**SUPPLEMENTARY INFORMATION**

# Eukaryotic DNA polymerases require an iron-sulfur cluster for active complex formation

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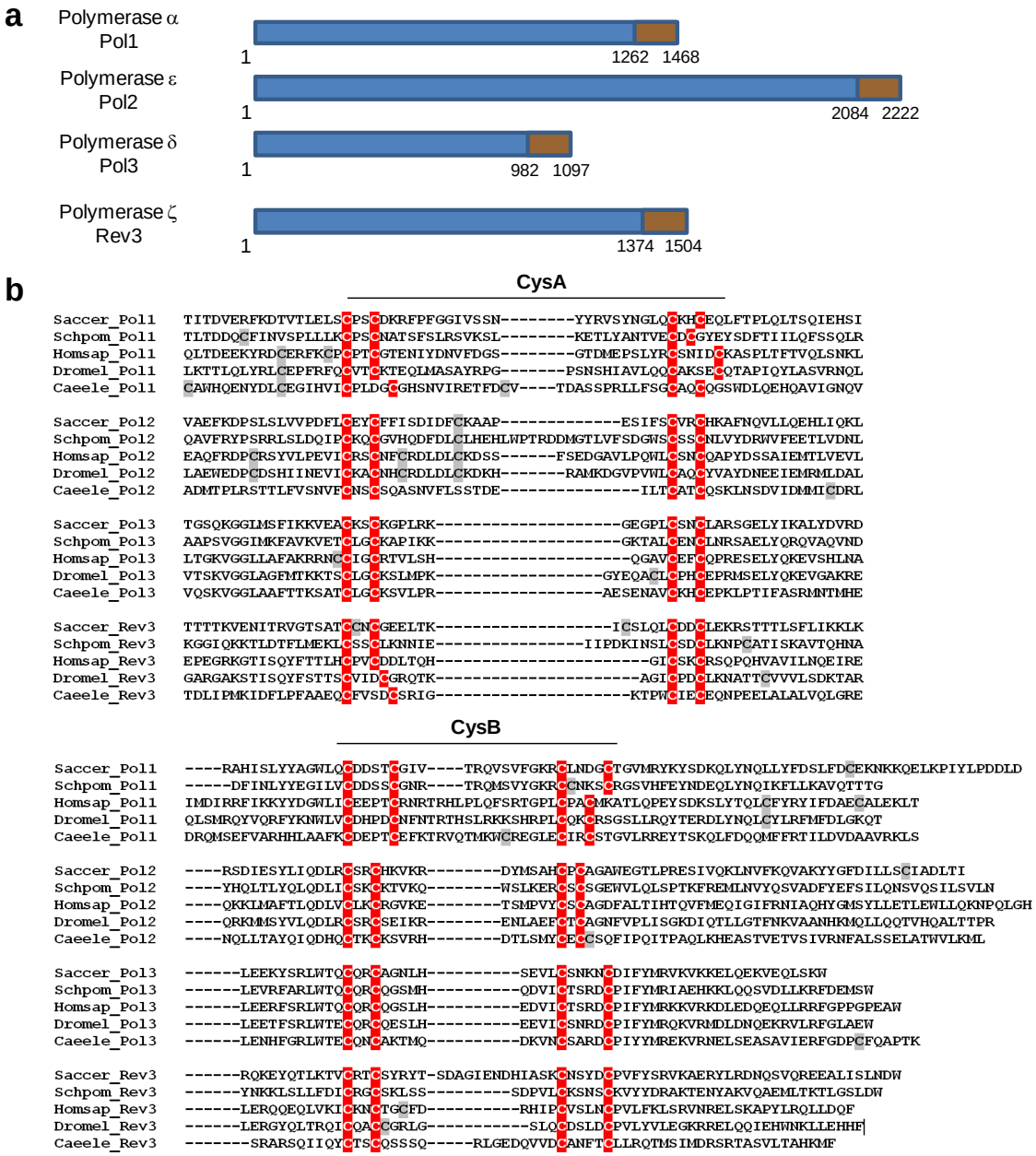
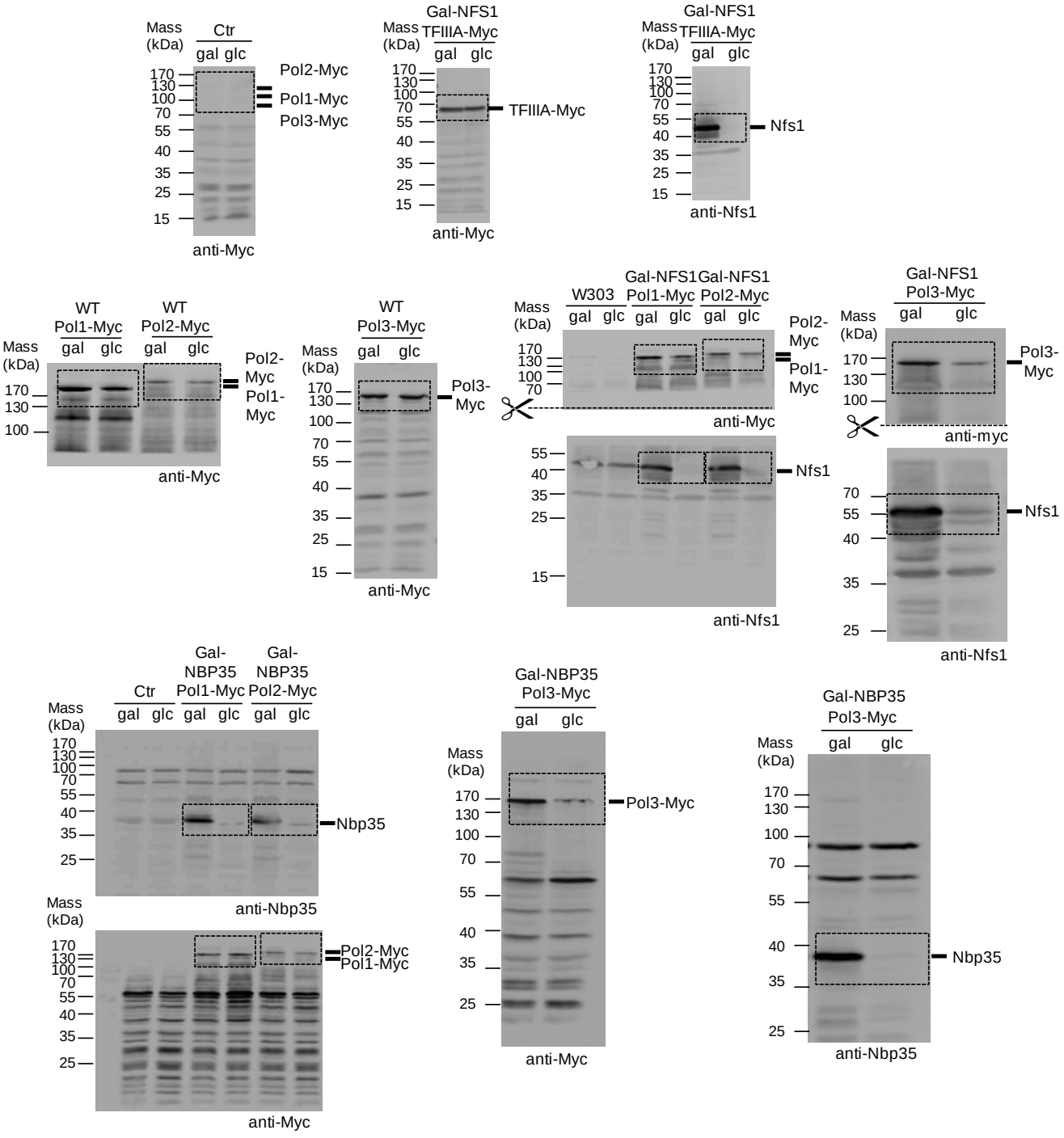
Supplementary Methods and References

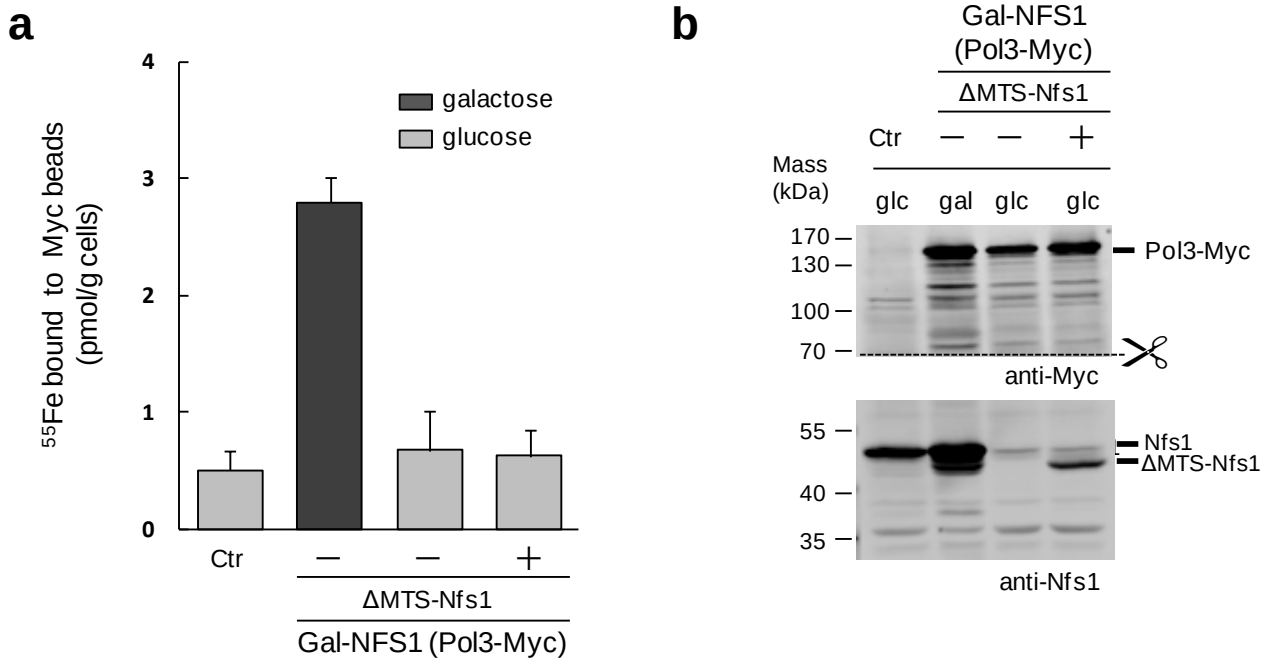
## Supplementary Results



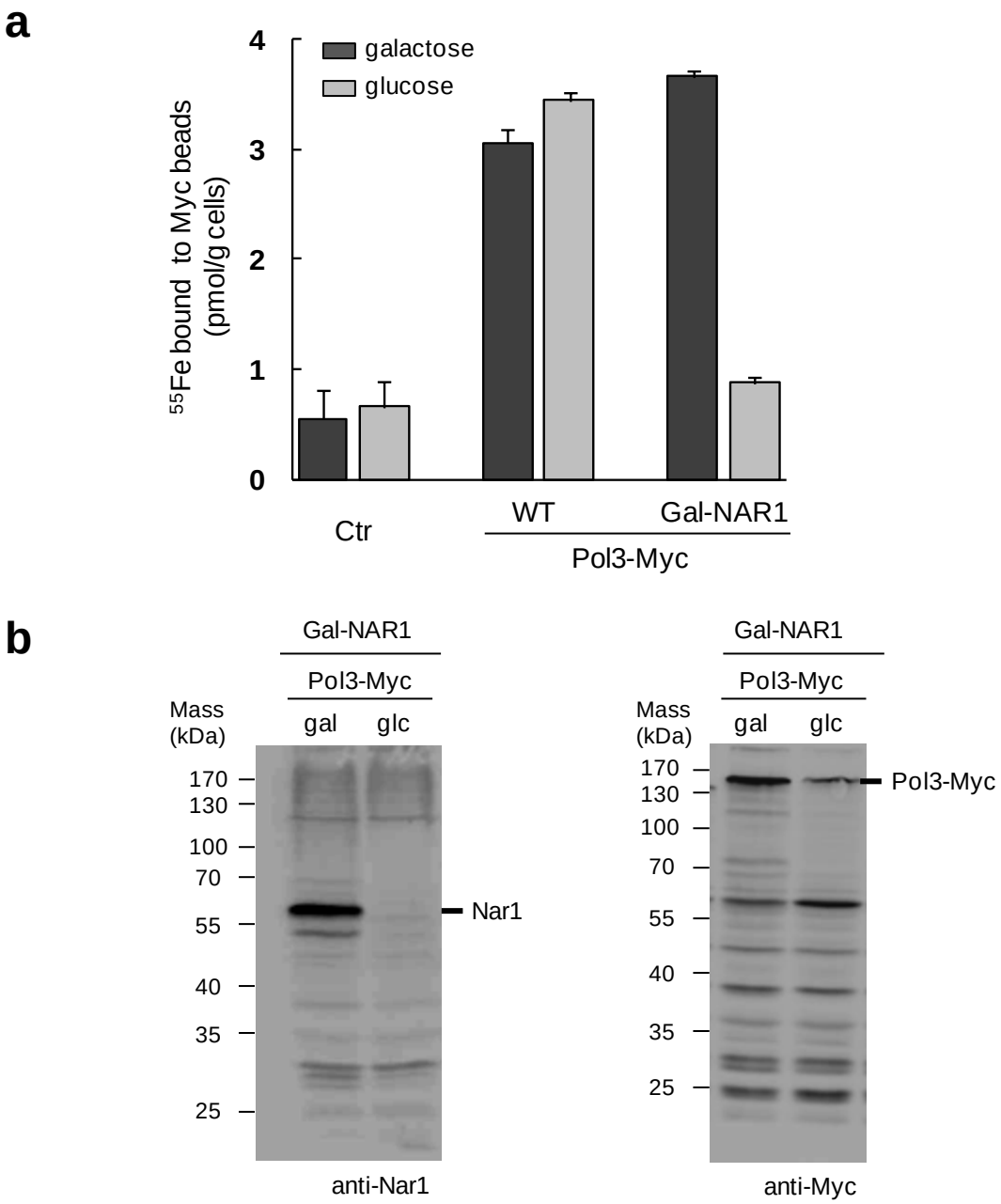

**Supplementary Figure 1. Eukaryotic B-family DNA polymerases contain eight conserved cysteine residues in their C-terminal domain (CTD). a,** Schematic representation of the *Saccharomyces cerevisiae* B-family DNA polymerases with amino acid positions demarking the CTDs (in brown). **b**, Amino acid sequence alignment of the CTDs shows two motifs of four cysteine residues, CysA and CysB which are involved in metal binding. Abbreviations: Saccer, *S. cerevisiae*; Schpom, *Schizosaccharomyces pombe*; Homsap, *Homo sapiens*; Dromel, *Drosophila melanogaster*; Caeele, *Caenorhabditis elegans*. Cysteine residues which are considered to coordinate metal ions have been highlighted in red, other cysteine residues in grey.



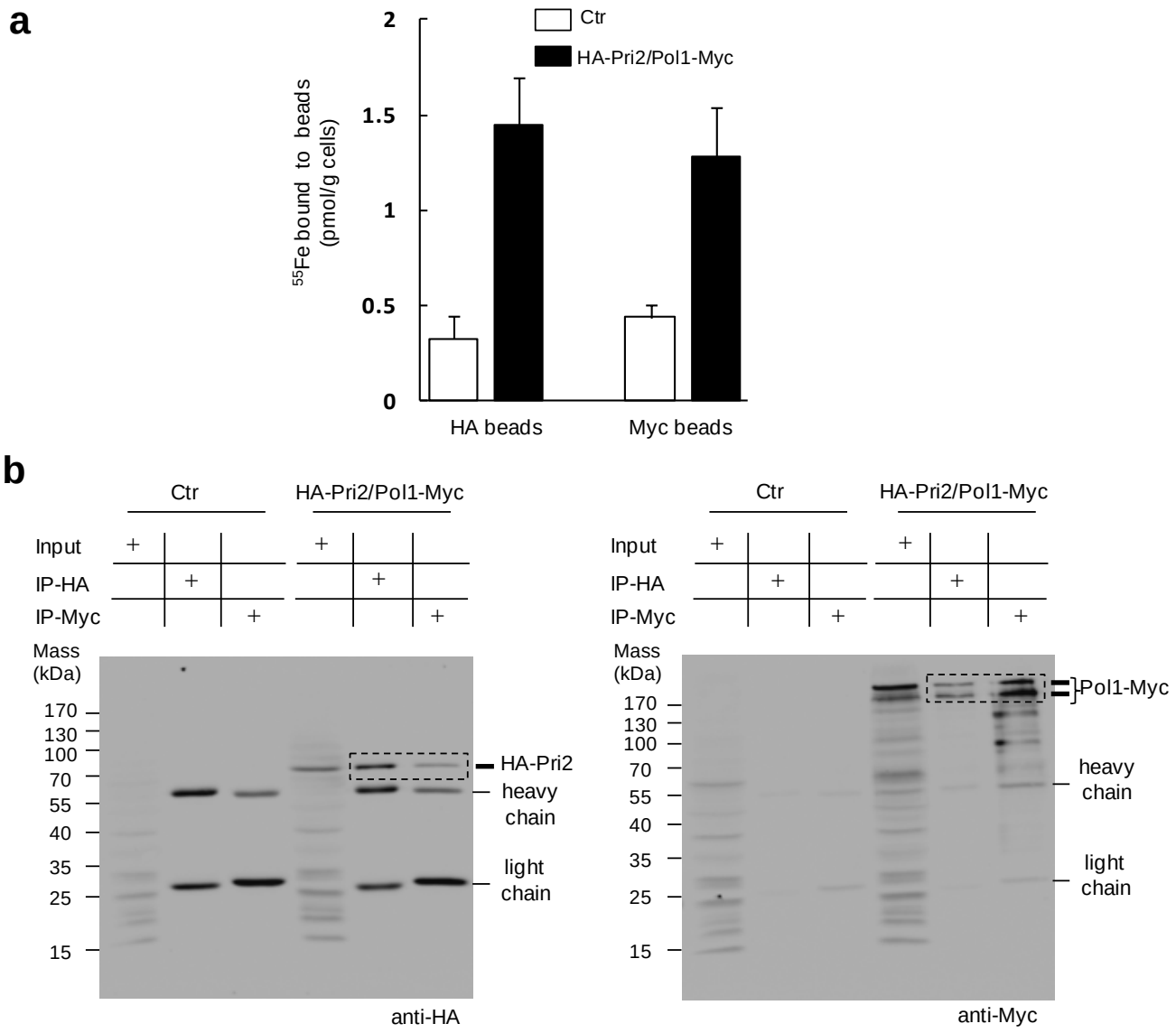

**Supplementary Figure 2.** Immunoblots for the indicated (tagged) proteins in the cell extracts subjected to $^{55}$Fe incorporation in Figure 1a. Proteins were visualized by immunostaining using specific antibodies and chemiluminescence detection by a CCD camera. Hatched boxes denote areas of particular interest (target of immunoprecipitation or protein depleted). The positions of molecular mass markers are indicated.



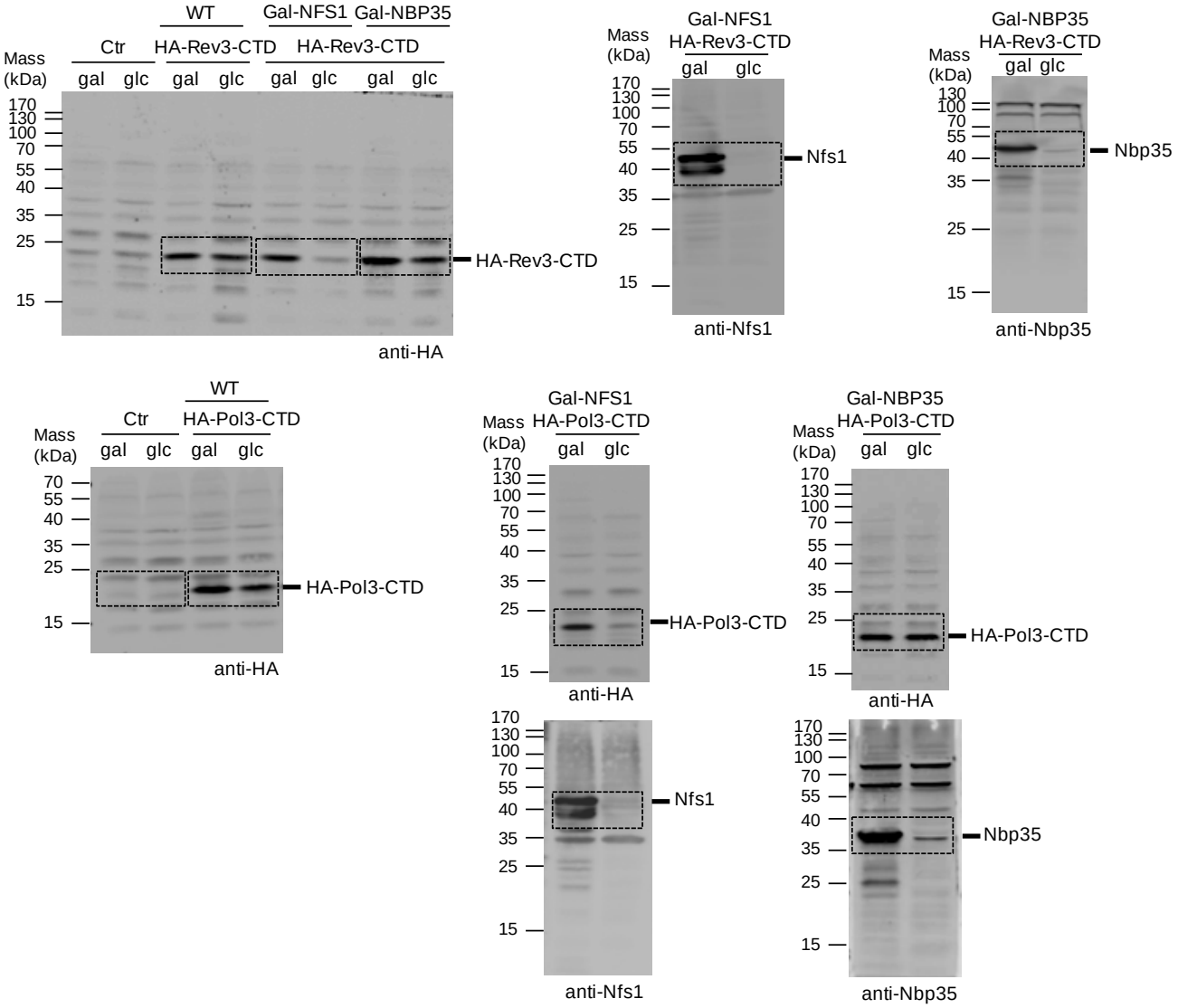

**Supplementary Figure 3. A cytosolic version of cysteine desulfurase Nfs1 does not support Fe-S cluster assembly on Pol3-Myc.** Gal-NFS1 cells with the *NFS1* gene under the control of the *GAL1-10* (Gal) promoter were transformed with a plasmid (+) encoding a cytosolic form[1] of Nfs1 (lacking amino acid residues 1-94, ΔMTS-Nfs1) or with an empty plasmid (-). **a,** $^{55}$Fe radiolabelling and immunoprecipitation was performed as in Fig. 1 using wild-type cells (Ctr) or the indicated Gal-NFS1 cells carrying genomically Myc-tagged Pol3. The data for wild-type cells and the galactose bar correspond to Fig. 1a in the main text. **b,** Cell extracts from **a** were analyzed by immunoblotting to show the presence of Pol3-Myc, the depletion of mitochondrial Nfs1, and the synthesis of the slightly shorter ΔMTS-Nfs1 protein. Error bars, s.d. ($n \geq 3$).



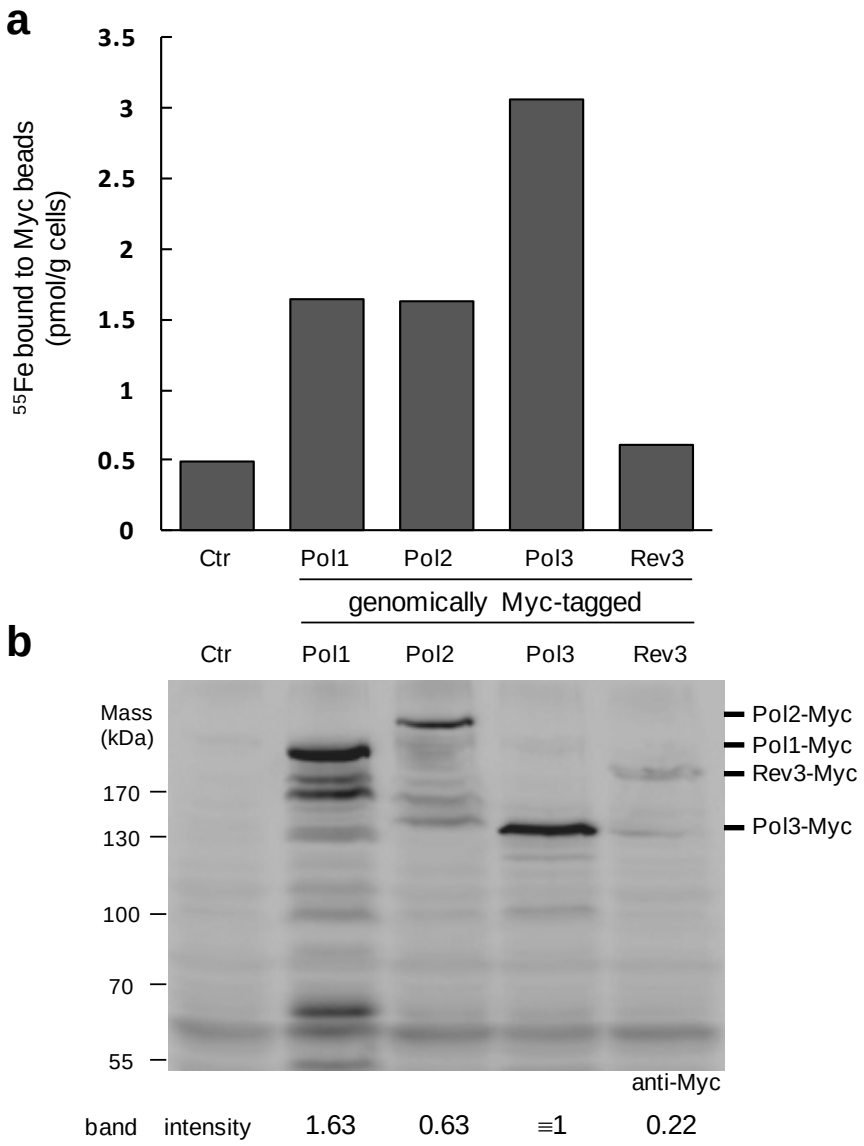

**Supplementary Figure 4. Depletion of the cytosolic CIA protein Nar1 leads to loss of radioactive iron associated with Pol3-Myc. a,** $^{55}$Fe radiolabelling and immunoprecipitation was performed as in Fig. 1 using wild-type cell extracts (Ctr), or extracts from wild-type (WT) and Gal-NAR1 cells carrying genomically Myc-tagged Pol3. Gal-NAR1 cells express the *NAR1* gene under the control of the *GAL1-10* (Gal) promoter. The data for Ctr and WT correspond to Fig. 1a in the main text. **b,** Immunoblots of cell extracts from **a**. The indicated (tagged) proteins were visualized by immunostaining using specific antibodies and chemiluminescence detection by a CCD camera. The positions of molecular mass markers are indicated. Error bars, s.d. ($n \geq 3$).



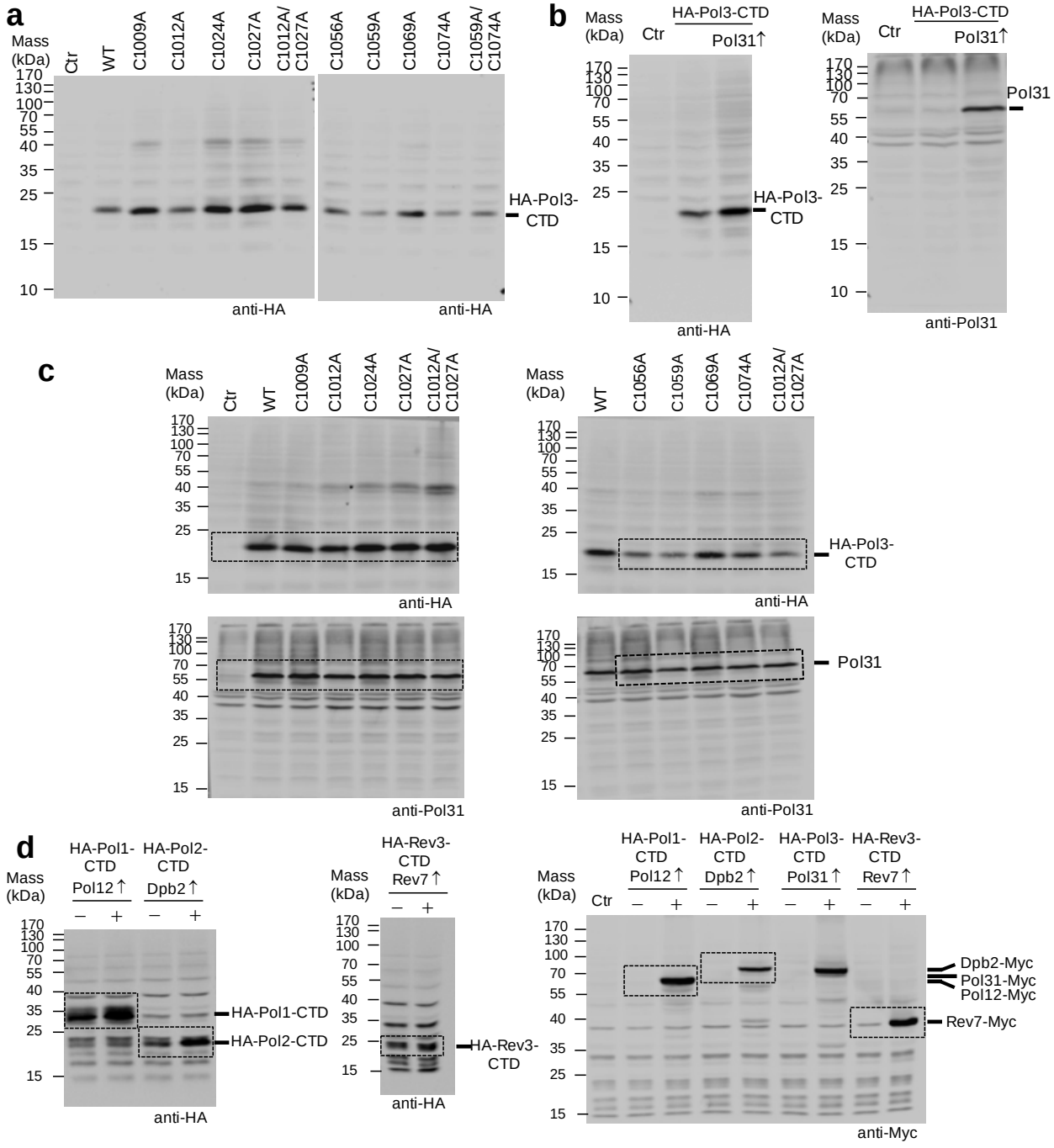

**Supplementary Figure 5. Comparison of $^{55}$Fe associated with endogenous levels of Pol1 and the [4Fe-4S] cluster-containing primase subunit Pri2. a,** $^{55}$Fe radiolabelling and immunoprecipitation was performed as in Fig. 1 using wild-type cells (Ctr) or a yeast strain with genomically integrated Pol1-Myc/HA-Pri2. Cells were grown in iron-free SC medium supplemented with galactose. **b,** Co-immunoprecipitation of HA-Pri2 and Pol1-Myc with anti-HA or anti-Myc beads. The strains described in **a** were grown on regular SC medium supplemented with galactose and lysates were prepared with glass beads (Input). After centrifugation, the obtained supernatants were separated into two aliquots and incubated with anti-HA or anti-Myc agarose beads. The beads were washed (IP-HA and IP-Myc) and analyzed by SDS-PAGE and immunoblotting using the indicated monoclonal antibodies. Bands labeled heavy and light chain are cross-reactive IgG subunits released from beads. The intensity of the HA-Pri2 band in the Myc beads was 20% of the same band in the HA beads (boxed bands in left blot) as quantified from the chemiluminescence recording of the CCD camera. Error bars, s.d. ($n \geq 3$).



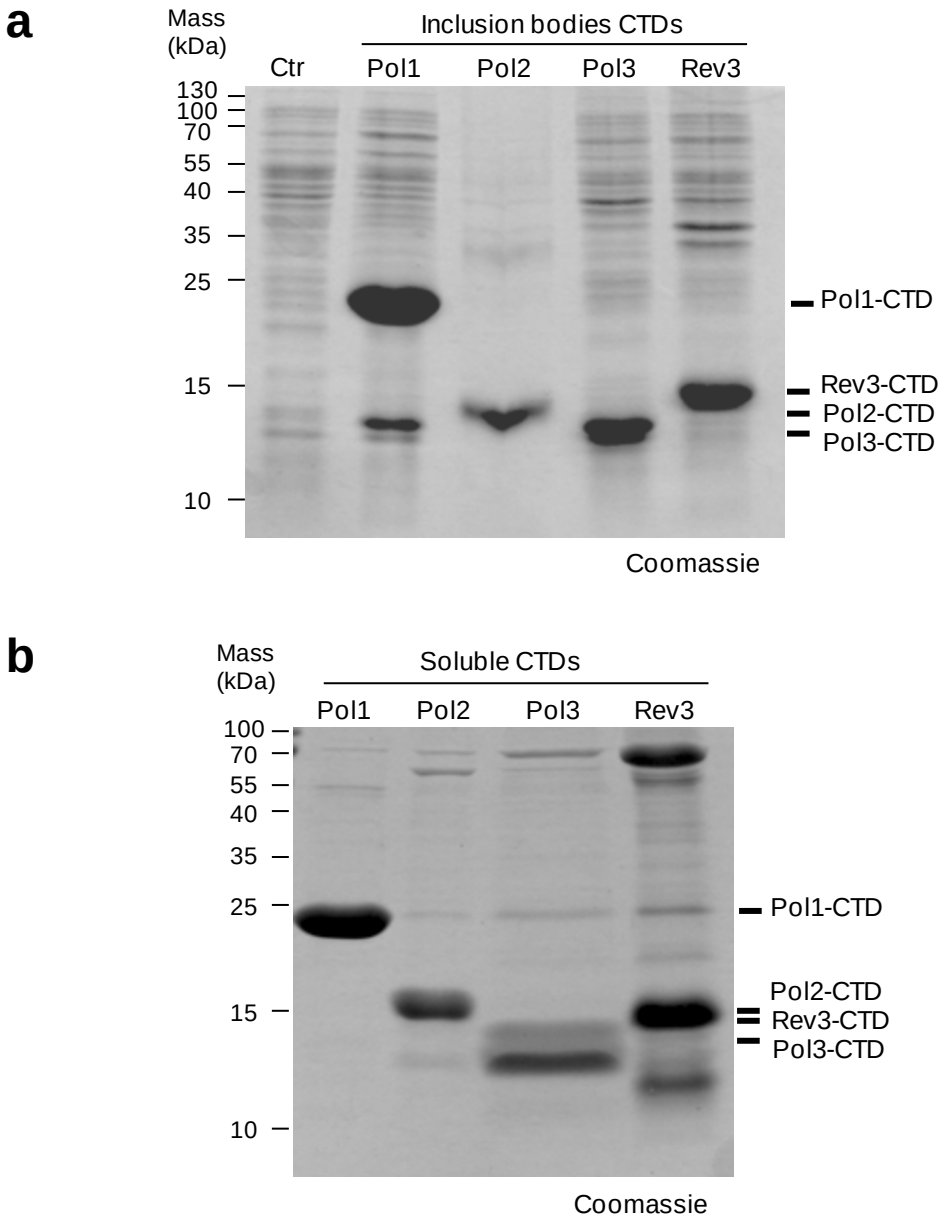

**Supplementary Figure 6.** Immunoblots for the indicated (tagged) proteins in the cell extracts subjected to $^{55}$Fe incorporation in Figure 1b. Proteins were visualized by immunostaining using specific antibodies and chemiluminescence detection by a CCD camera. Hatched boxes denote areas of particular interest (target of immunoprecipitation or protein depleted). The positions of molecular mass markers are indicated.



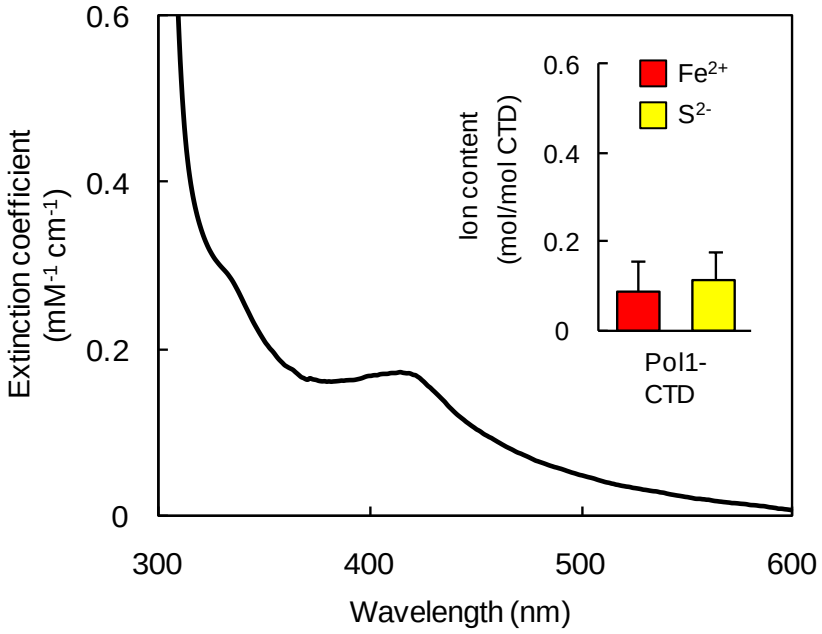

**Supplementary Figure 7. Comparison of the expression levels of yeast B-family DNA polymerases shows that Rev3 has a low abundance. a,** Wild-type yeast cells (Ctr) and cells carrying genomically Myc-tagged polymerases were grown in Fe-free galactose-containing SC medium. Cell extracts were prepared and an immunoprecipitation of the Myc-tagged, indicated polymerases was performed. Co-immunoprecipitated $^{55}$Fe was analyzed by scintillation counting. Data for Ctr, Pol1, Pol2 and Pol3 correspond to the Ctr and the WT bars of Fig. 1a in the main text. **b,** The indicated (tagged) proteins in cell extracts from **a** were visualized by immunostaining using specific antibodies and chemiluminescence detection by a CCD camera. The summed intensities (bottom) of discrete bands with molecular mass above 120 kDa were normalized to the intensity of a cross-reactive band at ~60 kDa. The positions of molecular mass markers are indicated. Error bars, s.d. ($n \geq 3$).



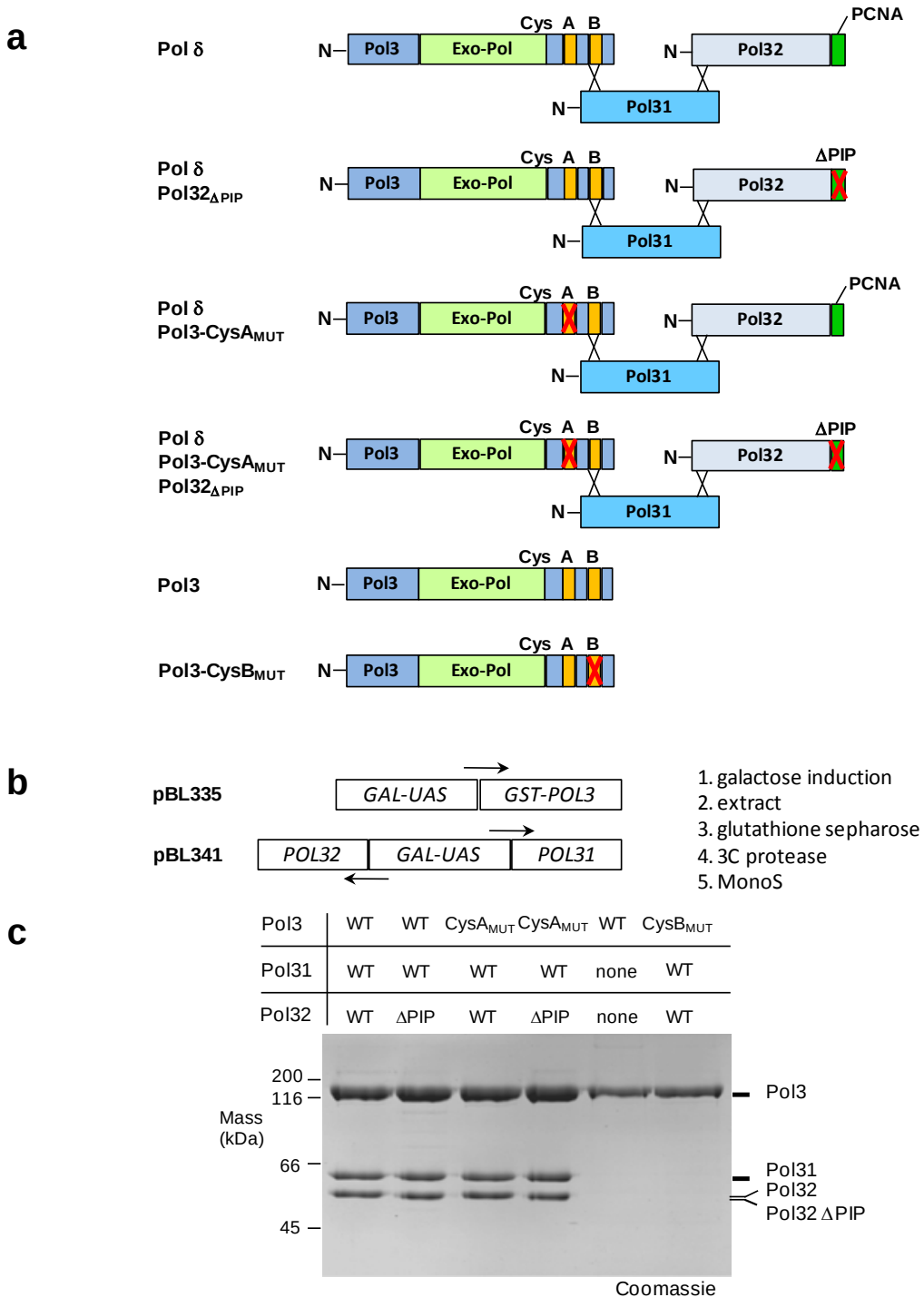

**Supplementary Figure 8. Immunostaining for the Pol3-CTD $^{55}$Fe incorporation experiment at endogenous Pol31 level and comparison of CTD and accessory subunit levels after over-expression. a,** HA-Pol3-CTDs in cell extracts were visualized by immunostaining using specific antibodies and chemiluminescence detection by a CCD camera. Nomenclature corresponds to Fig. 2, samples are from the light olive-green bars in Fig. 2. **b,** Idem, but with comparison of HA-Pol3-CTD and Pol31 upon Pol31 over-expression (Pol31↑), **c, d** Full-size immunoblots corresponding to the cropped data (hatched boxes) shown in Figure 2a and 2b, respectively. The positions of molecular mass markers are indicated.



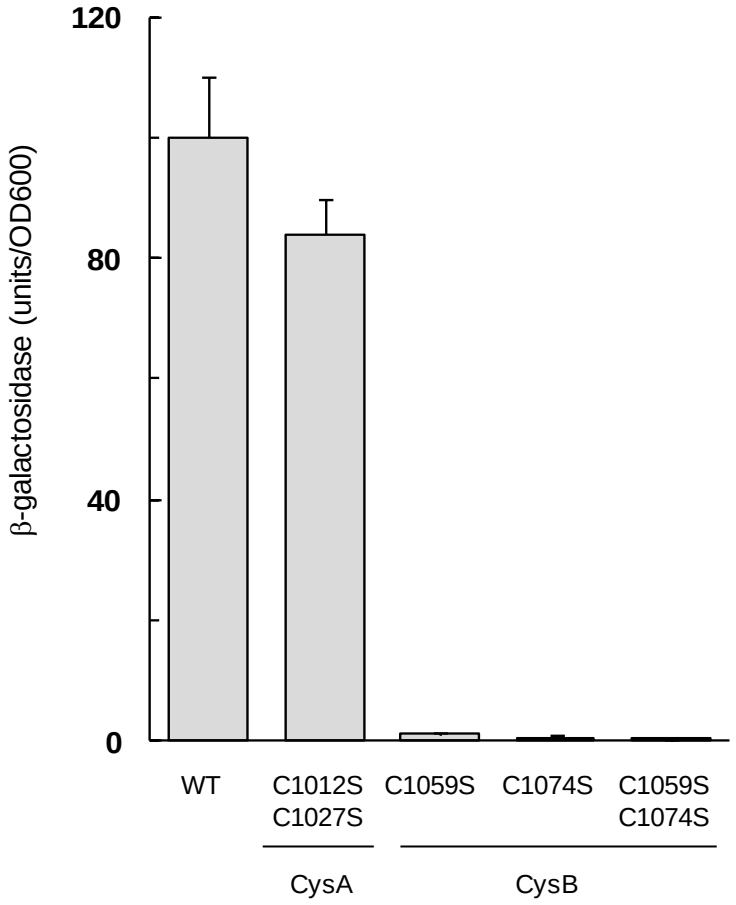

**Supplementary Figure 9. Purification of DNA polymerase CTDs after expression in *E. coli*. a,** Inclusion bodies solubilized with chaotropic agents (shown in Fig. 3a) were analyzed by SDS-PAGE and Coomassie staining. **b,** As in **a** but for soluble purified Pol1-, Pol2-, Pol3- and Rev3-CTDs used in Fig. 3 b,c and Supplementary Fig. 10.



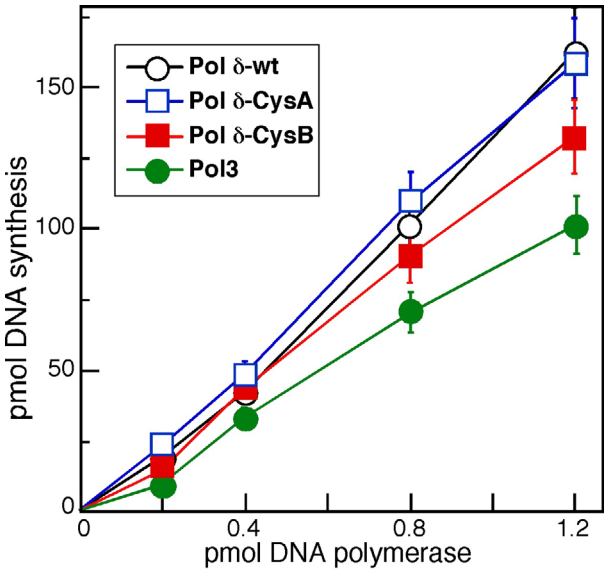

**Supplementary Figure 10. UV-Vis spectrum of soluble purified Pol1-CTD.** Pol1-CTD was purified as described in Methods. The inset shows the average non-heme iron and acid-labile sulfide content for three soluble Pol1-CTD preparations. Error bars, s.d. ($n \geq 3$).



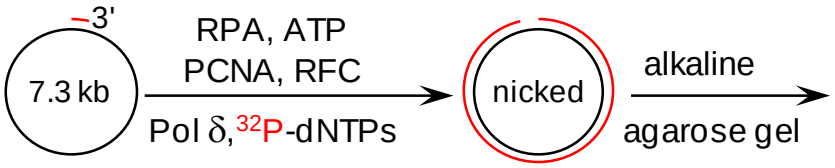

**Supplementary Figure 11. Pol δ and Pol3 protein preparations used in this study. a,** Overview of (mutant) Pol δ complexes used in Figs. 4 and 5. **b,** Scheme for overexpression (arrows indicate orientation of transcription) and protein purification. **c,** Photograph of the full length gel (SDS-PAGE) of purified proteins visualized by colloidal Coomassie staining. Fig. 4a in the main article contains cropped areas from the first, third and sixth lane of this gel image.




**Supplementary Figure 12. Pol3-CysB mutants are defective in binding Pol31.** Yeast two-hybrid interactions were measured employing plasmids with $LexA_{DBD}$-*POL3* (WT) or the indicated CysA and CysB mutants and with *GAL4*$_{AD}$-*POL31*. Averages of β-galactosidase activities are shown. Error bars, s.d. ($n \geq 3$).




**Supplementary Figure 13. Basal DNA polymerase activity of Pol δ is unaffected by CTD mutations.** The assay measures incorporation of dNTPs into activated salmon sperm DNA as described[2]. Note that the activity of the single Pol3 enzyme (lacking Pol31 and Pol32) is somewhat lower. We ascribe this to its tendency to aggregation. Error bars, s.d. ($n \geq 3$).




**Supplementary Figure 14. Outline of the assay for processive DNA replication.** The black circle represents single-stranded M13mp18 DNA primed with a 36 bp oligonucleotide (short red line) which is complementary to nucleotides 6330–6295. Polymerase replicates the single strand by extension of the primer with $^{32}$P-dNTPs to form double-stranded DNA with a single nick. Formation of the radioactive strand (red) is subsequently detected by alkaline agarose gel electrophoresis and phosphorimaging (see Fig. 5 a and b).

**Supplementary Table 1**. **Yeast strains used in this study**.

| Strain name | Background strain | Template for PCR (marker) | Tag and/or promoter introduced into genome | Source |
|---|---|---|---|---|
| Gal-NFS1 | W303 | pFA (His) | *GAL1-10* promoter | [3] |
| Gal-NBP35 | W303 | pFA (His) | *GAL1-10* promoter | [4] |
| Gal-NAR1 | W303 | pFA (His) | *GAL1-10* promoter | [5] |
| Pol1-Myc | W303 | pYM19 (His) | C-terminal 9xMyc | this study |
| Pol2-Myc | W303 | pYM19 (His) | C-terminal 9xMyc | this study |
| Pol3-Myc | W303 | pYM21 (Nat) | C-terminal 9xMyc | this study |
| Rev3-Myc | W303 | pYM19 (His) | C-terminal 9xMyc | this study |
| TFIIIA-Myc | Gal-NFS1 | pYM21 (Nat) | C-terminal 9xMyc | this study |
| Gal-NFS1 Pol1-Myc | Pol1-Myc | pYM-N23 (Nat) | *GAL1-10* promoter | this study |
| Gal-NBP35 Pol1-Myc | Pol1-Myc | pYM-N23 (Nat) | *GAL1-10* promoter | this study |
| Gal-NFS1 Pol2-Myc | Pol2-Myc | pYM-N23 (Nat) | *GAL1-10* promoter | this study |
| Gal-NBP35 Pol2-Myc | Pol2-Myc | pYM-N23 (Nat) | *GAL1-10* promoter | this study |
| Gal-NFS1 Pol3-Myc | Gal-NFS1 | pYM21 (Nat) | *GAL1-10* promoter | this study |
| Gal-NBP35 Pol3-Myc | Gal-NBP35 | pYM21 (Nat) | *GAL1-10* promoter | this study |
| Gal-NAR1 Pol3-Myc | Gal-NAR1 | pYM21 (Nat) | *GAL1-10* promoter | this study |
| GalL-HA-Pri2 | W303 | pYM-N28 (Nat) | *GALL* promoter & N-terminal 3xHA | this study |
| HA-Pri2 | GalL-HA-Pri2 | See text | Loss of *GALL* promoter | this study |
| HA-Pri2 Pol1-Myc | HA-Pri2 | pYM21 (Nat) | C-terminal 9xMyc | this study |

Abbreviations: pFA, pFA6a-HIS3MX6-Gal1-10; His, *Kluyveromyces lactis* His3; for pYM plasmids see[6]; Nat, nourseothricin acetyl transferase. TFIIIA is Transcription Factor IIIA (systematic name Pzf1).

**Supplementary Table 2**. **Plasmids used in this study.**

| Plasmid (promoter) | Encoding protein | Tag and position | | Use |
|---|---|---|---|---|
| 416 (*MET25*) | Pol3-CTD | 3HA | N | $^{55}$Fe incorporation |
| | Rev3-CTD | 3HA | N | $^{55}$Fe incorporation |
| | Pol1-CTD | 3HA | N | $^{55}$Fe incorporation |
| | Pol2-CTD | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1009A | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1012A | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1024A | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1027A | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1012A/C1027A | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1056A | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1059A | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1069A | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1074A | 3HA | N | $^{55}$Fe incorporation |
| | Pol3-CTD C1059A/C1074A | 3HA | N | $^{55}$Fe incorporation |
| | ΔMTS-Nfs1 | none | | $^{55}$Fe incorporation |
| 424 (*TDH*) | Pol12 | 3Myc | C | Co-expression with Pol1-CTD |
| | Dpb2 | 3Myc | C | Co-expression with Pol2-CTD |
| | Pol31 | none | | Co-expression with Pol3-CTD |
| | Rev7 | 3Myc | C | Co-expression with Rev3-CTD |
| pASK-IBA43plus (*TET*) | Pol1-CTD | Strep | C | Protein expression in *E.coli* |
| | Pol2-CTD | Strep | C | Protein expression in *E.coli* |
| | Pol3-CTD | Strep | C | Protein expression in *E.coli* |
| | Rev3-CTD | Strep | C | Protein expression in *E.coli* |
| pBL335 (*Gal1-10*) | GST-Pol3 | GST | N | Protein expression in yeast |
| pBL341 (*Gal1-10*) | Pol31 and Pol32 | none | | Protein expression in yeast |
| pBL322 (*ADH*) | Pol3 (mutants) | $LexA_{DBD}$ | N | Two-hybrid analysis |
| pBL364 (*ADH*) | Pol31 | $GAL4_{AD}$ | N | Two-hybrid analysis |

Abbreviations: N, N-terminal; C, C-terminal; $LexA_{DBD}$, bacterial LexA DNA-binding domain; $GAL4_{AD}$, yeast *GAL4* activating domain. ΔMTS-Nfs1 is Nfs1 lacking amino acids 1-94[1].

## Supplementary Methods

**Yeast strains and genetic manipulation.** Cassettes for the introduction of promoter sequences were amplified from pFA6a-HIS3MX6-Gal1-10[7], pYM-N23 or pYM-N27 templates[6]. pYM19 or pYM21 were used as template for C-terminal fusion of polymerase proteins with a nona-Myc tag sequence[6]. For these strains constructs the endogenous promoters remained unchanged. Exchange of the endogenous promoter for the *GalL* promoter with concomitant introduction of an N-terminal triple HA epitope tag for the construction of the GalL-HA-Pri2 strain was achieved with the pYM-N28 template[6]. Primers contained 20 nucleotides of the template plasmid and 50 nucleotides corresponding to the relevant genomic region used for homologous recombination. GalL-HA-Pri2 was converted to a strain with N-terminally tagged Pri2 under control of its natural promoter by homologous recombination and selection on glucose supplemented medium[8]. The fragment for recombination was obtained by PCR amplification of wild-type DNA with a promoter primer and the reverse complement of the 20 nucleotides directly 5' of the start ATG and 50 nucleotides encoding the HA tag. A complete list of strains and plasmids is presented in Supplementary Tables 1 and 2, respectively. Cells were grown in rich (YP) or minimal (SC) media at 30°C, containing the required carbon sources at a concentration of 2 % (w/v) and appropriate auxotrophic markers[9].

**Cloning and cysteine mutagenesis of polymerase CTDs**. A PCR fragment from pYM-N28[6] encoding an N-terminal triple HA tag was amplified with primers which added XbaI and SpeI restriction sites. The cut fragment was cloned into an XbaI-digested p416 plasmid with *Met25* promoter. Sequencing identified a clone with the correct orientation (p416-Met25-3HA). The coding regions of the CTDs of Pol3 (amino acids 982-1097), Rev3 (amino acids 1374-1504) Pol1, (amino acids 1262-1468) and Pol2 (amino acids 2084-2222) were PCR amplified from yeast chromosomal DNA with primers adding SpeI

(all) and EcoRI (Pol3 and Rev3) or HindIII (Pol1 and Pol2) restriction sites. After digestion, these fragments were cloned into the corresponding sites of p416-Met25-3HA. Pol3-CTD cysteine mutagenesis (see Supplementary Table 2 for plasmids) was carried out in p416-Met25-3HA-Pol3-CTD as template with primer design as described by Zheng et al.[10]. The Watcut tool (http://watcut.uwaterloo.ca/) from Michael Palmer (University of Waterloo, Canada) was used to introduce restriction sites with silent mutations. The coding regions of Pol31, Pol12, Dpb2 and Rev7 were PCR amplified from yeast chromosomal DNA with primers adding SpeI (all) and EcoRI (Pol31) or SalI (all other) restriction sites. After digestion, these fragments were cloned into the corresponding sites of p424-TDH (Pol31) or p424-TDH-3Myc (all other). The latter vector was prepared by cloning a fragment which was obtained by PCR amplification with primers adding SalI and XhoI restriction sites to the C-terminal 3Myc encoding region of pYM19[6] into p424-TDH.

Regions coding for the natural C terminus of the B-family polymerases were cloned into the NheI and NcoI restriction sites of pASK-IBA43-plus after amplification from yeast chromosomal DNA. This vector supplies a C-terminal Strep-tag. The constructs encompassed the following amino acids: Pol1, 1262-1468; Pol2, 2084-2222; Pol3, 982-1097 and Rev3, 1374-1504. All constructs and mutations were confirmed by DNA sequencing.

**$^{55}$Fe incorporation into yeast proteins.** The yeast strains listed in Supplementary Table 1 were grown in regular SC medium for 24 h, followed by growth in iron-free SC medium for 16 h. Depletion in glucose-containing medium led to less than 11 % decrease in cellular growth (wet cell mass) compared to wild-type cells. After washing with de-ionized water, cells (~0.5 g) were incubated for 2 h with $^{55}FeCl_3$ in iron-free SC medium. From this point onwards all steps were carried out below 4°C. Cells were collected by centrifugation and resuspended in an equal volume of TNETG buffer [10 mM Tris-

HCl pH 7.4, 150 mM NaCl, 2.5 mM EDTA, 0.5 % (v/v) Triton X-100, 10% (v/v) glycerol] with 2 mM PMSF and complete protease inhibitor tablets (Roche). Disruption of the cells was achieved by vortexing with glass beads in three one-min bursts, alternated with cooling on ice for 1 min. Cell debris were removed by centrifugation (1,500×*g*, 5 min), followed by clarification of the supernatant by centrifugation at 13,000×*g* for 12 min. Aliquots of 200 µl were incubated in 1.5 ml vials for 1 h with 20 µl suspension of agarose beads with coupled anti-Myc or anti-HA antibodies (Santa Cruz). After three washes with 0.5 ml TNTEG buffer, the beads were dispersed in 1 ml scintillation fluid and the amount of protein-associated $^{55}$Fe was measured by scintillation counting (LS 6500, Beckman Coulter Inc.). Presence of tagged proteins and/or depletion of proteins in the cell extracts were confirmed by SDS-PAGE and immunostaining analysis of aliquots taken before immunoprecipitation. Monoclonal antibodies against Myc or HA epitopes were from Santa Cruz. Polyclonal antibodies against yeast Nfs1, Nbp35, Nar1, Porin, Pol31 and Pol32 proteins were raised in rabbits in our laboratories.

**Yeast two-hybrid analysis.** The interaction of the Pol3-CysB mutants with Pol31 was analyzed by yeast two-hybrid assays carried out in strain L40 (*MAT*a, *his3*-Δ*200*, *trp1-901*, *leu2-3,112*, *ade2*, *LYS2*::(lexAop)$_4$-*HIS3*, *URA3*::(lexAop)$_8$-lacZ) as described before[2]. Pol3-Pol31 interactions were measured using plasmid pBL322 (LexA$_{DBD}$-*POL3* in 2µ vector pBTM116 with *TRP1* marker) or its corresponding CysA (C1012S, C1027S) or CysB (C1059S, C1074S) mutants, and plasmid pBL364 (*GAL4*$_{AD}$-*POL31* in 2µ vector pACT2 with *LEU2* marker). Quantitative β-galactosidase assays were carried out in triplicate and were corrected for the background obtained in absence of the pBL364 plasmid.

**Chemical analysis.** Non-heme iron bound to protein was determined by colorimetry with the iron chelator Ferene[11]. Iron in (mutant) yeast Pol δ preparations was determined with the chelator 2-(5-nitro-2-pyridylazo)-5-(*N*-propyl-*N*-sulfopropylamine)phenol (nitro-PAPS)[12]. Quantitative release of iron ions from polymerase (25 µl) was effected by addition of 125 µl of 7 M guanidine hydrochloride, 0.4 M sodium acetate, 100 mM sodium thioglycolate at pH 4.3. After denaturation 75 µl water and 25 µl 1 mM nitro-PAPS were added. Spectra of the samples (200 µl) were recorded in flat bottom Greiner 96 well microtiter plates. The low pH value, wavelength (690 nm minus background at 900 nm) and presence of thioglycolate eliminated interference of other metal ions.

Acid-labile sulfide content was measured[11] by formation of methylene blue (absorbing at 670 nm) from the reaction of *N*,*N*'-dimethyl-*p*-phenylenediamine with $H_2S$ and excess $FeCl_3$. Standardization was carried out with freshly purchased $Li_2S$. This method is highly specific for acid-labile sulfide and does not give a response with commonly encountered sulfur compounds including protein-bound cysteine or methionine[13]. The same method was downscaled 4-fold for determination of $S^{2-}$ in microtiter plates (200 µl sample volume) and to confirm by visible spectroscopy that methylene blue was produced.

Protein concentrations for assays, Fe/S analysis and calculation of extinction coefficients were determined by the Bradford method, using bovine serum albumin as standard. The protein quantities were insufficient for extensive quantification with quantitative amino acid analysis or the biuret method. Metal and sulfide contents could therefore be influenced by small differences [~20 %][14] in the relative extent of color development of Pol δ and CTDs in comparison to the bovine serum albumin standard.

**Statistical analysis**. All quoted values have standard deviations (error bars in figures) calculated for at least three independent experiments. Significant means that values differ from the control or blank according to non-paired Student's t-test ($p < 0.05$).

## References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