Supplementary Figure 1. Digestion of model DNA substrates. a, Linearized plasmid DNA (pIK31-PstI, lanes 1 and 2), supercoiled plasmid (pIK31, lanes 3 and 4), singly nicked plasmid (pIK31-Nb.BvCI, lanes 5 and 6) and M13mp18 ssDNA (lanes 7 and 8) was digested with S1 for 7.5 minutes at 37°C. b, A 100-mer circular substrate was hybridized with complementary oligonucleotides to generate 20 nt, 10 nt or 0 nt (nicked substrate) ssDNA gaps and then digested with S1 nuclease for the indicated times.
Supplementary Figure 2. Scheme of the polymerase release assay to monitor signal and collision release during replication. The lagging strand template (i.e. the leading strand product of a rolling circle reaction) is labeled using (α-32P)-dTTP and will be resistant to S1 (top diagram) unless a ssDNA gap is produced by signal release (bottom diagram). The expected results for 100% collision release and for 100% signal release, are illustrated to the right. Analysis of S1 digested lagging strand template DNA on denaturing agarose gels digests the lagging strand template in the event of signal release, which produces ssDNA gaps. In the case of 100% signal release, a ssDNA gap will be present between each Okazaki fragment, and S1 digestion will yield products that equal the size of Okazaki fragments. The products of lagging strand Okazaki fragment synthesis are monitored in a separate reaction by addition of (α-32P)-dATP. In the case of 100% collision release, only nicks will separate Okazaki fragments, and the lagging strand template will be resistant to S1 cleavage.
Supplementary Figure 3. Determination of product molecular weights and calculation of frequency of signal release. 

**a**, Lane profiles from Fig. 2b were normalized to the corresponding molecular weight at each pixel in order to correct for the fact that longer products incorporate more radiolabel (unbroken lines), and were analyzed by fitting to a single Gaussian distribution (dashed lines). The peak of the fit corresponds to the average length of DNA, which enables the percent of signal release to be calculated (see Supplemental Methods). The fit derived from a single Gaussian analysis reveals a small subpopulation of slower migrating products (a, upper panel), which we presume derive from asynchronously fired replisomes or prematurely terminated forks. **b**, To ensure that this subpopulation does not affect the outcome of the results, we compared results in which we included this subpopulation by fitting the data to a double Gaussian distribution (upper panel). **c**, Signal release was calculated using the length (kb) obtained from either the single (1G) or double (2G) Gaussian distribution analysis. The result was essentially unchanged; n = 7 for 2G, and n = 3 for 1G analysis, respectively. **d**, Mean of the product size was used to calculate signal and collision release as described in Supplemental Methods; n = 5 ± SEM.
Supplementary Figure 4. Control reactions for the bead-based S1 assay. 

**a**, Replication reactions containing (α-32P)-dTTP to label the lagging strand template were performed as in Fig. 2b, in the absence of primase (lane 1). The ssDNA product is degraded by S1 (lane 2). This control demonstrates that sufficient S1 is used to digest all ssDNA that can possibly be produced in the assay in the absence of lagging strand synthesis. 

**b**, Replication reactions performed in the presence of (α-32P)-dATP to label the lagging strand Okazaki fragments were treated with or S1 (lane 2) as described in Fig. 2. This control shows that duplex regions, formed by Okazaki fragment synthesis, are not digested by S1. 

**c**, Replication reactions containing (α-32P)-dTTP were quenched, the beads were washed and then resuspended in replication buffer in the presence of 20 nM Pol II and dNTPs to fill any ssDNA gaps prior to S1 analysis. The gap filling reaction shows that the lagging strand template becomes resistant to S1 when Okazaki fragments are complete, as the DNA products look the same in the absence (lane 1) or presence (lane 2) of S1 treatment. 

**d**, Replication reactions performed in the presence of 5’ phosphorylated oligonucleotides to initiate lagging strand synthesis and α-32P-dATP to label Okazaki fragments were quenched, the beads washed and incubated with a buffer control (lane 1) or T4 ligase (lane 2). The results show that a significant proportion of Okazaki fragments become ligated, demonstrating that they contain a nick produced by collision release.
Supplementary Figure 5. Polymerase release is independent of β clamp concentrations. a, Upper panel. S1 analysis of a β clamp titration into replication reactions as described in Fig. 2. Replication reactions contain either (α-32P)-dTTP (lanes 1-10), which labels the leading strand (lagging strand template), or (α-32P)-dATP (lanes 11-15), which labels Okazaki fragments. Lower panel. Quantification of percent signal release at different concentrations of β. b, Reactions were performed as in panel a, except equimolar amounts of both β clamp and γ-complex (γc) clamp loader were titrated into the replication reaction.
Supplementary Figure 6. Signal release occurs in the absence of primase. Quantification of the gel in Figure 2d. Percent signal release at different concentrations of DNA oligonucleotides; n = 3 ± SEM.

Supplementary Figure 7. Polymerase signal release at different concentrations of primase. a, S1 analysis of replication reactions was performed at the indicated concentrations of primase. b, Quantification of percent signal release at different concentrations of primase; n = 4 ± SEM.
Supplementary Figure 8. Long Okazaki fragments are preferentially terminated by signal release. a, Schematic illustration of the fill-in reaction to label gaps produced by signal release. b, Alkaline gel analysis of Okazaki fragments produced during rolling circle replication in the presence of 320 nM primase. Reactions were performed in the presence (lanes 1 and 3) or absence (lane 2) of (α\(^{32}\)P)-dATP to produce either radiolabeled or unlabeled Okazaki fragments, respectively. Quenched reactions were washed, and beads resuspended in replication buffer containing Pol II (lanes 2 and 3) and (α\(^{32}\)P)-dATP was added to label Okazaki fragments that are followed by a ssDNA gap, and thus are extended by Pol II. Lane 2 shows that Okazaki fragments that are unlabeled in the initial reaction, become labeled by Pol II, and are significantly longer than the bulk of Okazaki fragments labeled during replication (compare with lane 1). Lane 3, with radiolabel in both Pol III and Pol II reactions, shows that the overall distribution of Okazaki fragments becomes lengthened relative to lane 1, but that shorter Okazaki fragments essentially remain unchanged. The scan profiles of each lane are shown to the right of the alkaline gel. The data enable an estimate of the average size of the gaps left during signal release by comparing the signal generated during the fill-in reaction, which reflects the presence of all gaps, to the Okazaki fragment lengths (lanes 1 and 2), using the following calculation: Gaps = Length (fill-in) – Length (Okazaki fragments). The result of this calculation yields an average gap size of 800bp. The wide distribution of Okazaki fragment size is due to the fact that primase action is stochastic, resulting in a heterogeneous population of Okazaki fragment sizes.
Supplementary Figure 9. Representative line profiles of individual DNA strands as described in Fig. 4c and h. **a**, buffer flow was 10 µl/min. **b**, buffer flow was 100 µl/min. The data was averaged over a width of 3 pixels and 10 successive frames to minimize signal fluctuations due to lateral or vertical DNA strand motions in the flow. Therefore, the dips in the fluorescence intensity are directly reflecting a variation of the number of YoPro1 intercalators present on DNA, which correlates with the amount of dsDNA present.