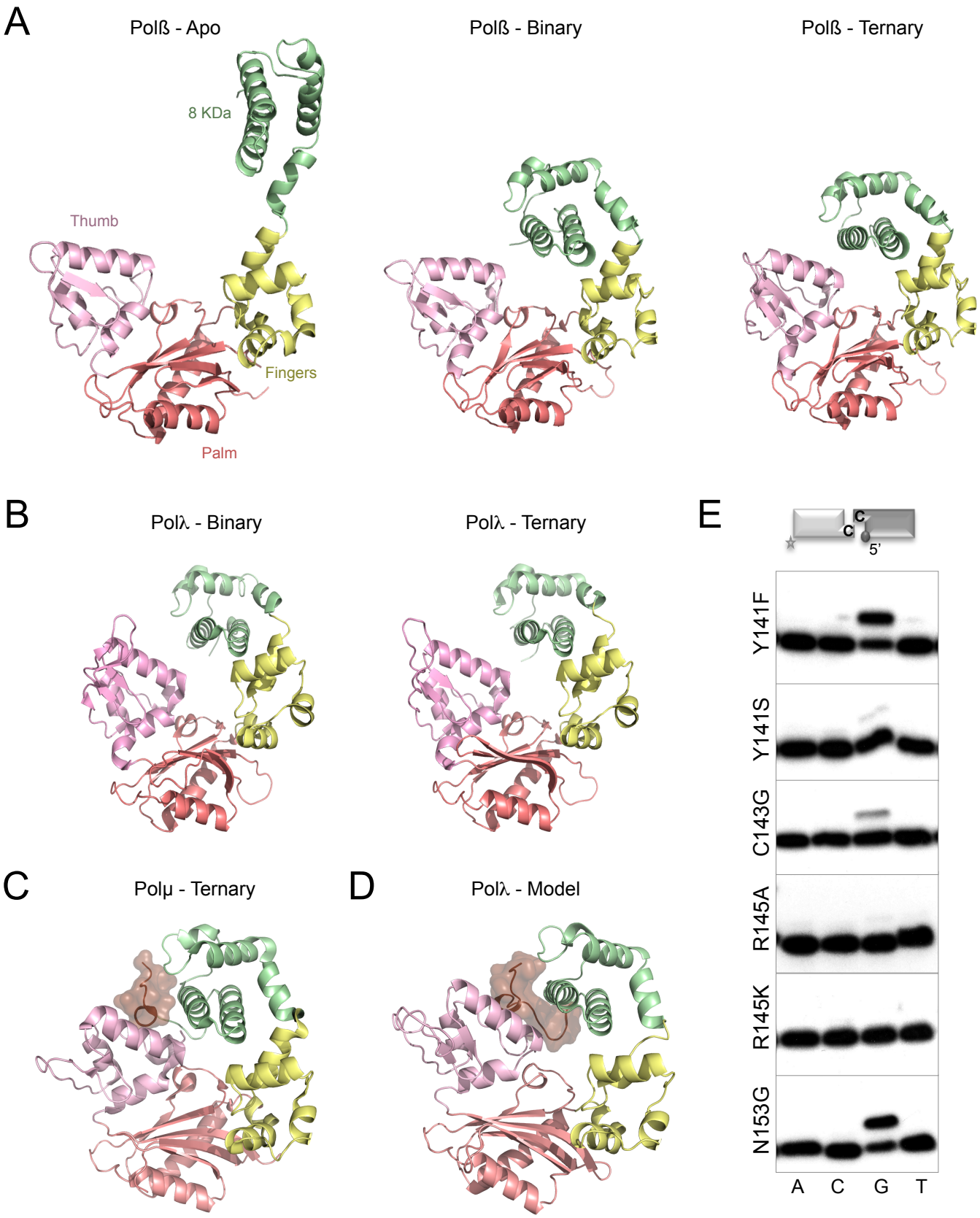
SUPPLEMENTARY MATERIALS AND METHODS

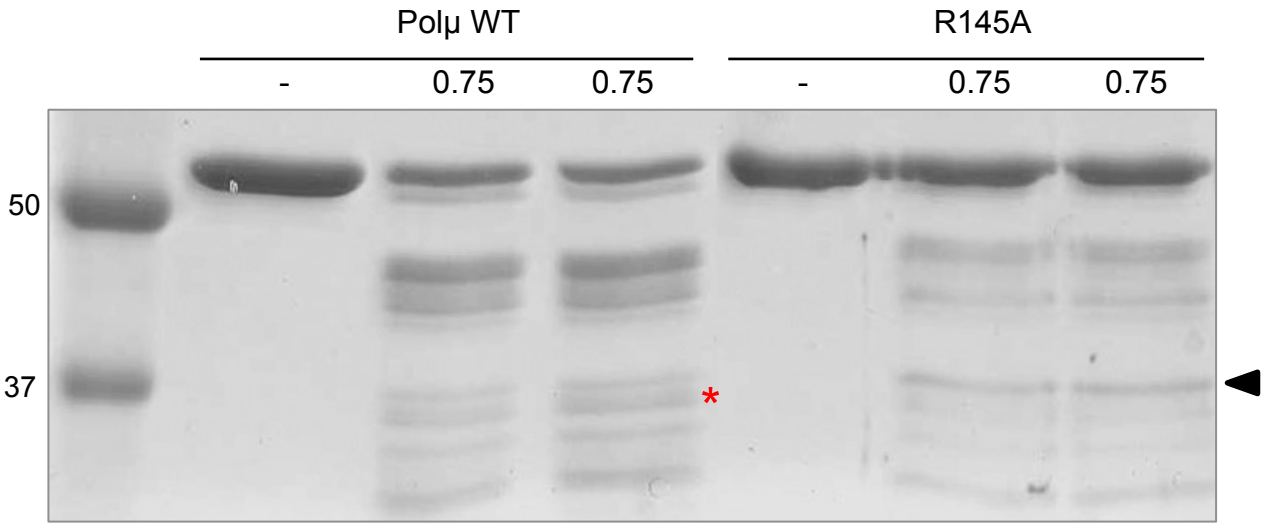
Partial proteolysis. Proteolytic digestion was performed in 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 10% (v/v) glycerol, the indicated amounts of purified polymerase and proteinase K. After incubation for 1 min at 25 ^{o}C the reaction was stopped by addition of 6x loading buffer and analyzed by SDS-12% PAGE for Polλ and 10% PAGE for Polμ. The gels were stained with Coomassie.

Mass spectrometry analysis. The proteomic Molecular Weight Analysis was carried out in the CBMSO protein chemistry facility (a member of ProteoRed network). For the reverse-phase liquid chromatography mass-spectrometry (RP-LC-MS/MS) analysis, gel bands were digested *in situ* with trypsin (5 ng/μl; Promega) for 1 h in an ice-bath. Stained protein gel bands were incubated in 50 mM NH_4HCO_3 (BA) prior to reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide, both in 50 mM BA. Digestion buffer was removed and gels were covered again with 50 mM BA and incubated for 12 h at 37ºC.
Whole supernatants were dried down and then desalted onto ZipTip C18 Pipette tips (Millipore) until the mass spectrometric analysis. Peptide mass fingerprinting was conducted using an Autoflex™(Bruker Daltonics, Bremen, Germany) mass spectrometer in a positive ion reflector mode employing 2,5-dihydroxybenzoic acid as matrix and an AnchorChip™surface target (Bruker Daltonics). Peak identification and monoisotopic peptide mass assignation were performed automatically using Flexanalysis™software, version 2.2 (Bruker Daltonics). Database searches were performed using MASCOT http://matrixscience.com against the NCBI non-redundant protein sequence database http://www.ncbi.nih.gov. The selected search parameters were as follows: tolerance of two missed cleavages, carbamidomethylation (Cys) and oxidation (Met) as fixed and variable modifications, respectively, and setting peptide tolerance to 100 ppm after close-external calibration. A significant MASCOT probability score ($p < 0.05$) was considered as condition for successful protein identification.


A
Polß - Apo
Polß - Binary
Polß - Ternary
8 KDa
Thumb
Fingers
Palm
B
Polλ - Binary
Polλ - Ternary
C
Polμ - Ternary
D
Polλ - Model
E
C
C
5'
Y141F
Y141S
C143G
R145A
R145K
N153G
A
C
G
T

Supplementary Figure 1. Comparison of domain movements during the catalytic cycle in different members of the X family. A) Cartoon representations of the apoenzyme, binary and ternary complexes of Polß, showing the movements of the different domains, colored as follows: 8 kDa in green, fingers in yellow, palm in red and thumb in pink. B) and C) Cartoon representations of the binary and ternary complex of Polλ and of the ternary complex of Polμ with the same domain coloring as in A). D) Structural model of Polλ core containing the brooch, with the same domain coloring as in A). The brooch is shown in cartoon and semitransparent surface colored in brown. E) NHEJ reactions were performed with 200 nM Polμ mutants and using two sets of substrates: the labelled substrates contain 1C and 1D-NHEJ, and the cold substrates, 2C and 2D-NHEJ. The dark spheres indicate the presence of a 5'-P group, which forces binding of this substrate to the 8 kDa domain of Polμ and thus its usage as the downstream part of the break. ddNTPs were added separately at 1 mM in the presence of 2.5 mM $MgCl_2$. After electrophoresis, labelled fragments were detected by autoradiography.

A



B

MLPKRRRARVGSPSGDAASSTPPSTRFPGVAIYLVEPRMGRSRRAFLTGLARSKGFRVLDACS
SEATHVVMEETSAEEAVSWQERRMAAAPPGCTPPALLDISWLTESLGAGQPVPVECRHRLEVA
GPRKGPLSPAWMPAYACQRPTPLTHHNTGLSEALEILAEAAGFEGSEGRLLTFCRAASVLKAL
PSPVTTLSQLQGLPHFGEHSSRVVQELLEHGVCEEVERVRRSERYQTMKLFTQIFGVGVKTAD
RWYREGLRTLDDLREQPQKLTQQQKAGLQHHQDLSTPVLRSDVDALQQVVEEAVGQALPGATV
TLTGGFRRGKLQGHDVDFLITHPKEGQEAGLLPRVMCRLQDQGLILYHQHQHSCCESPTRLAQ
QSHMDAFERSFCIFRLPQPPGAAVGGSTRPCPSWKAVRVDLVVAPVSQFPFALLGWTGSKLFQ
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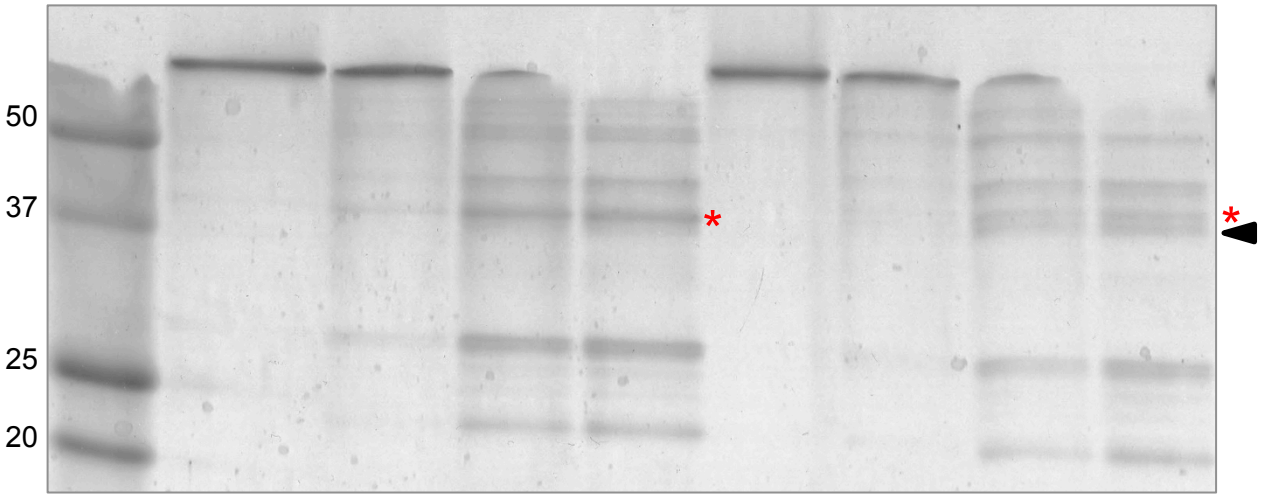
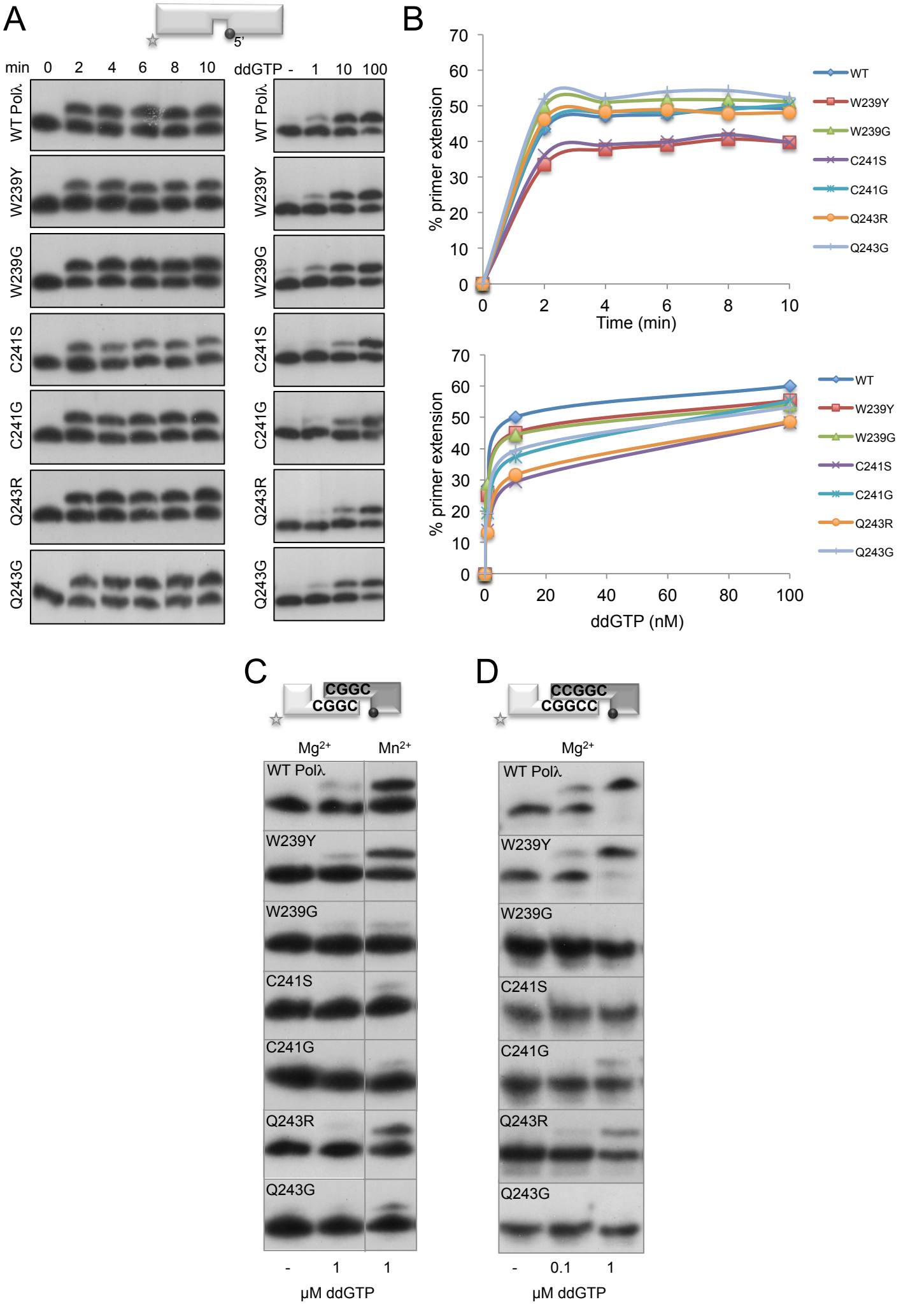
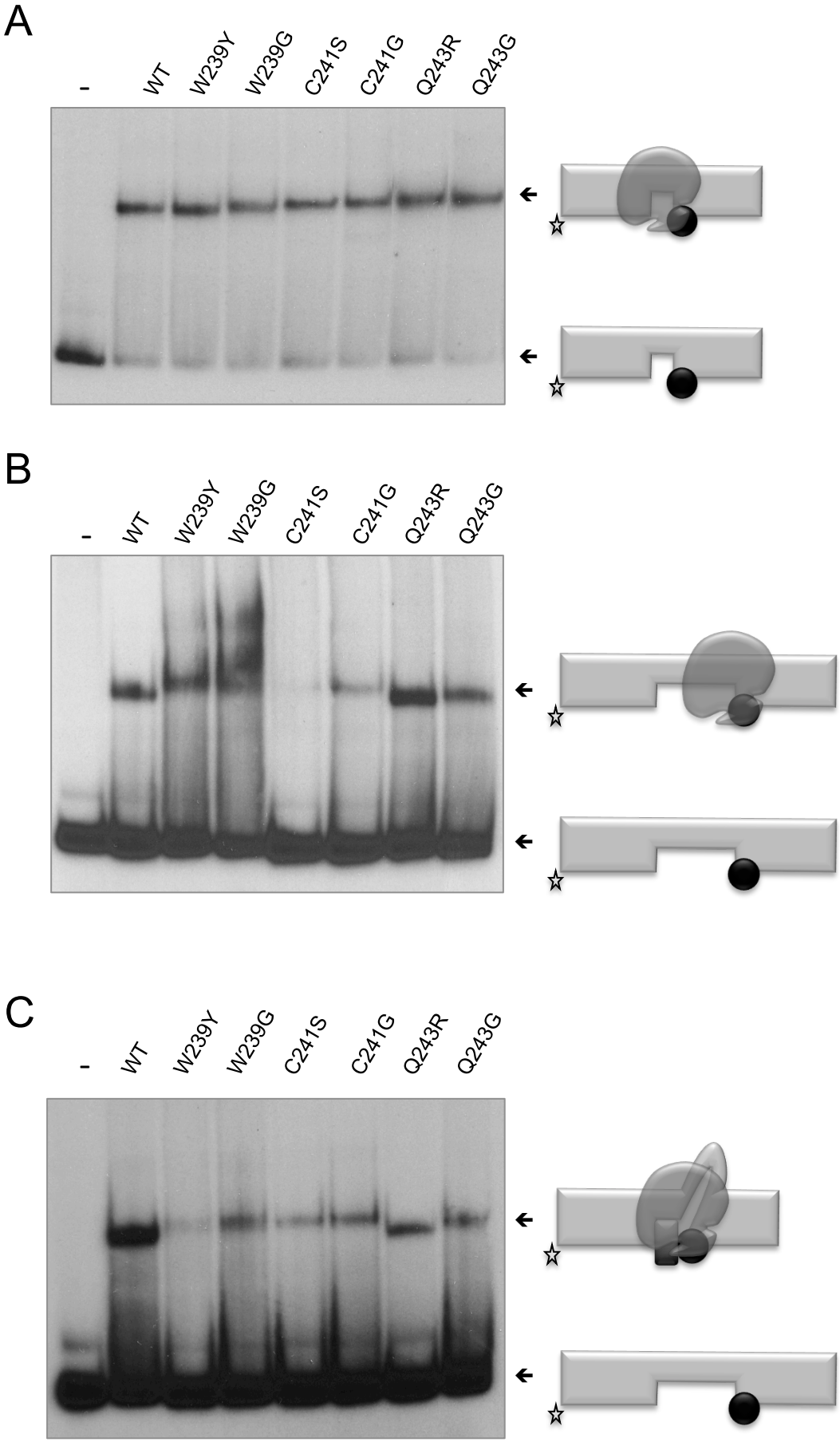
C



Supplementary Figure 2. Proteolytic analyses of brooch mutants. A) Partial proteolysis of purified wild-type Polμ and R145A mutant (4 μg each) were performed as indicated in Materials and Methods, incubation time was 1 min in the presence of the indicated amounts (ng) of proteinase K. The arrowhead indicates the new band appearing in the brooch mutant. The red asterisk indicates the size of the polymerase core. B) Sequence of wild-type Polμ, showing the peptides detected by mass-spec in bold. In black, the peptides found in both samples, wild-type and mutant. In red, the peptides that were only found in the wild-type sample but not in the mutant band indicated in A). In orange, the peptides found in this mutant band in very low amount. The brooch is indicated in blue&bold. C) Partial proteolysis of purified wild-type Polλ and W239G mutant (5 μg) was performed with the indicated amounts of proteinase K.


A
5'
min 0 2 4 6 8 10
ddGTP - 1 10 100
WT Polλ
W239Y
W239G
C241S
C241G
Q243R
Q243G
B
% primer extension
Time (min)
ddGTP (nM)
WT
W239Y
W239G
C241S
C241G
Q243R
Q243G
C
CGGC
CGGC
Mg2+
Mn2+
μM ddGTP
D
CCGGC
CGGCC
Mg2+
μM ddGTP

Supplementary Figure 3. The brooch in Polλ facilitates polymerization on NHEJ substrates. A) Gap-filling reactions were performed as described in Materials and Methods with the wild-type Polλ and the indicated mutants (50 nM) in the presence of 2.5 mM $MgCl_2$ at 30 ºC, using a gapped substrate containing the oligonucleotides SP1C, T13C and DG1-P. Left panels show time courses performed with 10 nM ddGTP, right panels show ddGTP concentration courses incubated for 20 min. B) Quantification of the experiments shown in A). C) NHEJ reactions were performed as described with the wild type Polλ and the indicated mutants (500 nM) in the presence of the indicated concentrations of ddGTP and either 2.5 mM $MgCl_2$ or 0.1 mM $MnCl_2$ at 30ºC for 60 min. Two separate sets of substrates were used: the labelled substrate (light grey) was formed by hybridization of D3 and D1, and the cold substrate (dark grey), by hybridization of D4 with D2-P. D) NHEJ reactions were performed as described in Materials and Methods with the wild type Polλ and the indicated mutants (500 nM) in the presence of the indicated concentrations of ddGTP and 2.5 mM $MgCl_2$ at 30ºC for 60 min. Two separate sets of substrates were used: the labelled substrates (light grey) were formed by hybridization of D3+C with D1, and the cold substrates (dark grey) by hybridization of D4GC with D2-P.


A
-
WT
W239Y
W239G
C241S
C241G
Q243R
Q243G
B
-
WT
W239Y
W239G
C241S
C241G
Q243R
Q243G
C
-
WT
W239Y
W239G
C241S
C241G
Q243R
Q243G

Supplementary Figure 4. The brooch in Polλ is implicated in correct binding to long gaps. A) EMSA was performed for the wild-type Polλ and the indicated mutants (50 nM) using 3 nM gapped substrate containing the oligonucleotides FP-P, FP-T and FP-D. Reactions were incubated for 15 min at 30 ºC. After electrophoresis, gel was dried and the labelled fragments were detected by autoradiography. B) EMSA was performed as in A) for the wild-type Polλ and the indicated mutants (300 nM) using 3 nM gapped substrate containing the oligonucleotides FP-P, FP-T5 and FP-D. C) EMSA was performed as in B) but adding 1 μM ddTTP and 2.5 mM $MgCl_2$.

FT-T (5'-GGCAGCTTGGATCTTGTCGAAAAACGTCAACATTCGCCTAGGCTTCGGCAAT ACTGAGGTCTTGTCGAAAAACGTCAACATTGCGTGGCC)

FT-P (5'-GGCCACGCAATGTTGACGTTTTTCGACAAGACCTCAGTAT)

FT-D (contains a 5'P group)
(5'-GCCGAAGCCTAGGCGAATGTTGACGTTTTTCGACAAGATCCAAGCTGCC)

FT-T5 (5'- GGCAGCTTGGATCTTGTCGAAAAACGTCAACATTCGCCTAGGCTTCGGCAG CATATACTGAGGTCTTGTCGAAAAACGTCAACATTGCGTGGCC).

A

Polμ

H.sapiens	YACQRPTPL
P.abelii	YACQRPTPL
P.anubis	YACQRPTPL
R.norvegicus	YACQRPSPL
M.musculus	YACQRPSPL
B.taurus	YACQRPTPL
S.scrofa	YACQRPTPL
C.griseus	YACQRPTAL
D.rerio	YACQRRTPL
X.tropicalis	YGCQRRTPL

TdT

H.sapiens	YACQRRTTL
P.abelii	YACQRRTTL
M.mulatta	YACQRRTTL
S.scrofa	YACQRRTTL
B.taurus	YACQRKTTL
C.lupus	YACQRRTTL
M.musculus	YACQRRTTL
M.domestica	YACQRRTTL
D.rerio	YACQRRTTL
X.laevis	YACQRCTTL

Polλ

H.sapiens	WVCAQPSSQ
P.abelii	WVCAQPSSQ
M.mulatta	WVCAQPSSQ
R.norvegicus	WVCAQPSSQ
M.musculus	WVCAQPSSQ
B.taurus	WVCAQPSSQ
M.domestica	WVCAQPSSQ
C.lupus	WVCAQPSSQ
D.rerio	WVCSQPSTT
X.tropicalis	WVCAHSSES

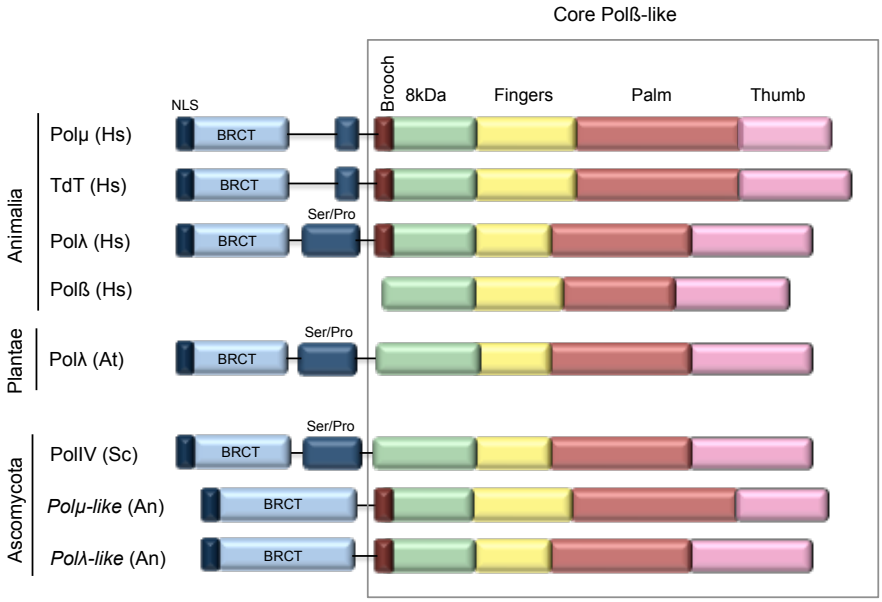
Ascomycota Polμ-like

S.pombe	YACQRKTPL
D.squalens	YACQRPSPL
S.hirsutum	FACERPSPL
C.cinerea	YVCQRASPL
T.versicolor	YACQRASPL
S.commune	YACCRASPL
P.indica	YCCQRQTPL
L.bicolor	YACKRASPL
U.maydis	YACRRPSPL
M.osmundae	YSCMRLSPR
C.militaris	YSCQRPTPM

Ascomycota Polλ-like

A.nidulans	FVCMQKHD
A.clavatus	FTCMQKHD
A.oryzae	FACVQKHD
A.flavus	FACVQKHD
A.niger	FACMQKHD
A.terreus	FACMRAHD
A.capsulatus	FSCMHKHD
A.otae	FSCMGKMD
T.rubrum	FTCMNKMD
V.dahliae	FACTRGGL

B



Supplementary Figure 5. Evolution of the brooch. A) Multiple sequence alignment of the N-terminal boundary of the 8 kDa domain (containing the brooch) of different PolXs belonging to the three higher eukaryotic kingdoms of life: animals, fungi and plants. These have been used to generate the sequence logos in figure 8. B) Schemes showing the domains present in PolXs from different kingdoms.