Supplemental Figure 1. Sequence analysis of the HCT116 PNKP$^{-/-}$ cell clone. 65 nucleotides were deleted and 10 nucleotides were rearranged in Allele 1; 78 nucleotides were inserted and 2 separate nucleotides were mutated in Allele 2. The termination codon in allele 1 is underlined. Targeted sequences in the WT allele corresponding to the guide RNA are also underlined.

```
E3-14-1         CTGGGAGTTAACCCCTCAACTACCGGGACCCAGGAGTTGAAGCCGGGGGTTGGAGGGCTCT
E3-14-2         CTGGGAGTTAACCCCTCAACTACCGGGACCCAGGAGTTGAAGCCGGGGGTTGGAGGGCTCT
PNKP-Exon3      CTGGGAGTTAACCCCTCAACTACCGGGACCCAGGAGTTGAAGCCGGGGGTTGGAGGGCTCT
                ************************************************************

E3-14-1         CTGGGGGTGGGGGACACACTGTATTTGGTCAATGG----------------------T
E3-14-2         CTGGGGGTGGGGGACACACTGTATTTGGTCAATGGCCTCCACCCACTGACCCTGCGCTGG
PNKP-Exon3      CTGGGGGTGGGGGACACACTGTATTTGGTCAATGGCCTCCACCCACTGACCCTGCGCTGG
                ************************************************************

E3-14-1         GAAGATGCC-----------------------------------------------------
E3-14-2         GAAGAGACCCGCACACCAGAATCCCAGCCAGATACTCCCGCACACCAGAATCCCAGAAGA
PNKP-Exon3      GAAGAGACCCGCACACCAGAATCCCAGCCAGATACTCC----------------------
                ************************************

E3-14-1         ------------------------------------------------------------
E3-14-2         GACCCGCACACCAGAATCCCAGCCAGATACTCCCGCACACCAGAATCCCAGAAGAGACCT
PNKP-Exon3      ------------------------------------------------------GCCT
                                                                      ***

E3-14-1         --------TCTGGTGTCCCAAGATGAGAAGAGAGATGCTGAGCTGCCGAAGAAGCATATG
E3-14-2         GGCACCCCTCTGGTGTCCCAAGATGAGAAGAGAGATGCTGAGCTGCCGAAGAAGCATATG
PNKP-Exon3      GGCACCCCTCTGGTGTCCCAAGATGAGAAGAGAGATGCTGAGCTGCCGAAGAAGCATATG
                ************************************************************

E3-14-1         CGGAAGTCAAACCCCGGCTGGGAGAACTTGGAGAAGTTGCTAGTGTTCACCGCAGCTGGG
E3-14-2         CGGAAGTCAAACCCCGGCTGGGAGAACTTGGAGAAGTTGCTAGTGTCCACCGCAGCTGGG
PNKP-Exon3      CGGAAGTCAAACCCCGGCTGGGAGAACTTGGAGAAGTTGCTAGTGTTCACCGCAGCTGGG
                ********************************************** *************

E3-14-1         GTGAAACCCCAGGGCAAG
E3-14-2         GTGAAACCCCAGGGCAAG
PNKP-Exon3      GTGAAACCCCAGGGCAAG
                ******************
```

Supplemental Figure 2. Sequence analysis of the HeLa PNKP$^{-/-}$ cell clone. Insertions of 1, 171 and 205 nucleotides were found at the target site. Alleles 1 and 3 harbor a termination codon immediately at the beginning of the insertion (underlined). Allele 2 is predicted to encode a 57-aa insertion. Targeted sequence in the WT allele corresponding to the guide RNA is also underlined. The entire region shown for allele 1 was sequenced in all cases; only the segment containing the insertion is shown.

Allele 1:

```
PNKP3(WT)        CACTACCGGGACCCAGGAGTTGAAGCCGGGGTTGGAGGGCTCTCTGGGGGTGGGGGACAC
PNKP3-1-1c       CACTACCGGGACCCAGGAGTTGAAGCCGGGGTTGGAGGGCTCTCTGGGGGTGGGGGACAC
                 ************************************************************

PNKP3            ACTGTATTTGGTCAATGGCCTCCACCCACTGACCCTGCGCTGGGAAGAGACCCGCACACC
PNKP3-1-1c       ACTGTATTTGGTCAATGGCCTCCACCCACTGACCCTGCGCTGGGAAGAGACCCGCACACC
                 ************************************************************

PNKP3            AGAATCCCAGCCAGATACTCCGCCTGGCACCCCTCTGGTGTCCCAAGATGAGAAGAGAGA
PNKP3-1-1c       AGAATCCCAGCCAGATACTCCGCCTGGCACCCCTCTGGTGTCCCAAGATGAGAAGAGAGA
                 ************************************************************

PNKP3            TGCTGAGCTGCCGAAG-AAGCGTATGCGGAAGTCAAACCCCGGCTGGGAGAACTTGGAGA
PNKP3-1-1c       TGCTGAGCTGCCGAAGTAAGCGTATGCGGAAGTCAAACCCCGGCTGGGAGAACTTGGAGA
                 *************** ****************************************

PNKP3            AGTTGCTAGTGTTCACCGCAGCTGGGGTGAAACCCCAGGGCAAGGTGAGGGCCACGCCGA
PNKP3-1-1c       AGTTGCTAGTGTTCACCGCAGCTGGGGTGAAACCCCAGGGCAAGGTGAGGGCCACGCCGA
                 ************************************************************

PNKP3            GGGCTGAGGGAGCCGCCACAGACTGGGACCCAATCCCACGTTTGTTGCGTGCTCTCA
PNKP3-1-1c       GGGCTGAGGGAGCCGCCACAGACTGGGACCCAATCCCACGTTTGTTGCGTGCTCTCA
                 ************************************************************
```

1 nucleotide was inserted.

Allele 2:

```
PNKP3 (WT)       GCTGAGCTGCCGAAGA------------------------------------------
PNKP3-1-1a       GCTGAGCTGCCGAAGACCTACAACCAGCTGTTCGAGGAAAACCCCATCAACGCCAGCGGC
                 ****************

PNKP3            ------------------------------------------------------------
PNKP3-1-1a       GTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGACGGCTGGAAAATCTG

PNKP3            ------------------------------------------------------------
PNKP3-1-1a       ATCGCCCAGCTGCCCGGCGAGAAGAAGAATGGCCTGTTCGGAAACCTGATTGCCCTGAGC

PNKP3            -------AGCGTATGCGGAAGTCAAACCCCGGCTGGGAGAACTTGGAGAAGTTGCTAGTG
PNKP3-1-1a       CTGGGCAAGCGTATGCGGAAGTCAAACCCCGGCTGGGAGAACTTGGAGAAGTTGCTAGTG
                        **************************************************
```

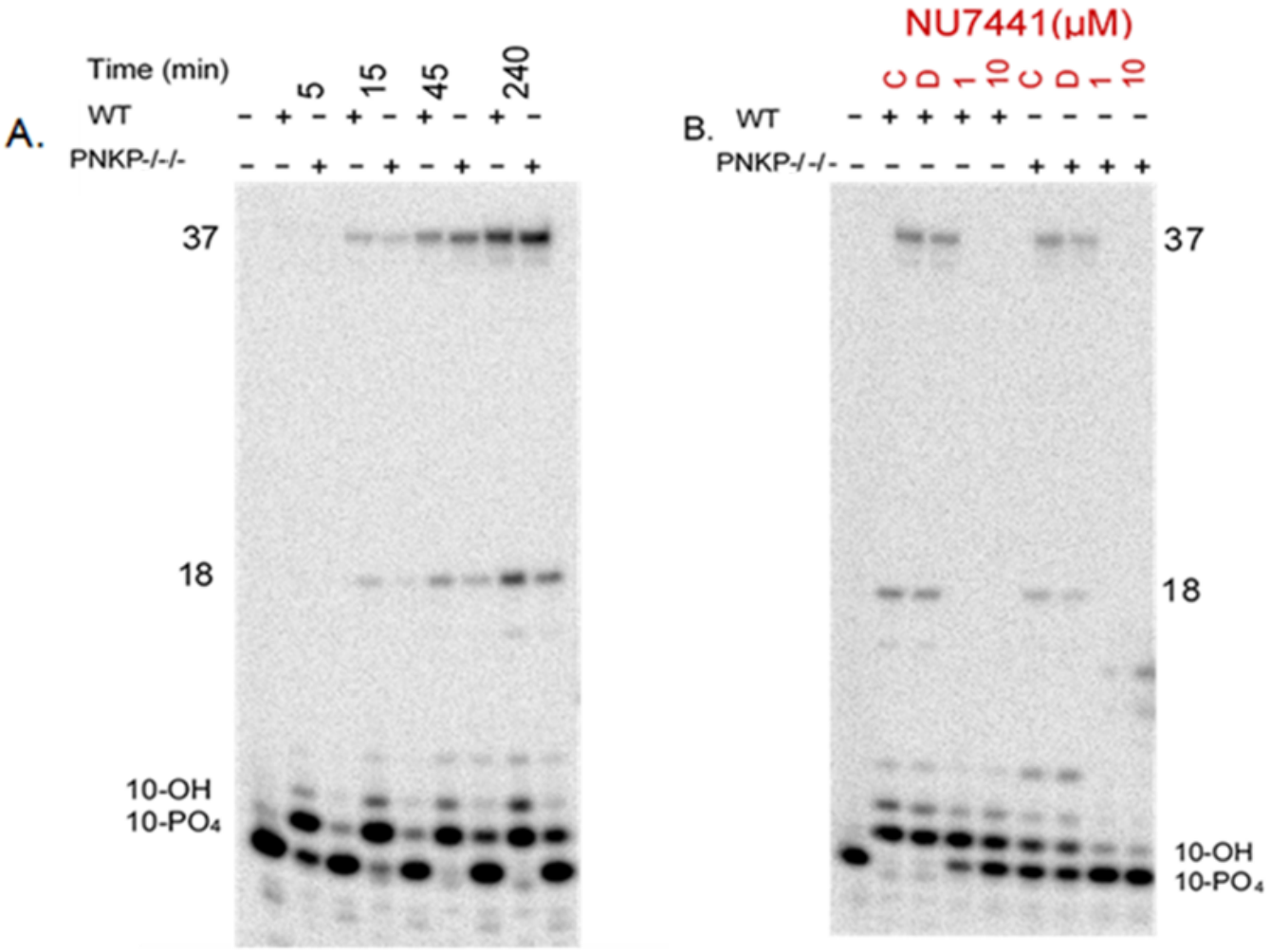
171 nucleotides were inserted. (continued)

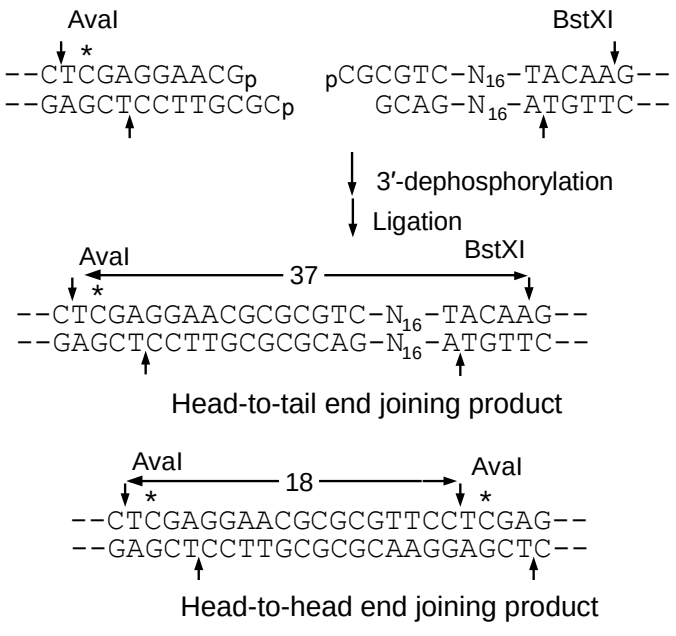
Allele 3:

```
PNKP-E3 (WT)     GGCACCCCTCTGGTGTCCCAAGATGAGAAGAGAGATGCTGAGCTGCCGAAG---------
PNKP3-1-1jR      GGCACCCCTCTGGTGTCCCAAGATGAGAAGAGAGATGCTGAGCTGCCGAAGTAAGCGCGG
                 **************************************************

PNKP-E3          ------------------------------------------------------------
PNKP3-1-1jR      CGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCTTAGCGCCCGCTC

PNKP-E3          ------------------------------------------------------------
PNKP3-1-1jR      CTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAA

PNKP-E3          ------------------------------------------------------------
PNKP3-1-1jR      ATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAAC


PNKP-E3          ----------------AAGCGTATGCGGAAGTCAAACCCCGGCTGGGAGAACTTGGAGAA
PNKP3-1-1jR      TTGATTTGGGCGATGGAAGCGTATGCGGAAGTCAAACCCCGGCTGGGAGAACTTGGAGAA
                                 ******************************************
```

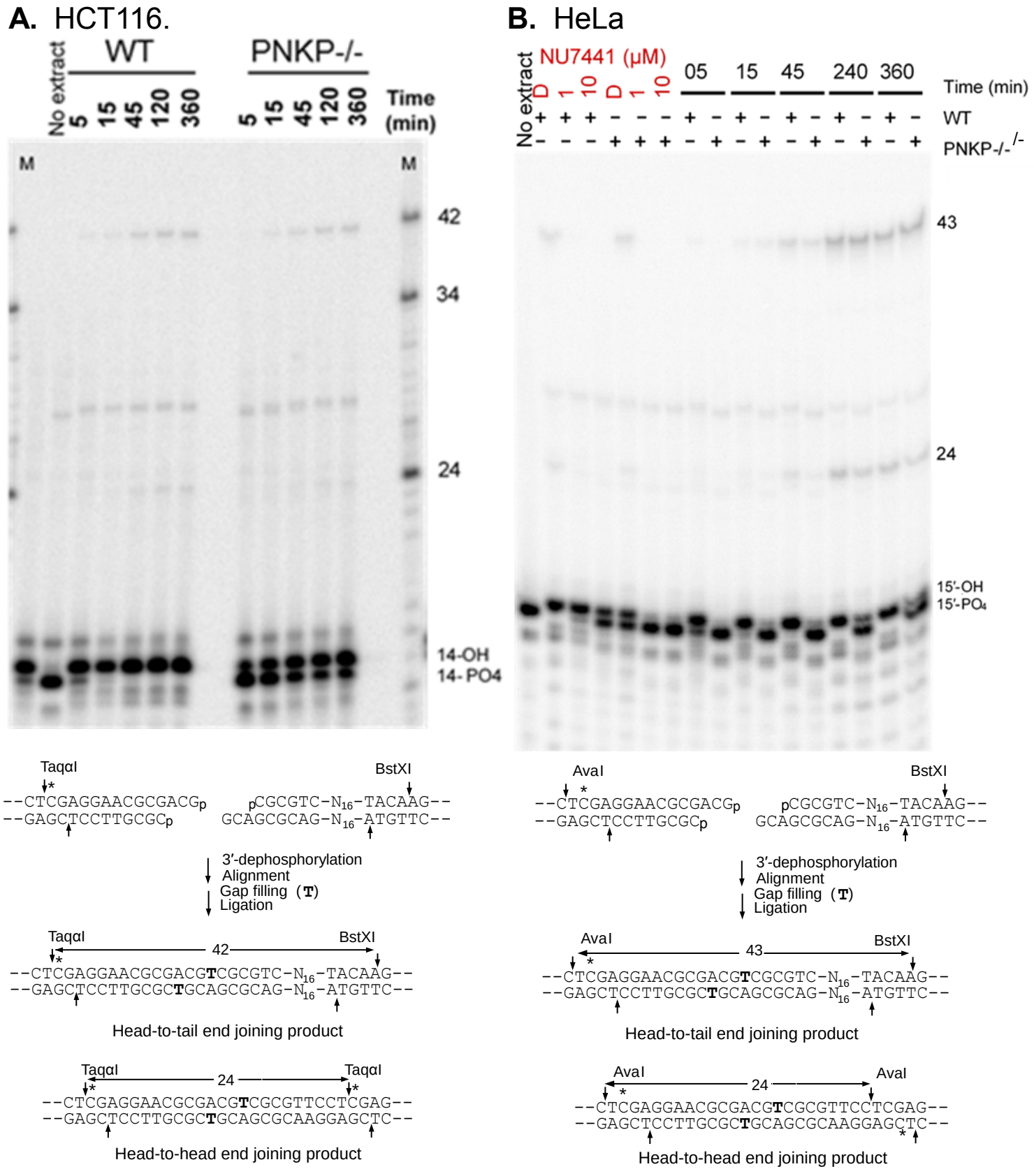
205 nucleotides were inserted.

Supplemental Figure 3. Dephosphorylation and end joining of a recessed 3′-phosphate DSB in HeLa and PNKP$^{-/-}$ whole-cell extracts and the effect of DNA-PKi. The substrate shown below was incubated in extracts for the indicated times (A), cut with AvaI and BstXI, and labeled products analyzed on a sequencing gel. In (B), NU7441 (DNA-PKi) was added 10 min before addition of substrate, followed by incubation for 6 hr. "C"= incubation with no DMSO; "D" = incubation in 1% DMSO (solvent for NU7441). The unprocessed substrate (10-mer) and the head-to-tail end joining product (37-mer) are one base longer than in Fig. 3 because of cleavage by AvaI instead of TaqI. See Fig. 3B for quantitation of these results.

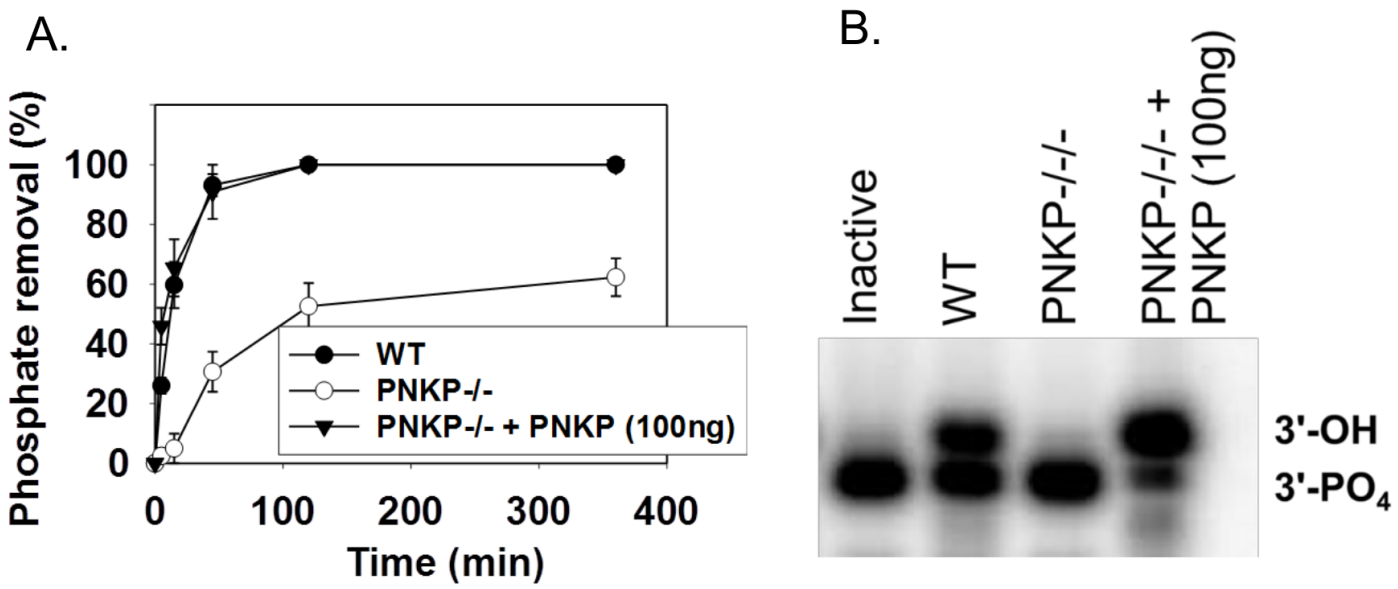




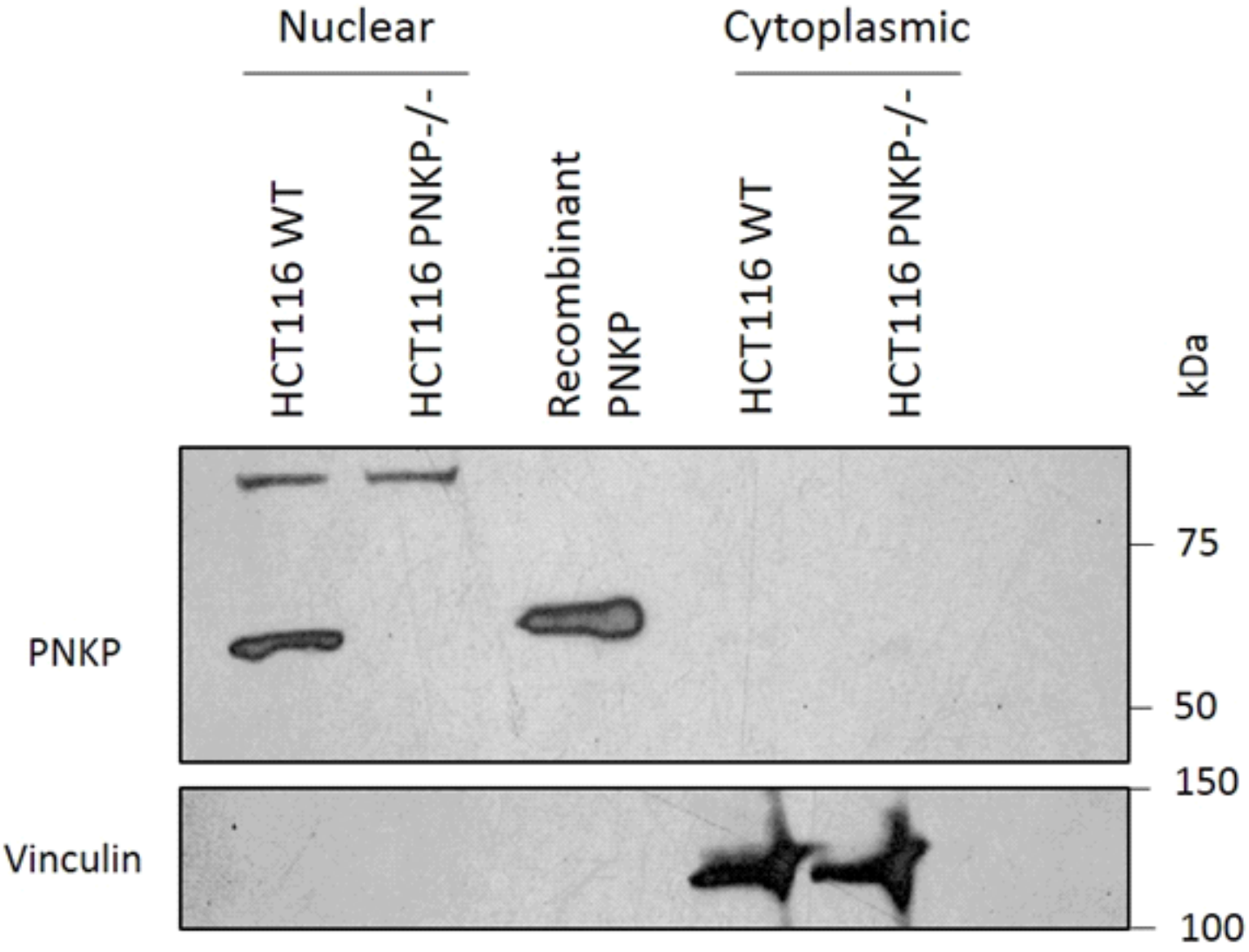
Supplemental Figure 4. Dephosphorylation and end joining of the 3′-overhang 3′-phosphate DSB substrate in whole cell extracts of HCT116 (A) or HeLa cells (B) and their PNKP-deficient derivatives, and the effect of DNA-PKi. The substrate shown below was incubated in extracts for the indicated times, cut with BstXI and either TaqI (A) or AvaI (B), and labeled products analyzed on a sequencing gel. In (B), NU7441 (DNA-PKi) was added 10 min before addition of substrate, followed by incubation for 6 hr. "D" = incubation in 1% DMSO (solvent for NU7441). The unprocessed substrate and the head-to-tail end joining product are one base longer in (B) than in (A) because of cleavage by AvaI instead of TaqI. See Fig. 4 and Fig. 7E-F for quantitation of these results.



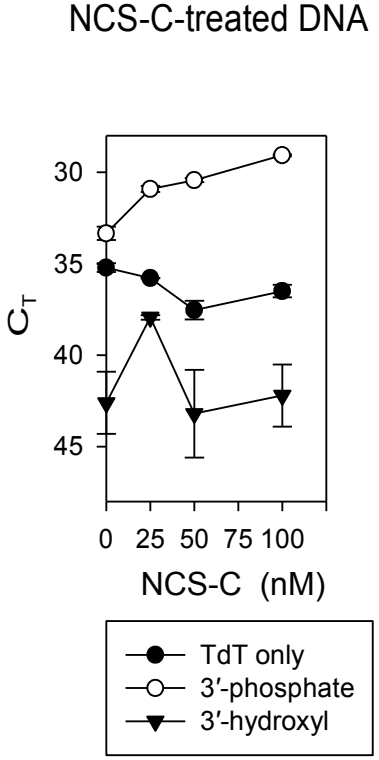
Supplemental Figure 5. Restoration of 3′-dephosphorylation activity in whole-cell extracts by addition of recombinant PNKP. **A.** A substrate bearing a 3′-phosphate on a 1-base 3′ overhang was incubated for the indicated times in WT and PNKP$^{-/-}$ HCT116 extracts, as well as PNKP$^{-/-}$ extracts supplemented with 100 ng PNKP. Dephosphorylation was assessed as in Fig. 4. Error bars indicate mean ± SEM for 3 experiments. **B.** A substrate with a 3′-phosphate on a 3-base 3′ overhang was incubated for 1.5 min in HeLa extract, PNKP$^{-/-}$ extract, or PNKP$^{-/-}$ extract supplemented with 100 ng PNKP, and dephosphorylation was similarly determined.



Supplemental Figure 6. Nuclear HCT116 and PNKP$^{-/-}$ extracts lack the cytoplasmic marker vinculin. Nuclear and cytoplasmic extracts were prepared using a BioVision fractionation kit, and 50 µg of each were analyzed by PAGE and western blot.



Supplemental Figure 7. Formation of 3′-hydroxyl and 3′-phosphate DSB termini following treatment of isolated human DNA with NCS-C. HCT116 DNA at 40 µg/ml was treated with the indicated concentrations of NCS-C in the presence of 5 mM glutathione at 22ºC for 1 hr. DSB termini were then analyzed by LMPCR as in Fig. 9. Results are from a single experiment and error bars represent mean ± SD for 2 PCR reactions. NCS-C concentrations were chosen to give approximately the same level of 3′-phosphate termini as treatment of cells with 5 µM NCS-C. Only 3′-phosphate ends and not 3′-hydroxyl ends show a concentration-dependent increase upon NCS-C treatment, and in contrast to DNA in treated cells, which contain roughly equal numbers of 3′-phosphate and 3′-hydroxyl ends, the treated isolated DNA contains at the highest NCS-C concentration 8,000 times as many 3′-phosphate as 3′-hydroxyl DSB termini (difference of 13 PCR cycles in C_T). Thus NCS-C does not directly induce any 3′-hydroxyl-terminated DSBs and any such termini must have been formed by 3′-dephosphorylation in cells.



Supplemental Fig. 8. Formation of 3′-hydroxyl and 3′-phosphate DSB termini following treatment of HeLa WT and PNKP$^{-/-}$ cells with NCS-C. Cells were incubated in suspension at 22ºC for the indicated times and DSB ends were analyzed by LMPCR as in Fig. 9. Results are from a single experiment and error bars represent mean ± SD for 2 PCR reactions.