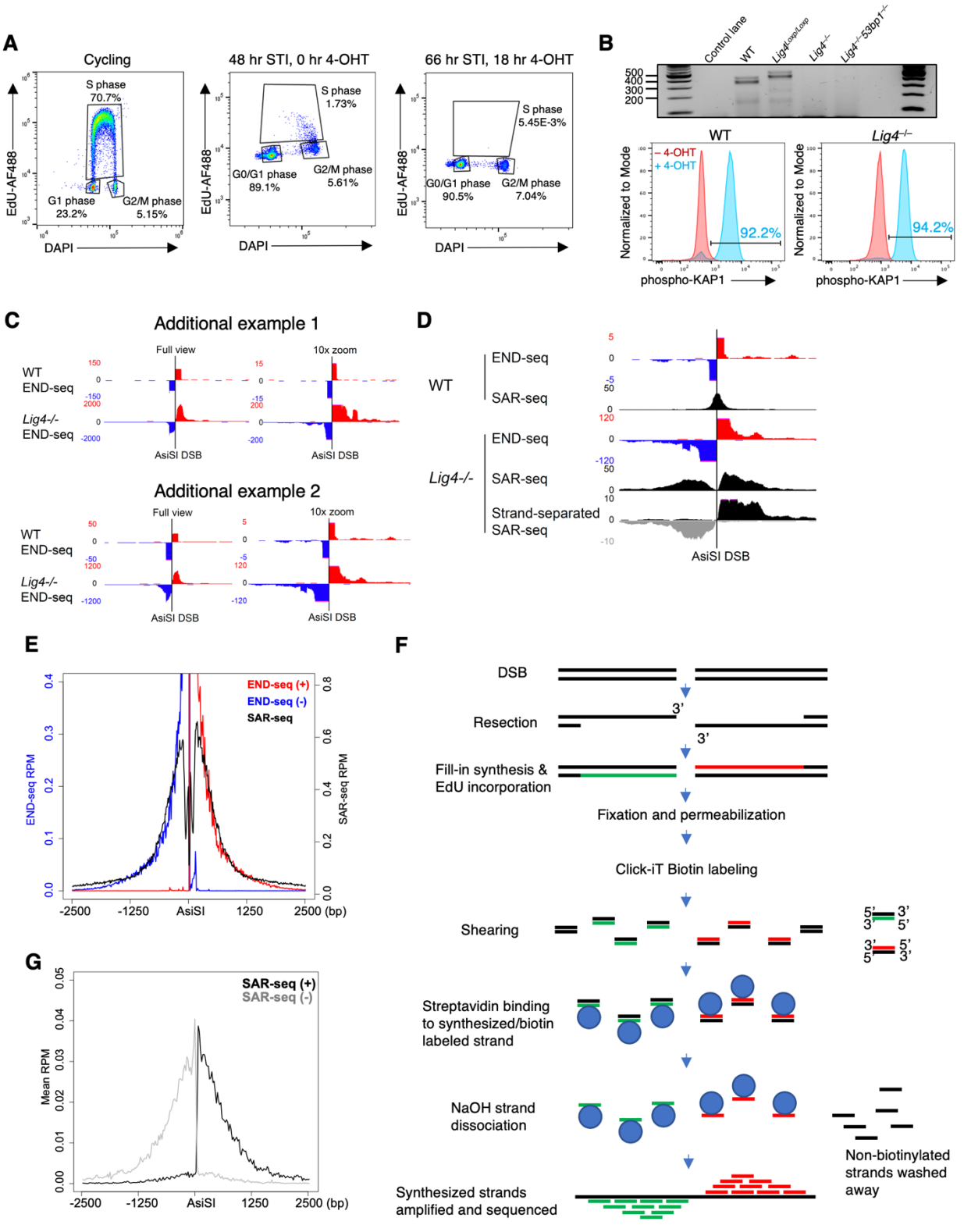
Paiano_Supp_FigS1



Supplemental Fig. S1. Fill-in synthesis occurs exclusively on resected 3' overhangs across the entire resected track. A) Cell cycle profiles of Lig4–/– G0/G1 arrest by EdU and DAPI flow cytometry measurement. While ~70% of cells are in S phase in cycling cultures, 90% of cells are G0/G1 arrested after 48 hr imatinib (STI) treatment and remain arrested 18 hr later after AsiSI breakage. **B)** Confirmation of conditional *Lig4* knockout in WT Abl pre-B cells (Lig4 band at 400 bp), *Lig4*$^{LoxP/LoxP}$ before Cre deletion, (Lig4 band at 500 bp with LoxP site), and *Lig4*$^{-/-}$ and *Lig4*$^{-/-}$*53bp1*$^{-/-}$ cells after Cre deletion. Phosphorylated KAP1 (pKAP1) FACS histograms of Abelson-transformed murine pre-B cells arrested in G0/G1 and induced to express synchronous AsiSI cutting. Positive pKAP1 staining indicates presence of DNA double-strand breaks (DSB) and indicates efficiency of AsiSI induction in the population. **C)** Genome browser snapshots of two additional examples of END-seq resection at AsiSI DSBs (Example 1: chr5:73292850-73292857; Example 2: chr2:32236003-32236010). **D)** Additional example of SAR-seq and strand-separated SAR-seq with END-seq resection profiles as references (chr2:32236003-32236010). **E)** *Lig4*$^{-/-}$ AsiSI-induced END-seq (strand-separated; red=positive strand, blue=negative strand) and SAR-seq (black line) reads per million (RPM) aggregated +/- 2.5 kb around the strongest 200 AsiSI cut sites, averaged across all breaks. Datasets are overlaid to visually demonstrate the identical nature of resection read distribution in END-seq and SAR-seq. This indicates that DNA synthesis, as defined by SAR-seq, totally coincides with ssDNA overhangs, as defined by END-seq dsDNA-ssDNA resection endpoints. **F)** Workflow of DNA strand-separated SAR-seq protocol modification in which only EdU-incorporated strands (i.e., synthesized

DNA) are sequenced. After AsiSI DSBs are induced, resected, and fill-in is EdU-labeled in vivo, cells are fixed/permeabilized and EdU is biotin-labeled via Click-iT reaction. A detailed description of the SAR-seq protocol can be found in Wu et al. 2021. After shearing and streptavidin enrichment on magnetic beads, dsDNA fragments are denatured by a brief NaOH digestion. Strands not bound to streptavidin-beads (i.e., non-biotinylated/non-synthesized strands) are then washed away before PCR amplification and sequencing. **G)** Aggregated reads per million (RPM), averaged across the top 200 AsiSI breaks, of strand-separated SAR-seq signal in *Lig4*$^{-/-}$ AsiSI-induced cells, +/- 2.5 kb around AsiSI cut sites. Strand-separated SAR-seq was performed once in *Lig4*$^{-/-}$. Reads aligning to the positive (black) or negative (grey) DNA strands are exclusive to either side of the break, indicating that DNA synthesis occurs only on 3' overhangs generated by resection. If synthesis occurred on 5' overhangs, then the opposite pattern would be observed. If synthesis occurred on a mixture of both 3' and 5' overhangs, then there would be a heterogeneous distribution of reads aligning to the positive strand and negative strand on either side of the DSB.