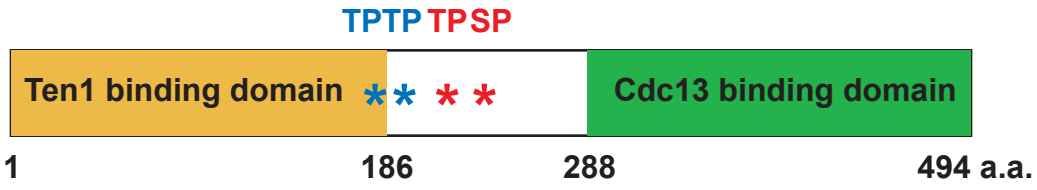
B



C

Stn1 (Fungal sequence alignment)

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Symbols                     .   :   *  :::   . .  :               .  .  :: . :

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S. castellii           232  QDENLRDELEITSPYFSSDPSFNFG--FTNPPSIEEETTEFELIEASQSR  279
S. Kluyveri            239  YSQNLWNSRSAEDLLVKGVENEFDVEDTHLMIPIHERKSATNDQRDNHIN  288
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Symbols: * = Identical; : = strong similarity; . = weak similarity

Figure S2

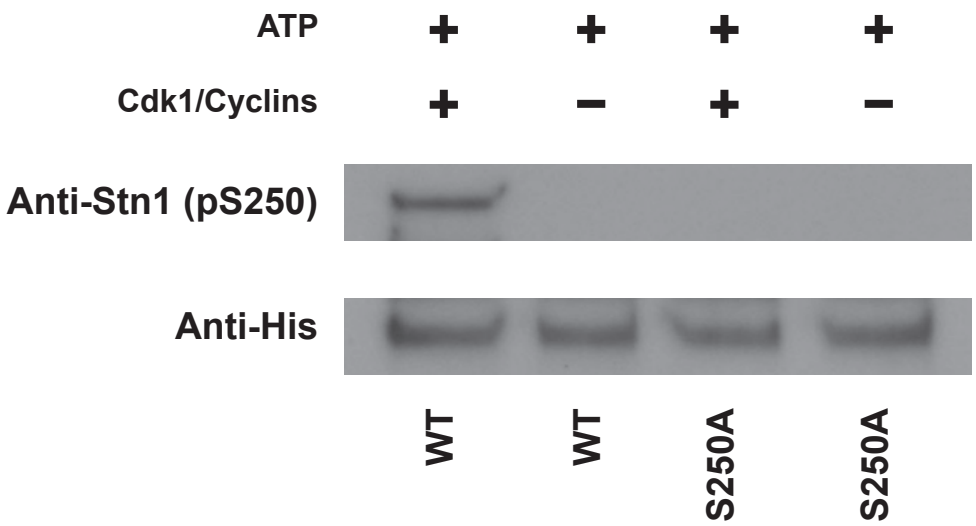
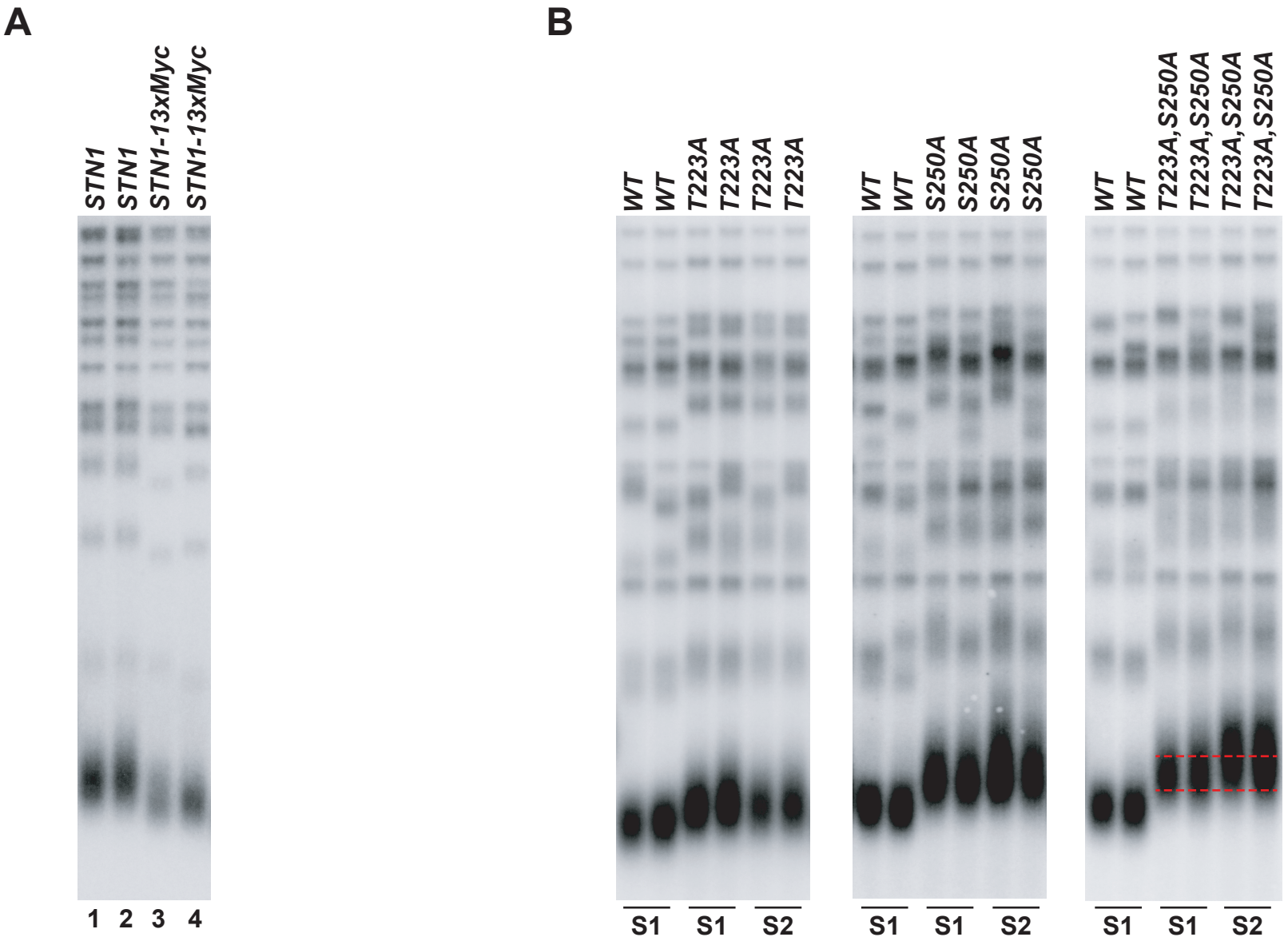


Figure S3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WT
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S250A
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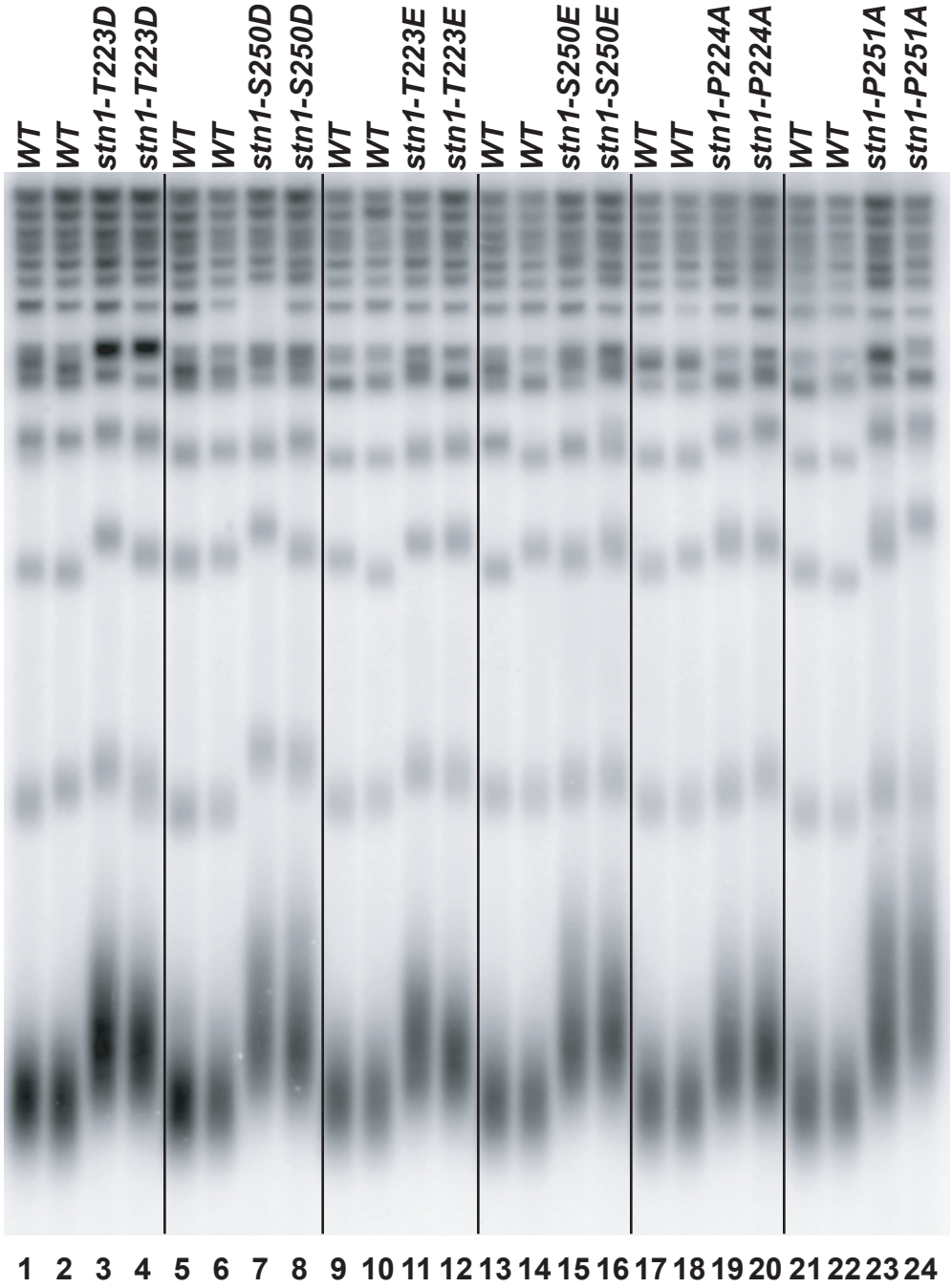
Figure S4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stn1-T223D
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WT
WT
stn1-S250E
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WT
WT
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stn1-P224A
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stn1-P251A
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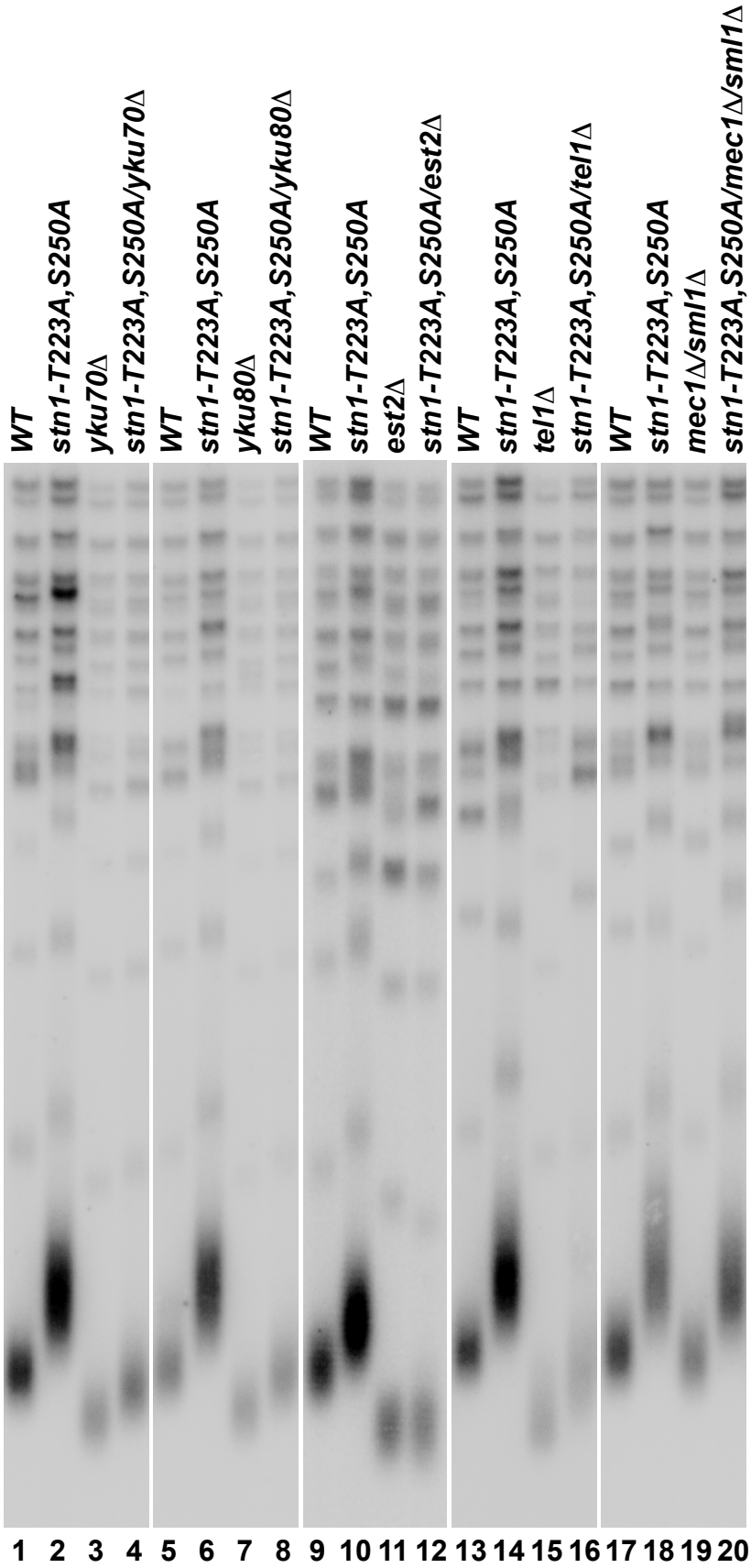
Figure S5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WT
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tel1Δ
stn1-T223A,S250A/tel1Δ
WT
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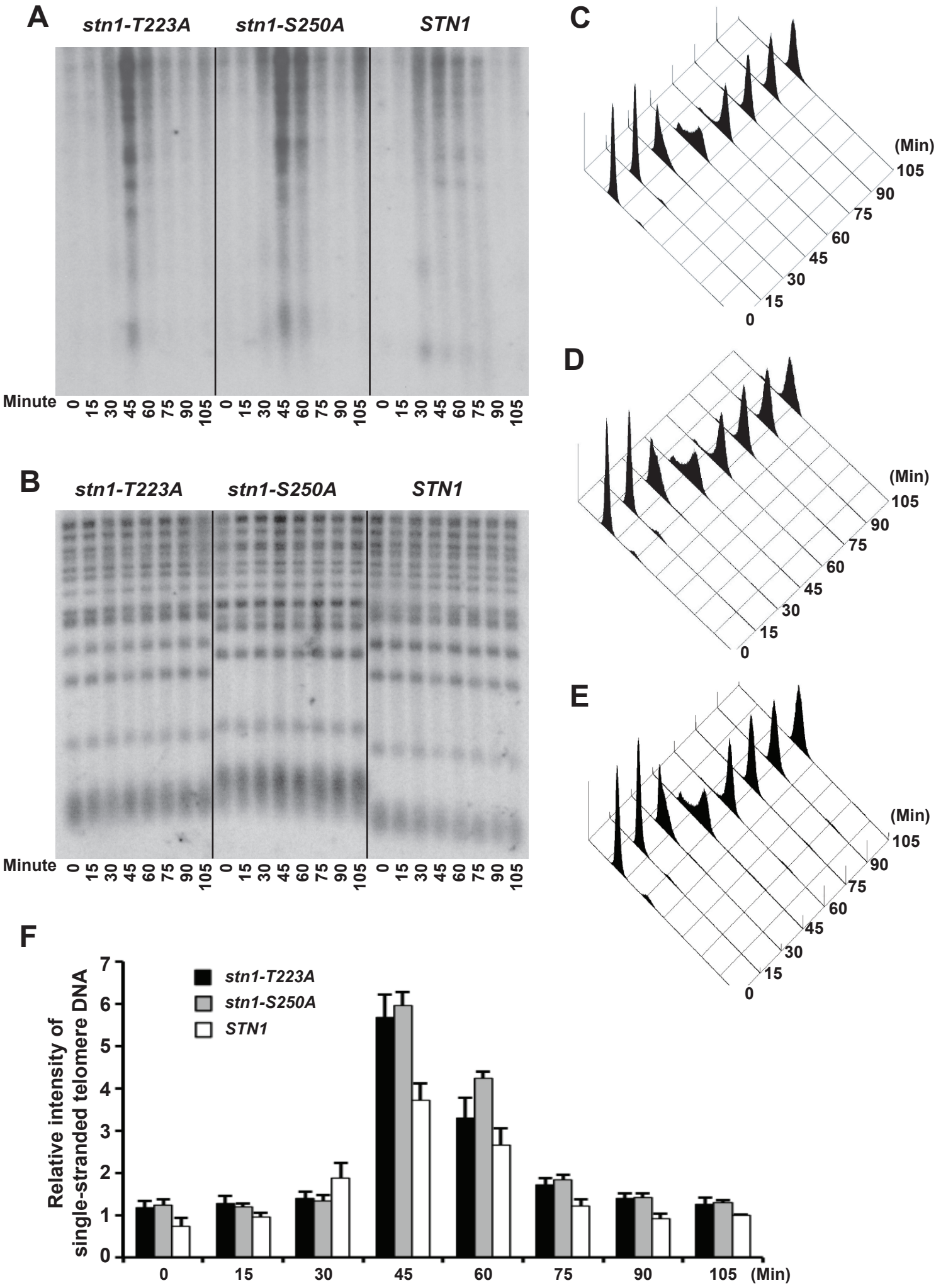
Figure S6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stn1-S250A
STN1
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B
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stn1-S250A
STN1
Minute
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C
(Min)
105
90
75
60
45
30
15
0
D
(Min)
105
90
75
60
45
30
15
0
E
(Min)
105
90
75
60
45
30
15
0
F
Relative intensity of single-stranded telomere DNA
stn1-T223A
stn1-S250A
STN1
0 1 2 3 4 5 6 7
0 15 30 45 60 75 90 105 (Min)

Figure S7

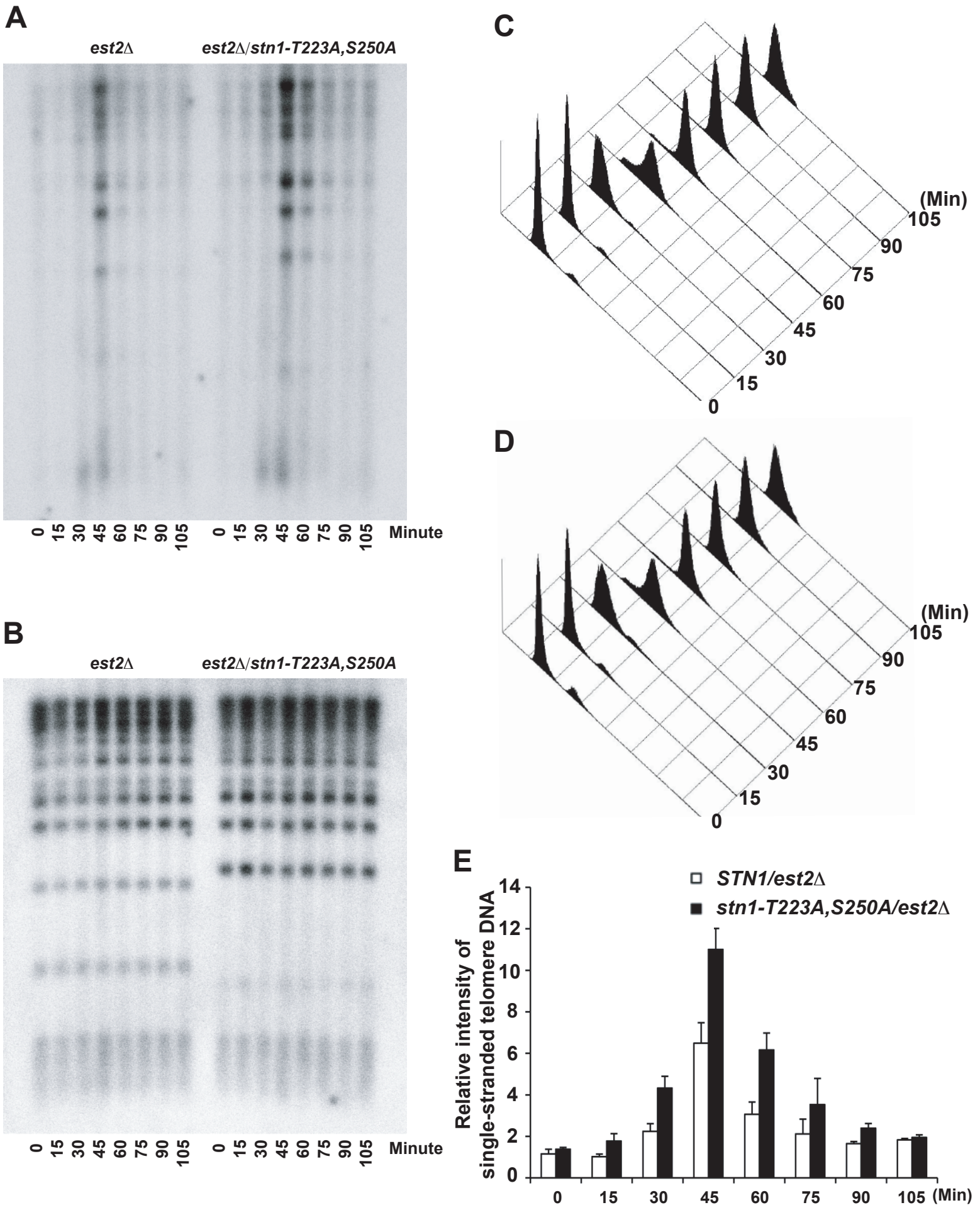

A
est2Δ
est2Δ/stn1-T223A,S250A
0 15 30 45 60 75 90 105
0 15 30 45 60 75 90 105
Minute
B
est2Δ
est2Δ/stn1-T223A,S250A
0 15 30 45 60 75 90 105
0 15 30 45 60 75 90 105
Minute
C
(Min)
105
90
75
60
45
30
15
0
D
(Min)
105
90
75
60
45
30
15
0
E
STN1/est2Δ
stn1-T223A,S250A/est2Δ
Relative intensity of single-stranded telomere DNA
0
2
4
6
8
10
12
14
0 15 30 45 60 75 90 105 (Min)

Figure S8

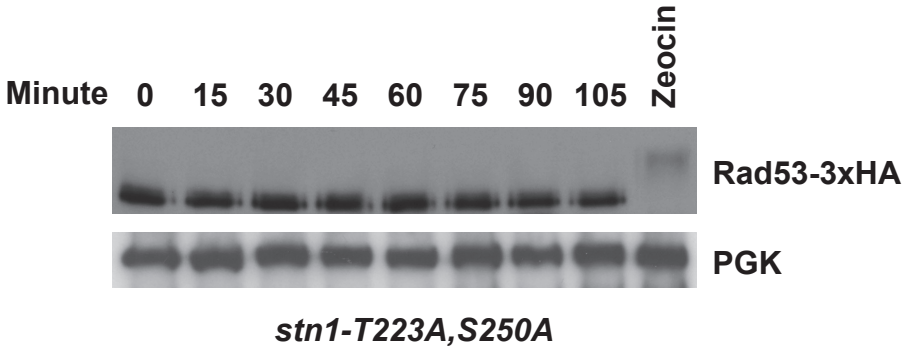

Minute
0
15
30
45
60
75
90
105
Zeocin
Rad53-3xHA
PGK
stn1-T223A,S250A

Figure S9

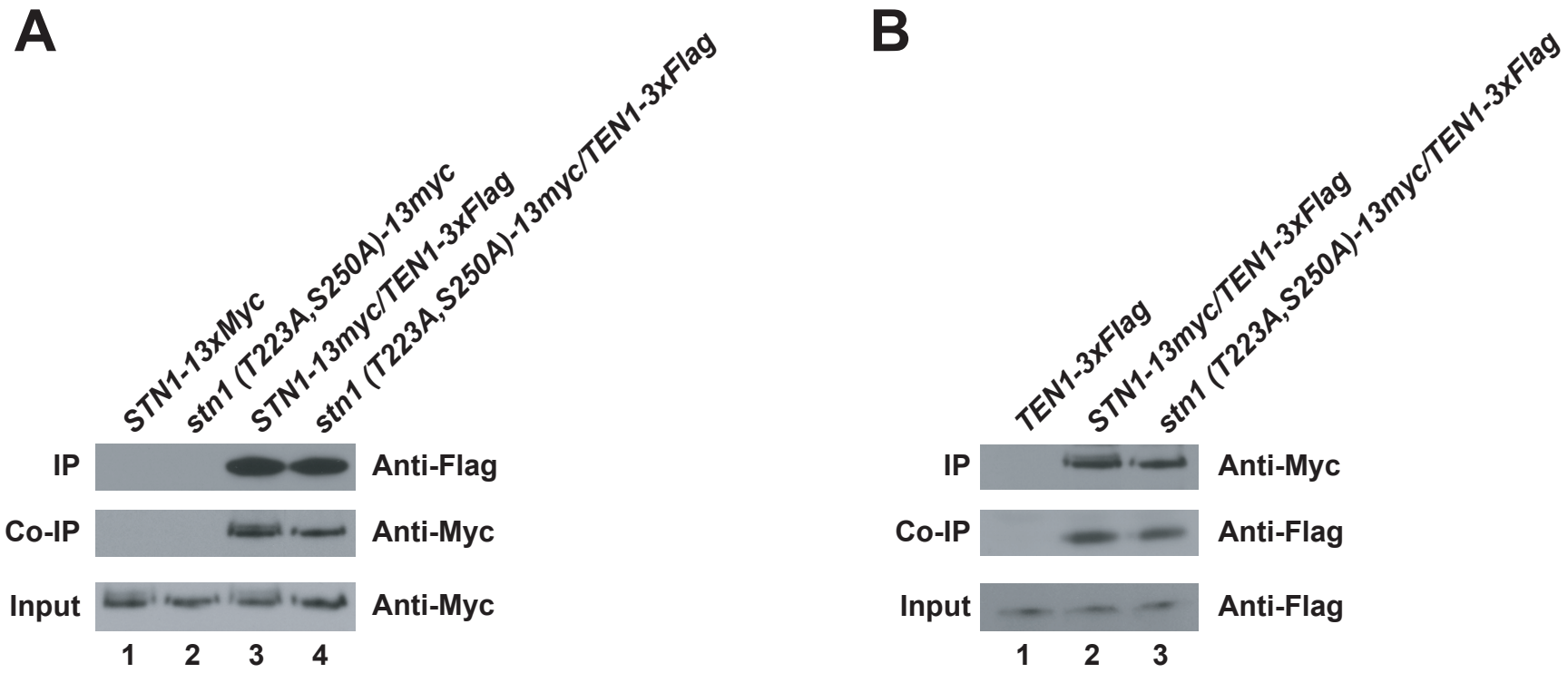

A
STN1-13xMyc
stn1 (T223A,S250A)-13myc
STN1-13myc/TEN1-3xFlag
stn1 (T223A,S250A)-13myc/TEN1-3xFlag
IP
Anti-Flag
Co-IP
Anti-Myc
Input
Anti-Myc
1 2 3 4
B
TEN1-3xFlag
STN1-13myc/TEN1-3xFlag
stn1 (T223A,S250A)-13myc/TEN1-3xFlag
IP
Anti-Myc
Co-IP
Anti-Flag
Input
Anti-Flag
1 2 3

Figure S10

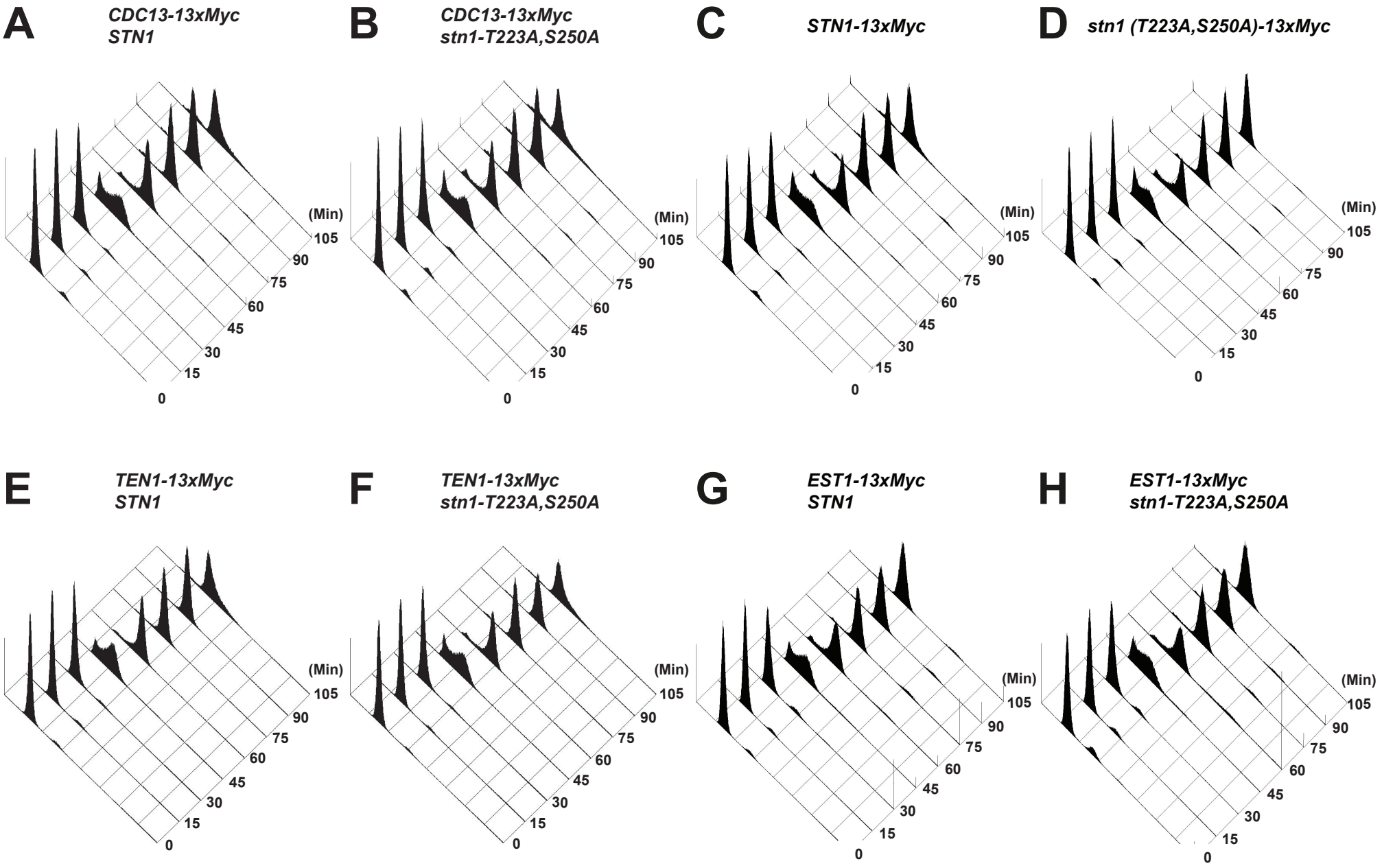

A
CDC13-13xMyc
STN1
B
CDC13-13xMyc
stn1-T223A,S250A
C
STN1-13xMyc
D
stn1 (T223A,S250A)-13xMyc
E
TEN1-13xMyc
STN1
F
TEN1-13xMyc
stn1-T223A,S250A
G
EST1-13xMyc
STN1
H
EST1-13xMyc
stn1-T223A,S250A
(Min)
0
15
30
45
60
75
90
105

Figure S11

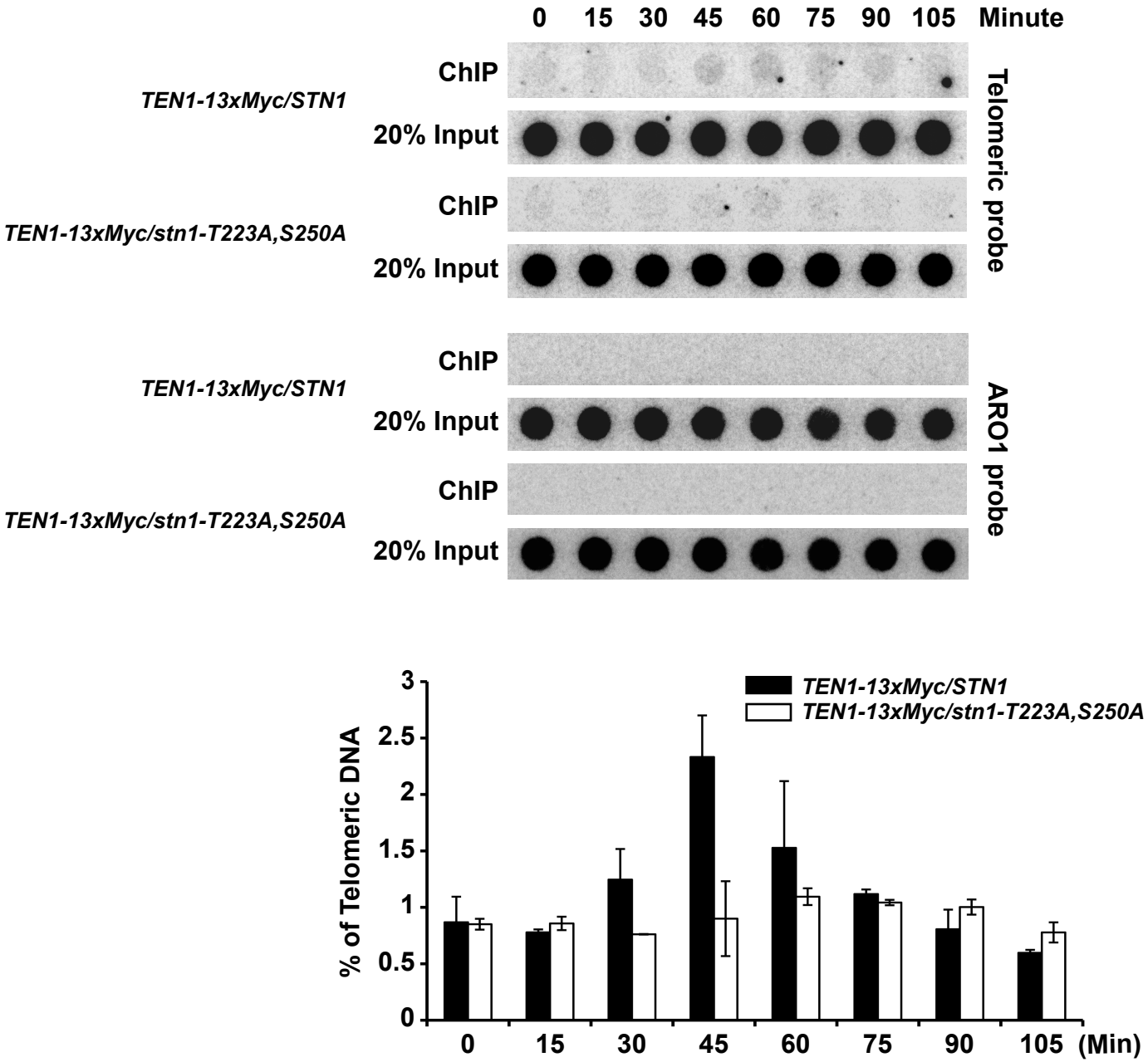

0 15 30 45 60 75 90 105 Minute
TEN1-13xMyc/STN1
ChIP
20% Input
TEN1-13xMyc/stn1-T223A,S250A
ChIP
20% Input
Telomeric probe
TEN1-13xMyc/STN1
ChIP
20% Input
TEN1-13xMyc/stn1-T223A,S250A
ChIP
20% Input
ARO1 probe
TEN1-13xMyc/STN1
TEN1-13xMyc/stn1-T223A,S250A
% of Telomeric DNA
3
2.5
2
1.5
1
0.5
0
0 15 30 45 60 75 90 105 (Min)

Figure S12

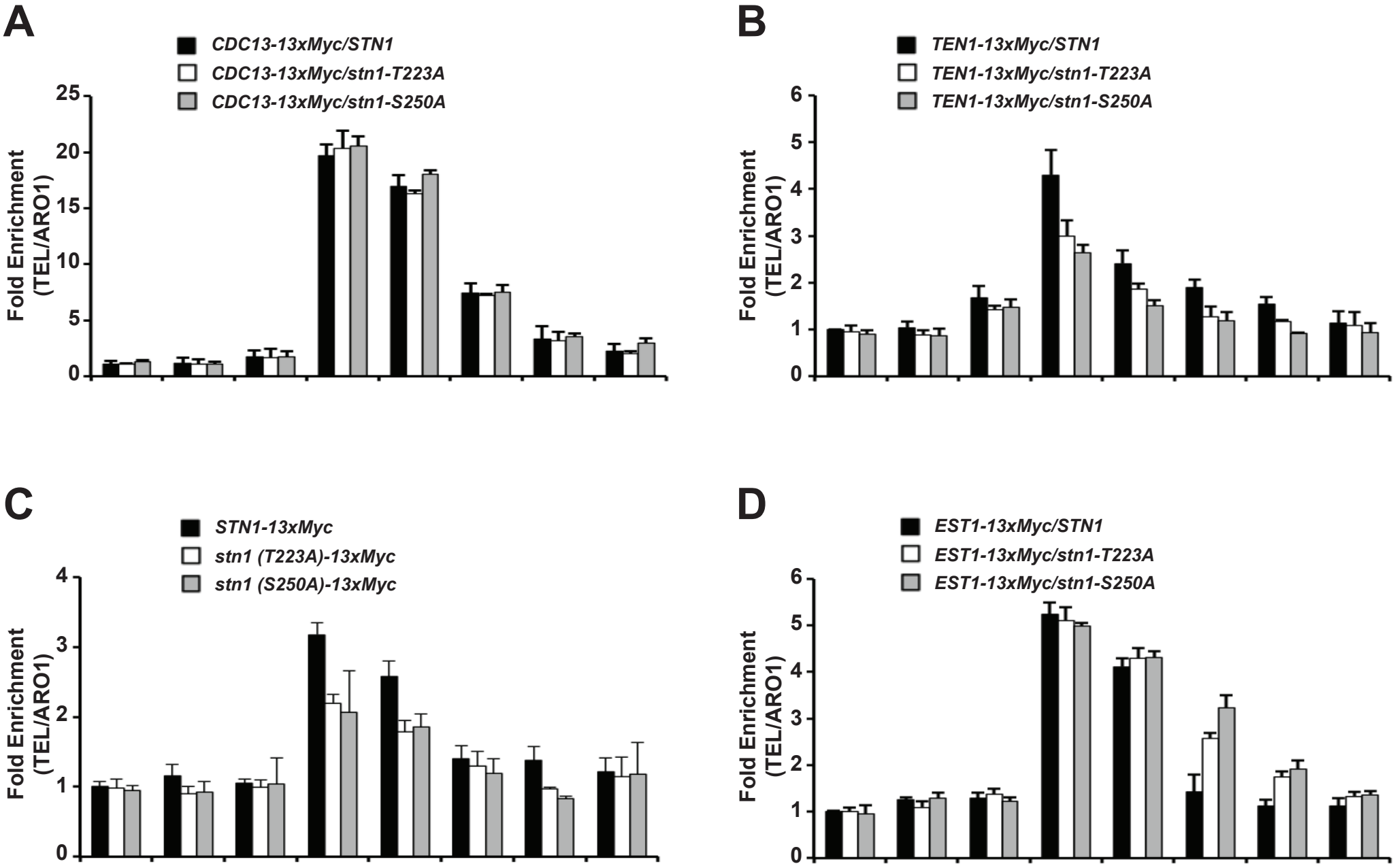
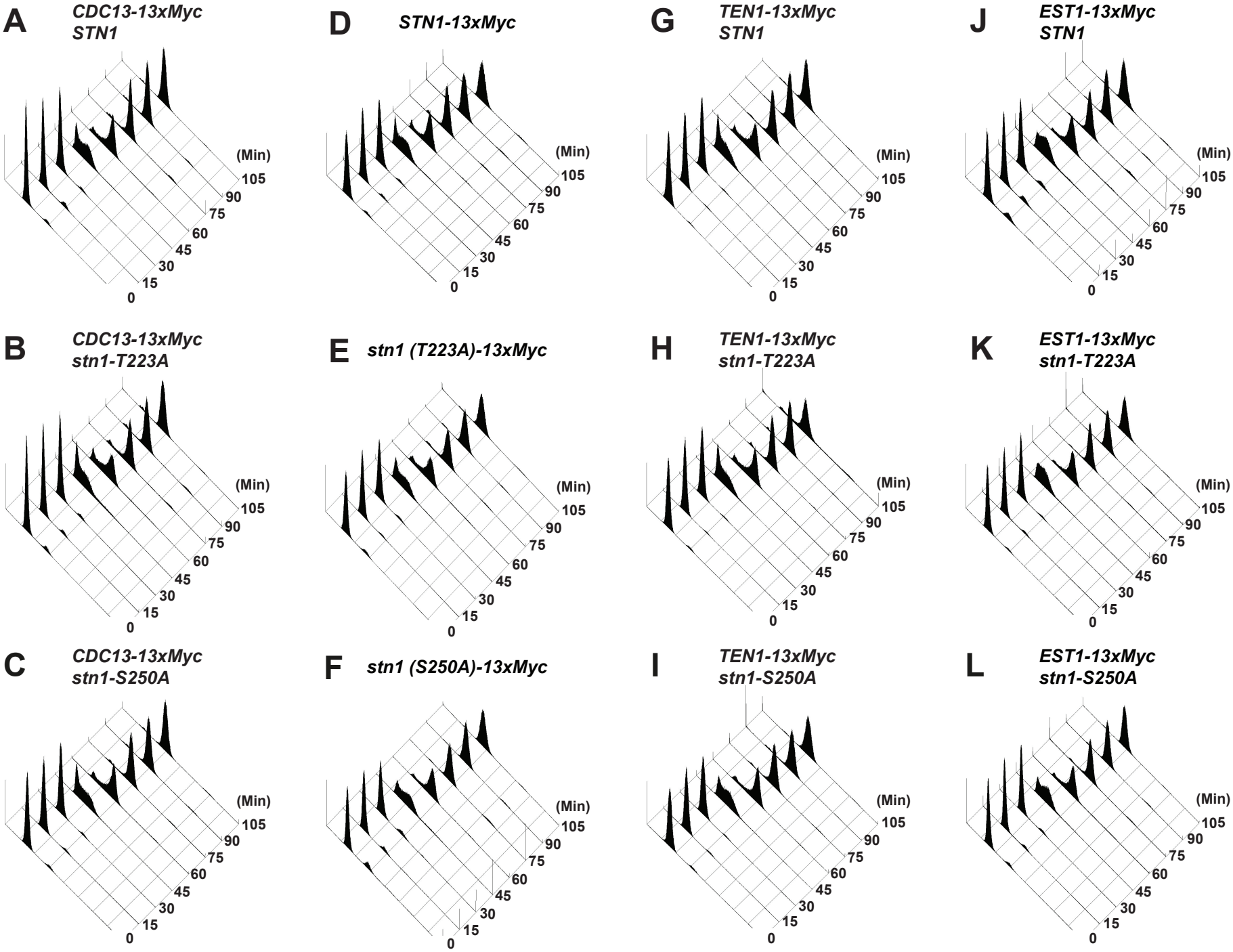

A
CDC13-13xMyc/STN1
CDC13-13xMyc/stn1-T223A
CDC13-13xMyc/stn1-S250A
Fold Enrichment (TEL/ARO1)
0
5
10
15
20
25
B
TEN1-13xMyc/STN1
TEN1-13xMyc/stn1-T223A
TEN1-13xMyc/stn1-S250A
Fold Enrichment (TEL/ARO1)
0
1
2
3
4
5
6
C
STN1-13xMyc
stn1 (T223A)-13xMyc
stn1 (S250A)-13xMyc
Fold Enrichment (TEL/ARO1)
0
1
2
3
4
D
EST1-13xMyc/STN1
EST1-13xMyc/stn1-T223A
EST1-13xMyc/stn1-S250A
Fold Enrichment (TEL/ARO1)
0
1
2
3
4
5
6

Figure S13


A
CDC13-13xMyc
STN1
D
STN1-13xMyc
G
TEN1-13xMyc
STN1
J
EST1-13xMyc
STN1
B
CDC13-13xMyc
stn1-T223A
E
stn1 (T223A)-13xMyc
H
TEN1-13xMyc
stn1-T223A
K
EST1-13xMyc
stn1-T223A
C
CDC13-13xMyc
stn1-S250A
F
stn1 (S250A)-13xMyc
I
TEN1-13xMyc
stn1-S250A
L
EST1-13xMyc
stn1-S250A
(Min)
0
15
30
45
60
75
90
105