Supplemental Material

Supplemental Fig. 1 . Interaction of PCNA with hPol ε and subunits. - Interactions of [^{32}P]-PCNA with hPol ε and various subunits (Supplemental Fig. 1). Though PCNA strongly interacted with the four-subunit complex, weaker PCNA binding was noted with the p261 N derivative, p59 subunit and p12. Interactions were not detected with the p261 C derivatives and the p17 subunit. Studies with the p261 full-length large subunit were compromised by its poor stability and insolubility and are not reported for these reasons. The sum of the amount of PCNA bound to the various subunits and truncated derivatives represented only 60% of that bound to the four-subunit complex. The reasons for this discrepancy are not clear. We noted the presence of a putative PCNA binding motif (PIP) in h261 (aa 1100-1107) and mutated this PIP motif as indicated in the truncated 261 N derivative. However, both wild-type and mutant p261-N derivatives were stimulated similarly by PCNA indicating that this putative PIP domain was not essential. The Sc Pol 2 large subunit contains a similar PIP sequence (aa 1193-1200) and its alteration did not affect the biological activity of the Sc Pol ε (59). These findings suggest that Pol ε subunits may contain multiple PCNA binding sites but their role in supporting DNA replication is unclear.

Supplemental Fig. 2. Influence of hGINS on hPol α-polymerase activity. - As shown in Fig. 7A, hGINS stimulated the Pol α-catalyzed elongation of a primed oligonucleotide template. This experiment was carried out using the two-subunit hPol α-complex (p180-p70) and similar effects were observed with the four-subunit hPol α-primase complex (p180-p70-p58-p48) (Fig. S1). It should be noted that the conditions used in these two experiments differ leading to variations in the observed polymerase activity. As shown in Fig. S2, the stimulatory effects of hGINS were more pronounced at low concentrations of Pol α-primase. Full-length chain extension of the primed oligonucleotide (50 mer primer elongated to the 90 mer) was observed at all levels of Pol α added. Influence of hGINS on the hPol α-catalyzed elongation of primed DNA templates. Reaction mixtures (20 μl) contained 40 mM Tris-HCl, pH 8.0 10 mM magnesium acetate, 1 mM DTT, 75 μg/ml BSA, 40 mM NaCl, 25 μM [α^{32}P]-dATP ($8x10^3$ cpm/pmol). 75 μM dCTP, 2.5 pmol of primed oligonucleotide substrate (90/50; $(TG)_{20}$ single strand region), GINS (250 nmol), where indicated, and various levels of Pol α-primase. After 30 min at 37ºC, 4 μl aliquots were subjected to 6M urea 10% PAGE separation followed by phosphorimaging.

Supplemental Fig. 3. Influence of hGINS on SV40 DNA replication - Replication of SV40 DNA has been studied extensively and shown to require a duplex DNA containing the SV40 core origin, the SV40 encoded T antigen, RPA, hPol α-primase complex and the Pol δ holoenzyme (including RFC and PCNA). Two different reaction conditions were used to evaluate the effects of hGINS in this system. These included the monopolymerase reaction which is dependent on SV40 T antigen, hRPA and hPol α-primase as well as the dipolymerase reaction in which the monopolymerase system is supplemented with the Pol δ holoenzyme (60). The monopolymerase reaction requires higher levels of Pol α-primase to support DNA synthesis than the dipolymerase reaction due to extensive elongation synthesis catalyzed by the Pol δ holoenzyme (60). As shown in Supplemental Fig. 3, monopolymerase reactions containing 40 and 20 fmol of the hPol α-primase complex supported synthesis of relatively short DNA chains; at lower Pol α-primase levels, dNTP incorporation was not detected. In the presence of hGINS, DNA synthesis was stimulated 2-3-fold, most evident in reactions containing 20 fmol of enzyme (lane 5). The addition of Pol δ holoenzyme stimulated both the level and length of DNA products synthesized. Stimulation was more apparent at low Pol α-primase levels (compare lanes 11 with 12 and lanes 13 with 14). In the absence of Pol α-primase (lanes 15 and 16), small amounts of labeled full-length DNA products were detected due to incorporation of dNTPs onto nicked DNA by the Pol δ holoenzyme. These findings indicate that hGINS stimulated the activity of Pol α-primase uder replication conditions, albeit using a

system dependent on the SV40 T antigen. Stimulation of SV40 ori$^+$DNA replication by GINS. (A). Monopolymerase system. Reaction mixtures (30 μl) were as described in Eki *et al* (60). Where indicated, reactions contained 0.8 μg of SV40 T antigen, 8 pmol of GINS and the four-subunit Pol α-primase (40 fmol, lanes 2, 3 and 6; 20 fmol, lanes 4 and 5). After 90 min at 37ºC, aliquots were subjected to alkaline agarose gel electrophoresis. (B). Dipolymerase reaction. Reaction mixtures were supplemented with Pol δ, RFC and PCNA (40, 15 and 500 fmol, respectively) and levels of Pol α-primase, as indicated.

Supplemental Fig. 4. Influence of GINS and subcomplexes on Pol α-catalyzed elongation of singly-primed M13. Reaction mixtures (15 μl) contained 40 mM HEPES-NaOH buffer, pH 7.5, 1 mM DTT, 70 μg/ml BSA, 10 μM [α^{32}P]-dATP (15,740 cpm/pmol, 50 μM each of dCTP, dGTP and dTTP, 2.4 nM singly-primed M13 DNA, 20 mM sodium glutamate, various levels of the GINS complexes indicated, and 14 nM of the two-subunit (p180-p70) hPol α complex. Reactions were incubated for 45 min at 37ºC and aliquots used to measure nucleotide incorporation and the size of DNA products following 1% alkaline agarose gel electrophoresis.

Supplemental Fig. 5 siRNA depletion of hPol δ and hPol ε. (A). Extent of depletion of hPol δ (B) and hPol ε in HeLa cells by siRNAs. Treatment of HeLa cells (as described in Fig. 8A) with 50 nM siRNA #06 reduced the level of hPol δ (p125 subunit) by 90% while the other siRNAs were less efficient. siRNA #08 (50 nM) directed against Pol ε (p261 subunit) reduced the level of Pol ε to 93%. Since higher levels of either siRNA were not more effective, 50 nM of each siRNA was used in all experiments. The percentage of S-phase and G1-phase cells observed with each siRNA (carried out as described in Fig. 8A) are also summarized.

Supplemental Fig. 6 Quantification of the amount of hPol δ and hPol ε in HeLa cells. Monolayer of HeLa were grown in DMEM supplemented with 10% FBS at 37ºC and in 5% CO_2 atmosphere. HeLa cells (2 x 10^6) were washed with ice-cold PBS and then scraped, lysed, sonicated and boiled in 0.2 ml of SDS loading buffer. Indicated amounts of the HeLa extract was loaded along side known amounts of purified recombinant hPol δ and hPol ε on 8% SDS-polyacrylamide. After electrophoresis and Western blotting onto nitrocellulose membrane, the large catalytic subunits of the Pols were detected with specific antibodies followed by chemiluminescence. The bands were quantified by using ImageGauge Software (FujiFilm).

References:

1. Dua, R., Levy, D. L., Li, C. M., Snow, P. M., and Campbell, J. L. (2002) *J Biol Chem* **277**, 7889-7896
2. Eki, T., Matsumoto, T., Murakami, Y., and Hurwitz, J. (1992) *J Biol Chem* **267**, 7284-7294

Supplemental Table 1

Influence of hGINS and subcomplexes on hPol α and hPol ε activities

hGINS complex added	Concentration (μM)	Activity observed with hPol α Fold stimulation	Activity observed with hPol ε Fold stimulation
four-subunit (Sld5, Psf3, Psf2, Psf1)	240	18.1	14.7
	120	10.6	10.0
three-subunit (Sld5-Psf3-Psf2)	240	3.21	3.67
	120	2.21	1.51
two-subunit (Sld5-Psf2)	240	3.12	2.90
	120	2.12	1.79

Reactions were as described in Fig. 7A with 50 mM NaCl and included the Pol α (p180-p70) complex and 50 mM of potassium glutamate. The fold stimulation reported above refers to the activity observed in the presence of GINS (or subcomplex) divided by the activity observed in the absence of GINS and subcomplexes.

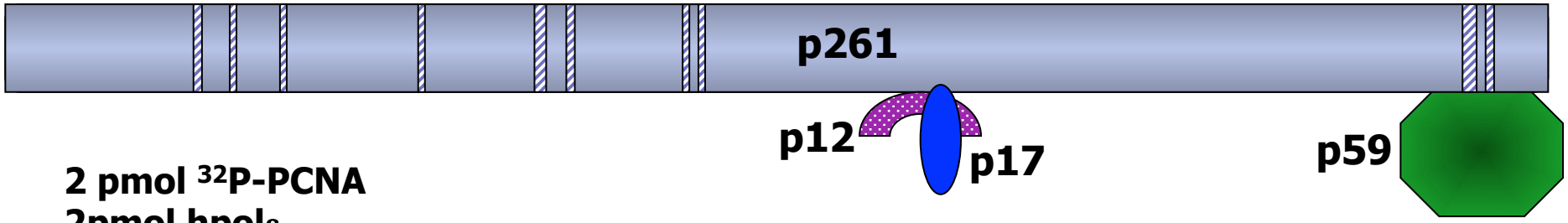
Figure S1: Binding of PCNA to hPol ε subunits

PCNA binding motif (PIP)	Q x x h x x a a
Scpol2	^{1193}Q T S L T D F F^{1200}
hp261	^{1180}Q K K I S E L F^{1187}
p21	Q T S L T D F Y

x - is any amino acid
h - hydrophobic (L,I,M)
a - aromatic (F,Y)

PIP	Qxxhxxaa
p59	^{189}QLKEGKFF^{196}
	^{205}QLDLSKAQ^{112}
	^{112}QFHSGLYT^{119}
	^{233}QVFHVNAF^{300}
	^{357}QSSRFVFV^{364}
	^{391}QRVPFSVF^{398}

PIP	Qxxhxxaa
p12	^{61}QEAIFILA^{68}



2 pmol ^{32}P-PCNA
2pmol hpolε

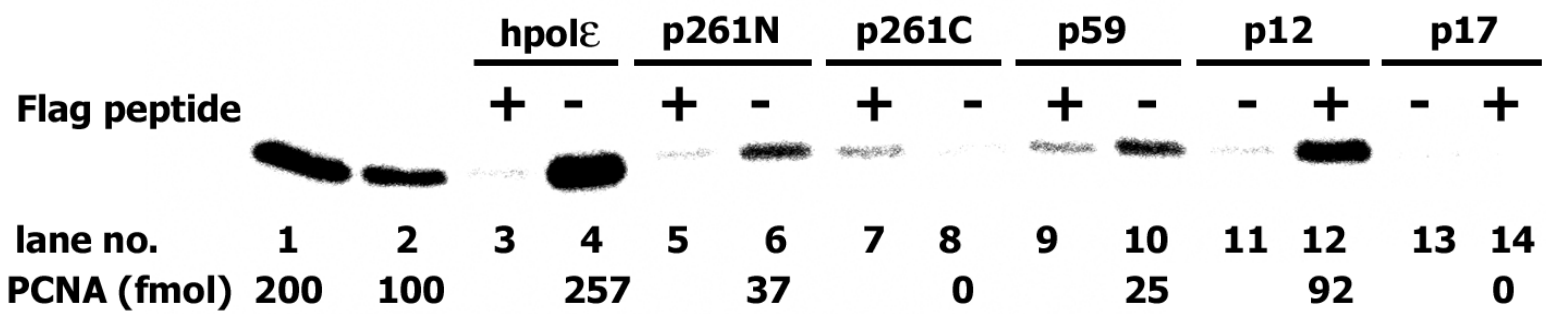


Figure S2: Influence of GINS on the Pol α-catalyzed elongation of primed DNA templates

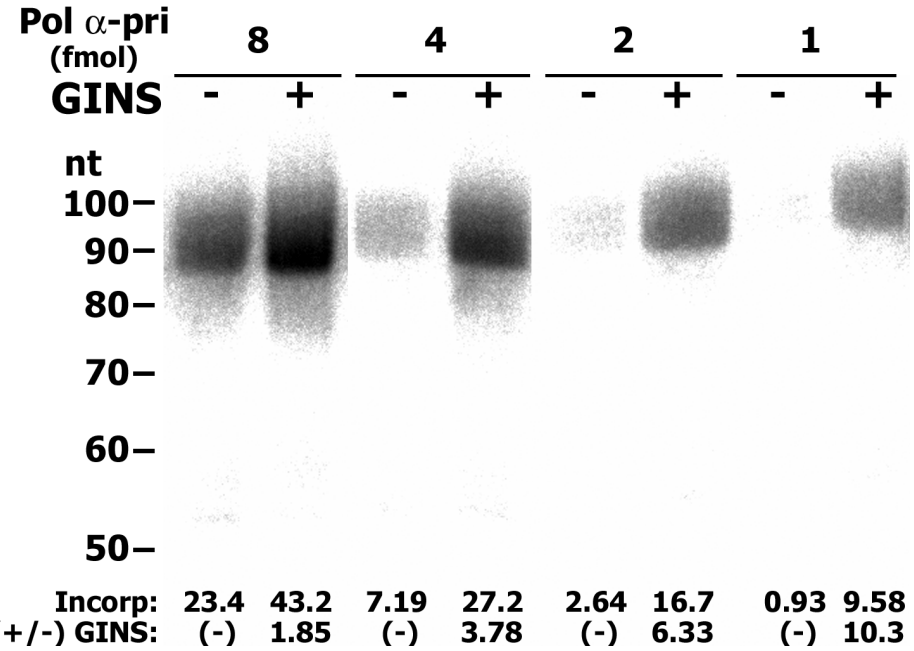


Figure S3: Influence of hGINS on SV40 DNA replication by GINS

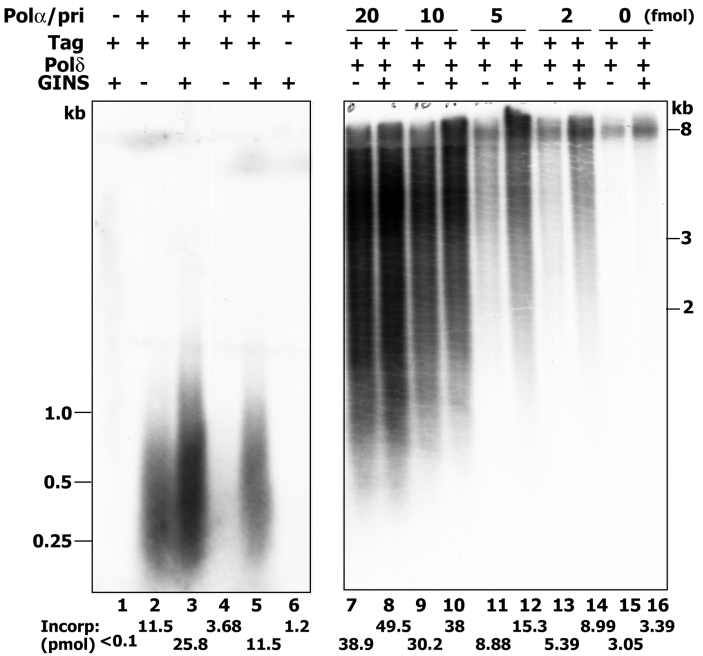


Figure S4: Effect of GINS derivatives on Pol α activity

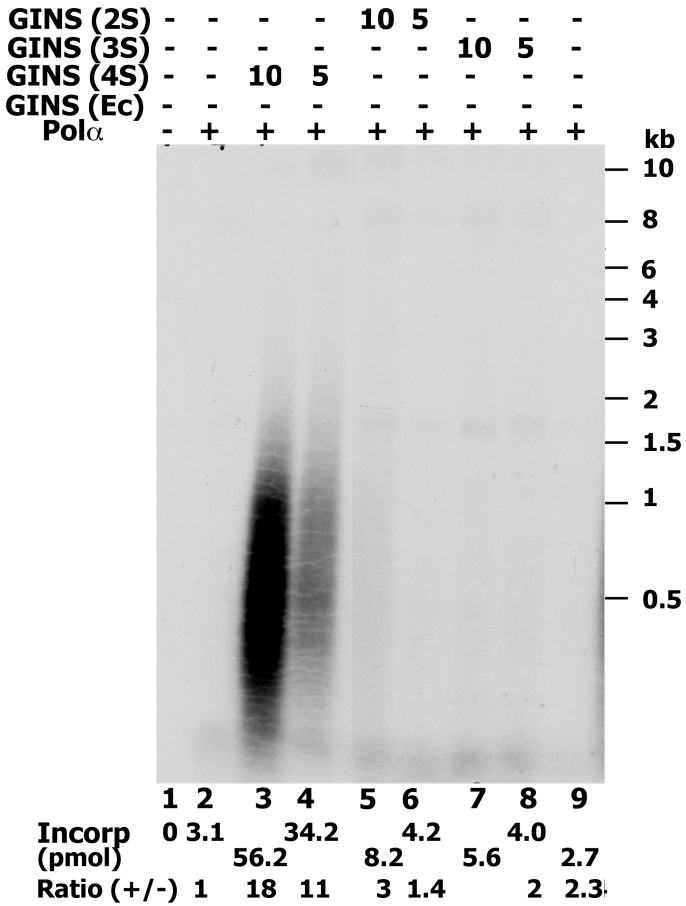
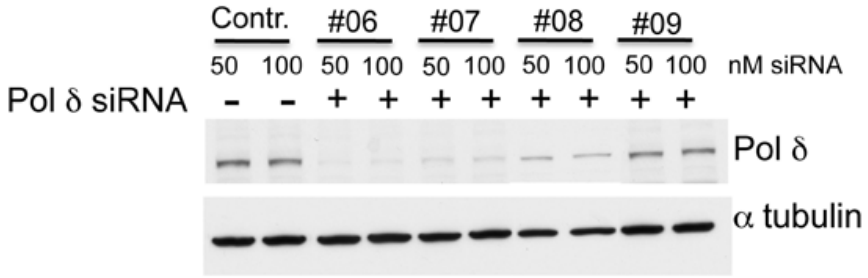
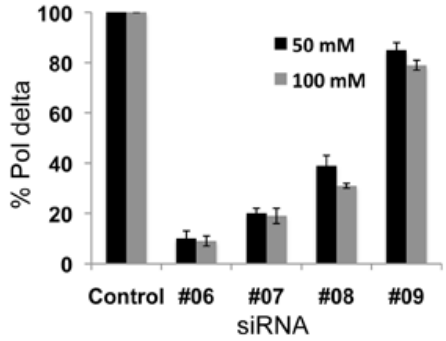


Figure S5A: Pol δ (p125 subunit) knock down in HeLa cells by siRNA



	#06	#07	#08	#09
50 nM	10%	20%	39%	85%
100 nM	9%	19%	31%	74%



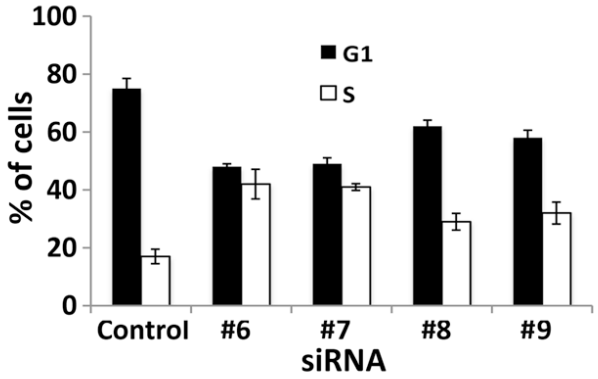
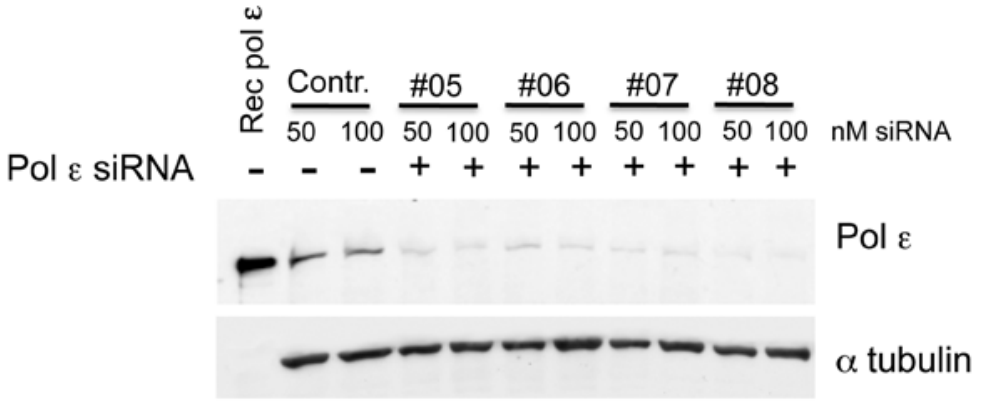
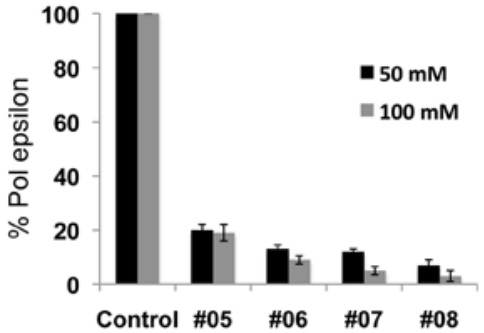


Figure S5B: Pol ε (p261 subunit) knock down in HeLa cells by siRNA



	#05	#06	#07	#08
50 nM	20%	13%	12%	7%
100 nM	19%	9%	5%	3%



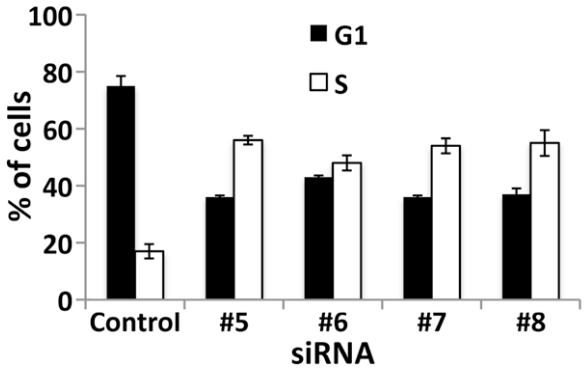


Figure S6: Quantitation of the number of molecules of Pol ε and Pol δ per each HeLa cell