SUPPLEMENTARY FIGURES

Supplementary Figure 1. The gel filtration profile from the final step of POLGβ purification produced 2 peaks (panel A); coomassie staining of fractions separated by SDS-PAGE indicated that the second peak contained no detectable contaminating protein (panel B).

Supplementary Figure 2. The overwhelming majority of POLGβ siRNA treated cells (t = 120 and t = 140) had a marked decrease in Picogreen staining compared to mock transfected cells: the figure illustrates other examples of the phenomenon shown in Fig. 4A of the main paper at two different magnifications.

Supplementary Figure 3. The component parts of the images in Fig. 4E, and enlargements of the merged images. U2OS cells were transfected with plasmid carrying the POLGβ gene tagged with HA. Immunocytochemistry using anti-HA antibody identified cells expressing the transgene (red), whereas DNA was labelled with an anti-DNA antibody (green).

Supplementary Figure 4. A 40-50% decrease in mtDNA does not, of itself, alter the number of nucleoids in HOS cells. In theory, the increase in mitochondrial nucleoid number associated with POLGβ siRNA (Fig. 4B) could have been an indirect effect of the accompanying 40% decrease in copy number (Fig. 1B). Dideoxycytidine (ddC) is a chain terminator that is particularly efficient at inhibiting mtDNA replication as it accumulates in mitochondria (26 and references therein). A range of ddC

concentrations and incubation times was tested for their effect on mtDNA copy number in HOS cells and 5 μM was found to cause a decrease in copy number slightly greater than that achieved after 120 hours of POLGβ siRNA (Panel A-1). Therefore, mitochondrial nucleoid number was quantified in HOS cells treated for 24 hours with and without 5 μM ddC and found to be unchanged; n = 2 experiments (Panel A-2). Furthermore, Twinkle siRNA produced a similar decrease in mtDNA copy number to POLGB siRNA (2,23), without any apparent increase in nucleoid number. Mitochondrial DNA copy number was determined by Q-PCR after transfecting HOS cells with no dsRNA (mock) or dsRNA targeting Twinkle DNA helicase (panel B-1). Cells treated with or without dsRNA targeting Twinkle were analysed by immunocytochemistry using a DNA specific antibody (panel B-2), (see materials and methods and also (2,23) for details of the procedures).

Supplementary Figure 5. PolGα protein level is unaffected by changes in the expression of PolGβ. Panel A; whole cell protein lysates from HOS cells treated with and without dsRNA targeting POLGβ were fractionated by SDS-PAGE and transferred to solid support. Membranes were incubated with antibody to POLGα, POLGβ or GAPDH, antibody binding was visualized by enhanced chemilluminescence (GE Healthcare) and the signals quantified using ImageJ software. Panel B; POLGα, POLGβ and GAPDH protein levels were determined for HEK cells without transgene (Ctrl), and HEK cells carrying a POLGβ.HA transgene cultured for 72 hours without (N.I.) and with (POLGβ o/e) 10 ng/ml doxycyclin. Panel C. Over-expression of POLGβ transgene had no effect on mtDNA copy number. Q-PCR analysis was performed on DNA from HEK cells carrying

POLGβ.HA cultured in the presence of absence of 10 ng/ml doxycyclin for 6 days, and HEK cells lacking the transgene.

Supplementary Figure 6. HEK Flp-In T-Rex cells (Invitrogen) carrying a POLGβ transgene were expanded and maintained without drug or cultured with 10 ng/mL doxycylin for 6 days to induce transgene expression (see supplementary Fig. 5). Mitochondria were isolated, and lysed with detergent before fractionating on a 20-45% iodixanol gradient, as previously described (2). Protein and DNA were recovered from each fraction and analysed by Southern and Western blotting to determine the distribution of mtDNA and the proteins POLGα, POLGβ.HA, mtSSB, and Tfam on the gradient; POLGβ.HA was detected using an antibody specific to the haemagglutinin tag.

Supplementary Figure 7. Panel A. Synthetic D-loop was incubated with increasing amounts of $ATAD3B_{44-247}$ and separated by 1D-AGE; the protein showed a clear preference for fast (f) over slow (s) migrating D-loops. Previously it has been shown that more tightly supercoiled D-loops are refractive to restriction digestion (28) and here *Xho*I failed to cleave the faster migrating D-loop (f), whereas slow migrating D-loop (s) was cut by the enzyme, as was supercoiled DNA without a D-loop (panel B). s/c – supercoiled plasmid, oc – open circles, l – linear DNA.

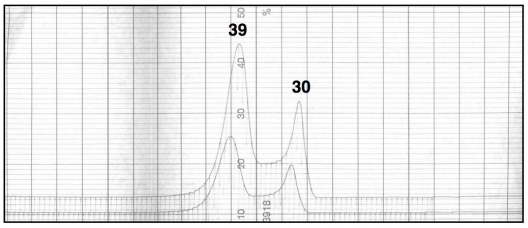
Supplementary Figure 8. Five nanogram aliquots of plasmid DNA lacking (pBluescript) or containing the non-coding region of human mtDNA (pNCR) were incubated with 0, 22.5, 45, 90 or 180nM of PolGβ for 30 minutes at 37 ^{o}C before fractionating on a non-denaturing 1 % agarose gel. After electrophoresis the DNA

was transferred to solid support and hybridized to radiolabelled pBluescript. Band intensities were quantified using a Typhoon phosphorimager and the values expressed as a percentage of the signal resolving as a discreet band in the absence of protein.

Di Re et al., Supplementary Fig. 1

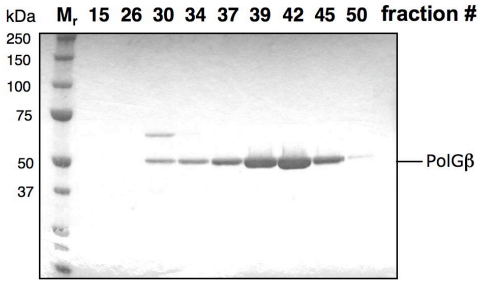
A

Gel filtration profile

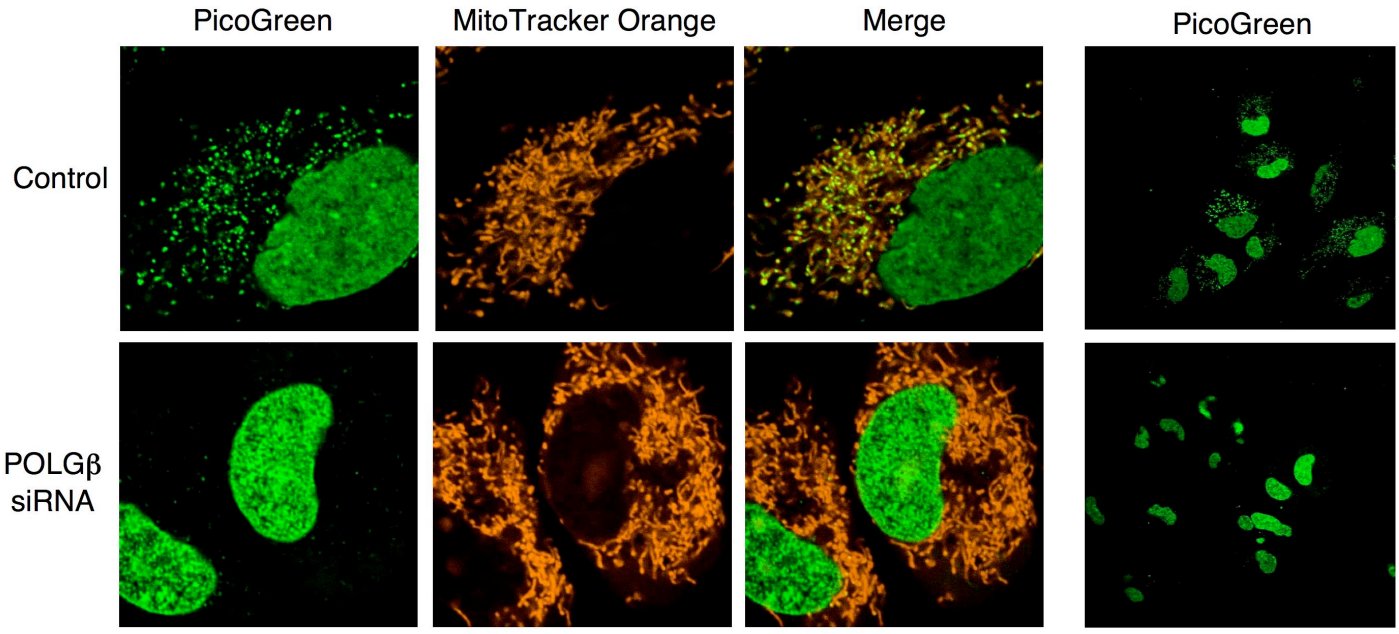


B

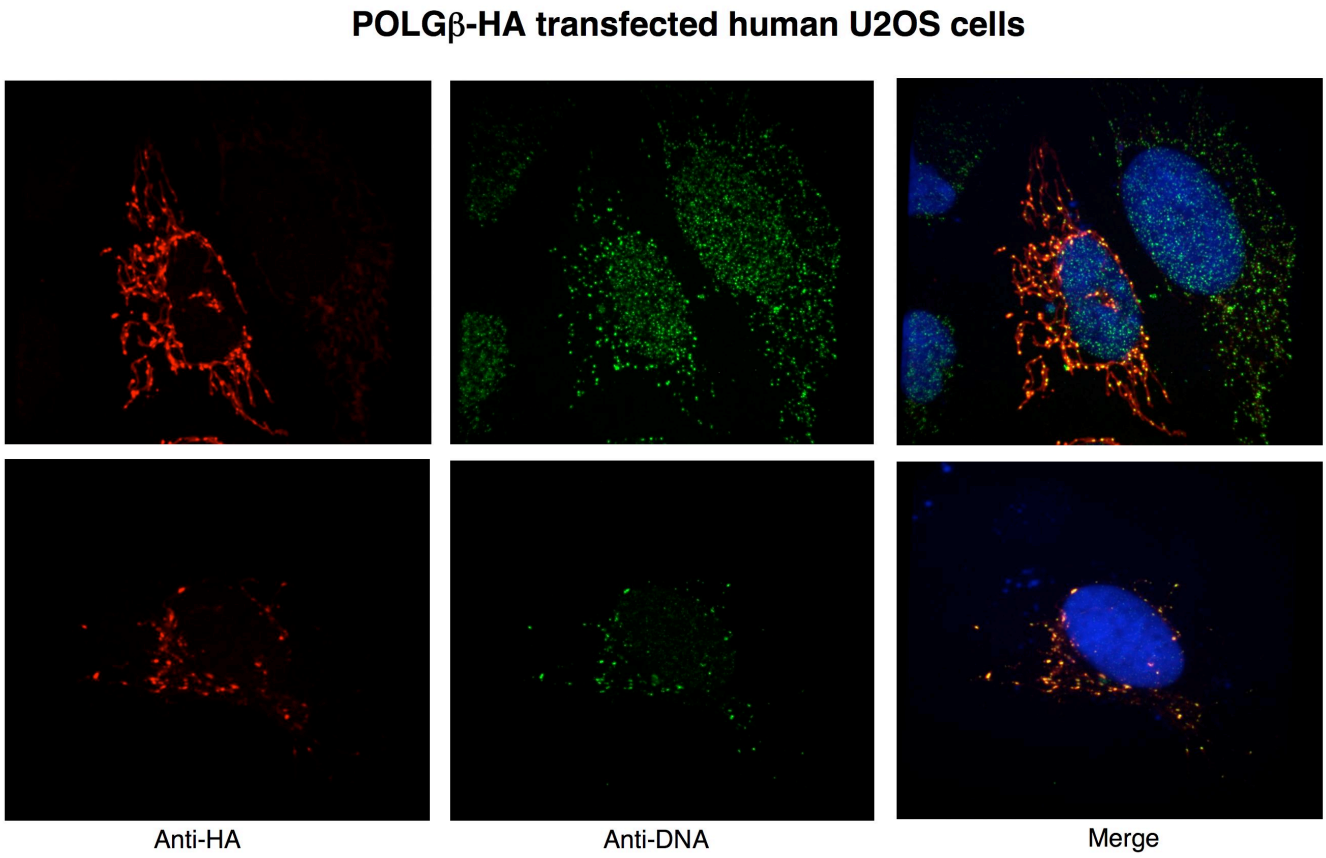
Coomassie stained 4-15% SDS-PAGE



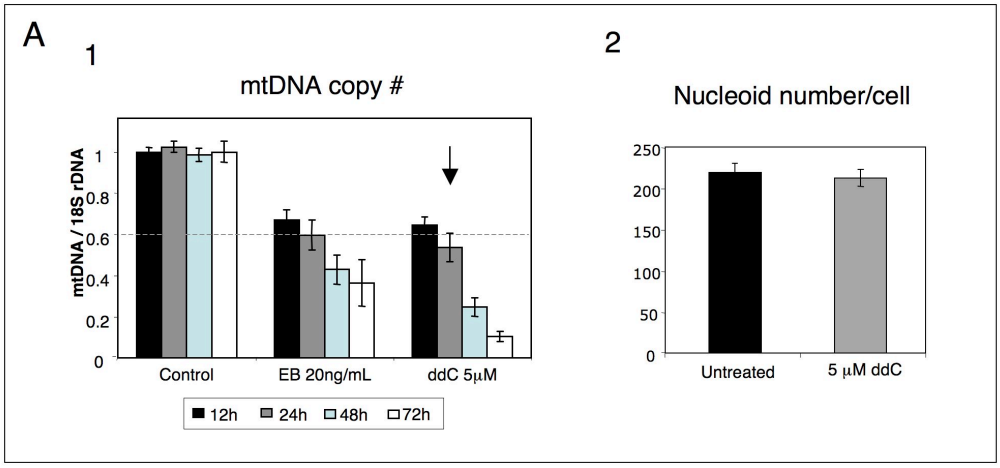
Di Re et al., Supplementary Fig. 2

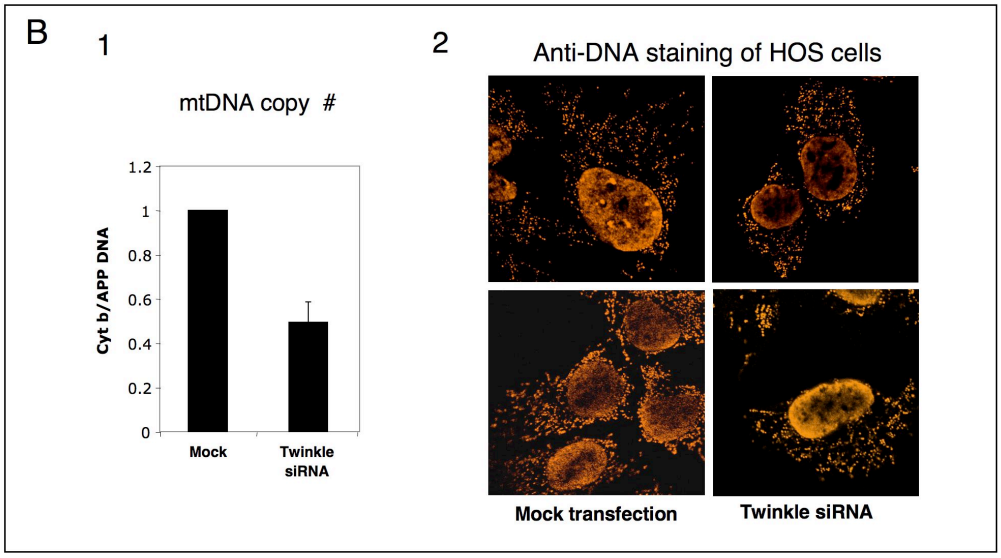


Di Re et al., Supplementary Fig. 3

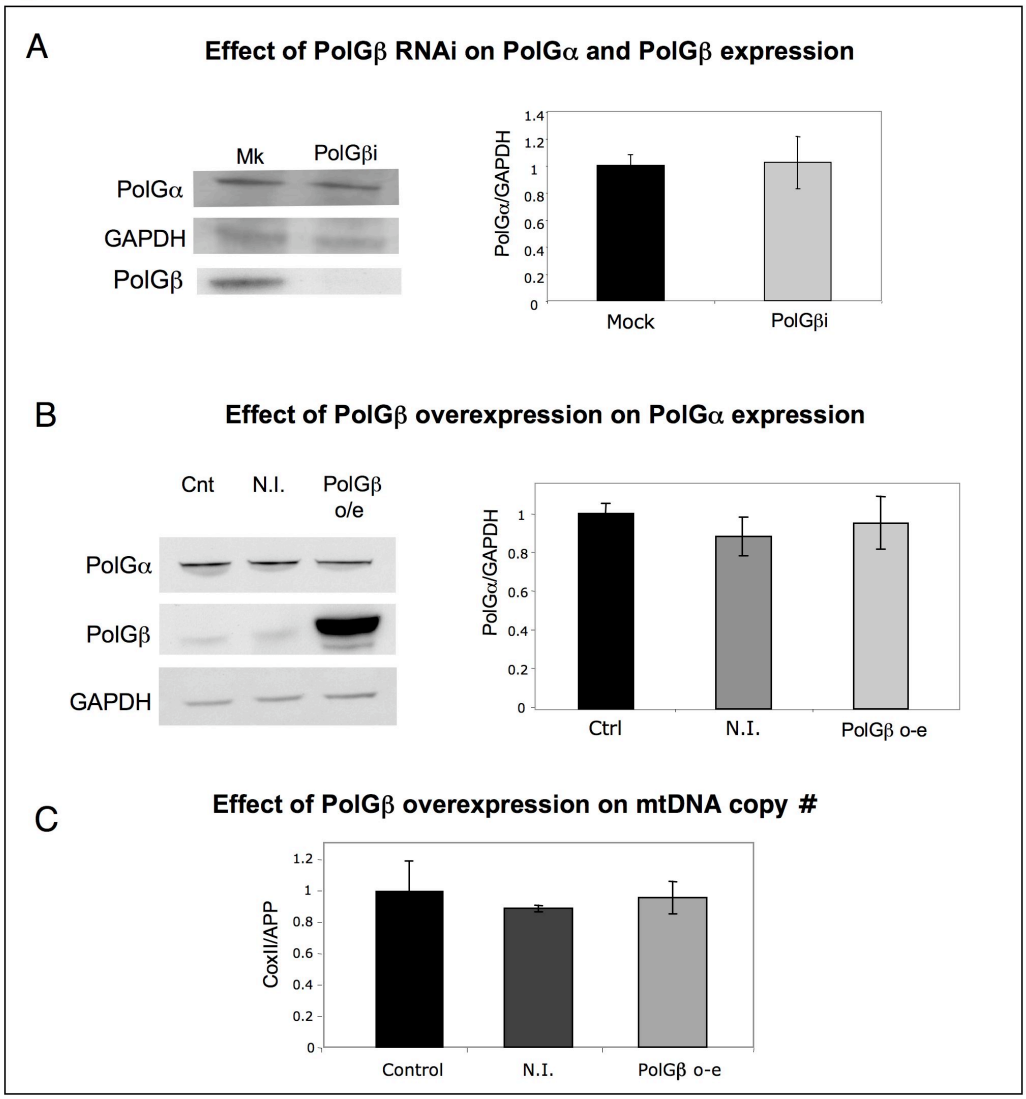


Di Re et al., Supplementary Fig. 4

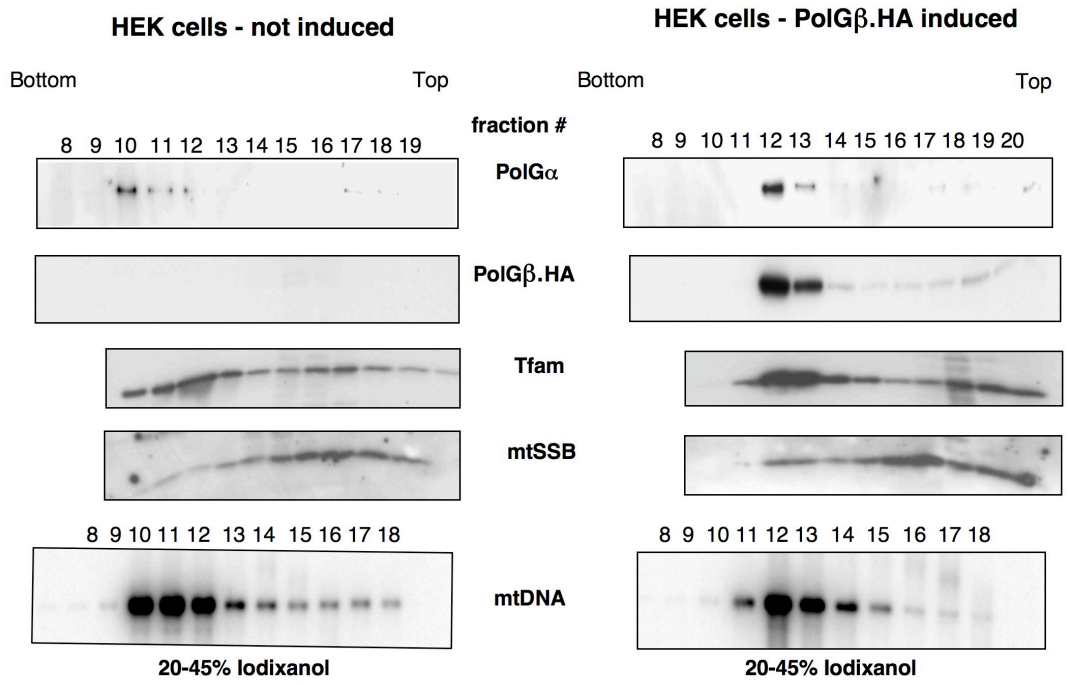




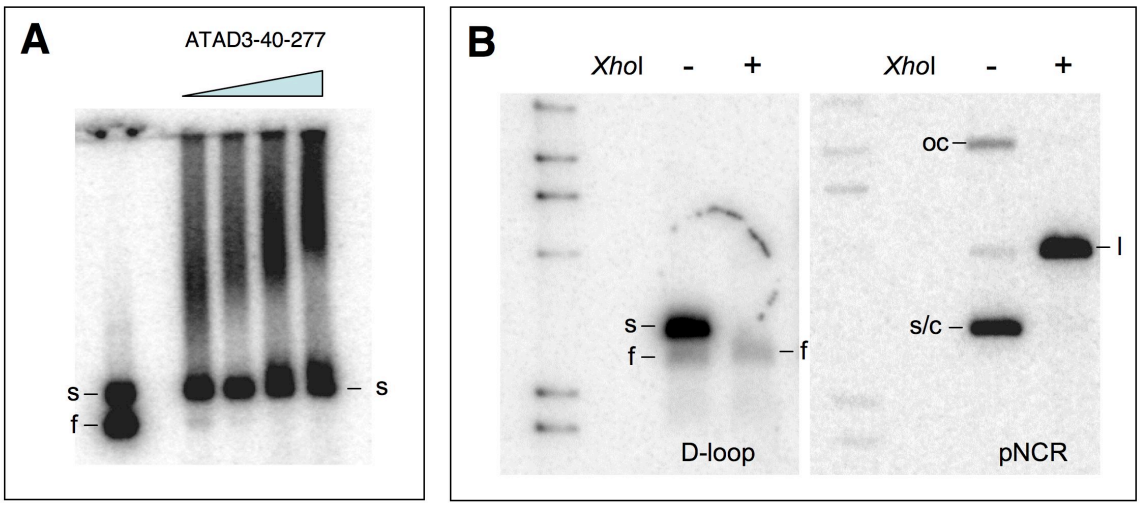
Di Re et al., Supplementary Fig. 5



Di Re et al., Supplementary Fig. 6



Di Re et al., Supplementary Fig. 7



Di Re et al., Supplementary Fig. 8