ONLINE METHODS

Microscope

Flow cells were mounted on an inverted microscope (IX-71, Olympus) and fluorescence was excited with objective-type TIR illumination through a high-N.A. objective (PLAPON 60X OTIRMF, N.A. 1.45, Olympus) [7,15]. For PhADE imaging of mKikR, a multi-line Argon and Krypton laser (I-70 Spectrum, Coherent) provided the 568-nm excitation line while the photoactivation line was from a diode-pumped, 405-nm laser (maximal output: 50 mW, CrystaLaser). After magnification and filtering, the 568-nm and 405-nm lines were merged via a dichroic mirror (z405bcm, Chroma) and collimated light was focused on the back aperture of the objective with a converging lens (Thor Labs). TIR excitation with 405-nm and 568-nm lines was aligned empirically by off setting the back-focal lens during imaging of an aqueous solution of 100 nM rhodamine 110 and sulforhodamine 101. Fluorescence was collected through the same objective and passed through a dichroic mirror (T585lp, Chroma) and emission filter (ET620/60, Chroma) before 1.6x magnification onto an EM-CCD (iXon, Andor). The pixel size in sample space was 166 nm x 166 nm. Pulses of excitation and activation light were shutter-controlled (Uniblitz) by home-built code (LabView) [36]. The 488-nm line from the Argon and Krypton laser was used to image fluorescein and mKik**G** and the 647-nm line was used to image AlexaFluor647 through standard FITC and Cy5 filter sets (Chroma). SYTOX Orange was excited with 568-nm light and imaged through the mKikR filter set.

Cloning, site-direct mutagenesis, and protein expression

Recombinant *Xenopus laevis* Fen1^{KikGR} was cloned in two steps. First, the mKikGR open reading frame (ORF) was amplified from pRSETb-6xhis-mKikGR [7] using primers A (5′-TAG GGA TCC GAT GAG TGT GAT TAC ATC AGA AAT GAA G-3′) and B (5′ATG ATG CTC

GAG GGC TTC AAA TTC ATA CTT GGC GCC-3′) (IDT DNA) with flanking restriction sites, and the double digest was cloned into pET28b between the BamHI and XhoI sites. The ORF of the resulting plasmid pET28b-mKikGR-6xhis was verified by sequencing. Second, *Xenopus laevis* Fen1a (xFen1) was amplified from pET28 2xFLAG-Fen1-GST-His [37] using primers C (5′-CTA TCC ATG GGA ATT CAC GGT TTG GCC AAA C-3′) and D (5′-GTC ATG CTA GCT TTA CCC CTC TTG AAC TTT CCT GC-3′) with flanking restriction sites, and the double digest was cloned into pET28b-mKikGR-6xhis between the NcoI and NheI sites. *Xenopus laevis* has a pseudo-tetraploid genome and, thus, two non-allelic genes xFen-1a and xFen-1b that differ by 4.5% of their amino acids [38]. Bibikova *et al.* have shown that both protein products are active but that xFen1a is more abundantly expressed in oocytes. The ORF of the resulting plasmid pET28b-xFen1-mKikGR-6xhis was verified by sequencing. Point mutants of Fen1^{KikGR} were made in plasmid pET28b-xFen1-mKikGR-6xhis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the following primers pairs (Operon and IDT DNA): ΔPIP Fen1 (5′-CCG GCT GGA TGA CGC TGC CAA AGT GAC CGG ATC C-3′ and complement) [37], D179A Fen1 (5′- GCA GCA ACA GAA GCC ATG GAT GCC CTG AC-3′ and complement), D181A Fen1 (5′-CAA CAG AAG ACA TGG CTG CCC TGA CCT TTG GC-3′ and complement), and the full ORFs were once again sequenced.

Fen1^{KikGR} were over-expressed and purified via Ni-NTA affinity with some modifications from the previously published protocol [37]. The expression plasmids were transformed in BL21(DE3) cells and selected with 50 μg/ml Kanamycin. 0.5 L of cells were grown at 37ºC in LB with 50 μg/ml Kanamycin to OD600 whereupon the temperature was turned down to 19ºC and overexpression was induced with 1 mM IPTG. From this step onwards, care was taken to protect cells or protein containing fractions from prolonged exposure to room light to limit

premature photoconversion of the KikGR fluorophore. After 6 hours of growth, cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at –80ºC. For purification, the cells were thawed in Buffer A (20 mM Hepes pH 8, 500 mM NaCl, 10% Glycerol, 2 mM BME, 0.1% Ipegal CA-630, 1 mM PMSF, 1 mM Benzamidine) supplemented with cOmplete EDTA-free Cocktail Tablet Inhibitor (Roche) and 1 mg/ml lysozyme and were lysed by sonication. All further steps were carried out at 4ºC. The lysates were cleared at 14,000 rpm in an SS-34 rotor (Sorvall) for 30 minutes, supplemented with 7.5 mM imidazole pH 8, and incubated in batch with 1 ml of Ni-NTA (Qiagen) for 1 hour. The Ni-NTA beads were washed in batch twice for 5 minutes with 25 bed volumes of Buffer A + 20 mM imidazole pH 8. The beads were then loaded onto a column and washed with 10 additional bed volumes of Buffer A + 20 mM imidazole pH 8. Finally, the protein was eluted with 1.5 bed volumes of Buffer A + 250 mM imidazole pH 8. Concentrated fractions, distinguishable by the green color of mKikGR, were pooled and dialyzed into Storage Buffer (50 mM Tris pH 8, 300 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% Glycerol) and stored at –80°C. Protein concentration was assessed by gel densitometry and Bradford assay with a BSA standard (Biorad). These concentration measurements were compared to and found to agree with the concentration determined from the extinction coefficient for 505-nm absorbance peak of the mKikG fluorophore. The photoactivation of KikG to KikR involves a beta elimination reaction that fragments the peptide backbone, allowing the extent of photoactivation to be monitored via SDS-PAGE [7]. Thus, the fraction of the $Fen1^{KikGR}$ that could be photoconverted was determined by exposing a 0.1 mg/ml solution the fusion protein to 405-nm light (0.01 W cm^{-2}) in a quartz cuvette for up to an hour. Aliquots were taken at various time points, separated by for SDS-PAGE, stained with Coomassie, and the ratio of

fragmented versus full-length Fen1^{KikGR} determined by band densitometry. We found that 80% of wild-type Fen1^{KikG} could be converted to Fen1^{KikR} (**Supplementary Fig. 2c**).

Purification and characterization of p27^{Kip}, geminin, and 6xhis-mKikGR were previously described [7,39,40]. 6xhis-mKikGR was biotinylated with EZ-link NHS-PEG$_4$-Biotin per manufacturer's instructions (Thermo Scientific).

Flow cell, DNA templates, and surface tethering

Streptavidin-coated and PEG-passivated flow cells were assembled as detailed in [41] except that inlet tubing was limited to 5 cm in length (flow cell volume including inlet tubing ~6 μl) and (2) that ELB++ (10 mM Hepes-KCl 7.7, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mg ml^{-1} BSA Fraction V (OmniPur, EMD) was used to block non-specific binding to the glass and to flow in DNA. Two DNA templates were used. Biotin-λ-biotin DNA was prepared by ligating 12-mer oligonucleotides modified with 3′ Biotin-TEG (IDT) (BL1: 5′-AGGTCGCCGCCC-TEG-Biotin-3′ and BL2: 5′-GGGCGGCGACT-TEG-Biotin-3′) to the complementary, single-stranded ends of Lambda DNA (N^6-methyl-adenine free) (NEB) [41]. Biotin-λ-fill was prepared similarly except BL2 was replaced with a non-biotinylated oligo, L2 (5′-GGGCGGCGACT-3′). A 30 pM solution of either DNA in ELB++ was drawn into the flow cell at 50 μl min^{-1} for 15 minutes to assemble double or single-tethers, respectively. In real-time imaging experiments, biotinylated QDot605 (Invitrogen) were sparsely immobilized on the surface for use as fiducial markers. In either case, the flow cell was washed extensively (100 flow cell volumes) with a replication-compatible buffer, ELB++, before extracts were added to start licensing and replication.

Single-molecule replication reactions

High-speed supernatant (HSS) extract and nucleoplasmic extract (NPE) were prepared from unfertilized *Xenopus laevis* eggs as described previously [11,14]. After DNA substrates were assembled and washed, the DNA was licensed in HSS. HSS was supplemented with an ATP regeneration system (2 mM ATP, 20 mM phosphocreatine, 5 μg ml^{-1} creatine kinase), 15 μg ml^{-1} nocadazole and, to rescue licensing of the low concentration of λ DNA [15,42], 30 ng μl^{-1} of a DNA oligo duplex (same as QuikChange oligo pair for D179A (see below) annealed in 10 mM Tris-HCl pH 7.5, 50 mM NaCl and 0.1 mM EDTA by slow cooling from 95ºC to room temperature for 1 hour). This oligo duplex was too short to be licensed itself [43]. 20 μl of supplemented HSS was drawn into the flow cell at 10 μl min^{-1} for 2 minutes, flow was stopped, and the reaction was allowed to proceed for 20 minutes at 22ºC.

Next, to promote efficient initiation and elongation only on the licensed λ DNA, a "replication mix" of HSS and NPE containing non-replicating carrier plasmid [42] was introduced into the flow cell. First, HSS was supplemented with ATP regeneration system and nocodazole as describe above and 400 nM geminin. After 5 minutes at room temperature, carrier plasmid (pBS(–)KS II) was added at a final concentration of 15 ng μl^{-1}. Meanwhile, NPE was diluted to either 25% or 50% with buffer supplemented with the ATP regeneration system, 6.7 μM digoxigenin-dUTP (dig-dUTP) (Roche) and, if used, the appropriate concentration of $Fen1^{KikGR}$. Finally, after 5 minutes at 22ºC, 2 volumes of the NPE reaction were mixed with 1 volume of HSS supplemented with Geminin and pBS(–)KS II (non-licensed carrier plasmid). 15-30 μl of this replication mix was drawn into the flow cell at 10 μl min^{-1} to start replication.

To limit initiation on licensed λ DNA, the replication mix was drawn in for only 30-90s and immediately replaced, by flowing at 10 μl min^{-1} for 1 minute, the same mix supplemented with the Cdk inhibitor $p27^{Kip}$ at 70 ng μl^{-1} as described previously [15].

α-Dig staining and measuring of replication bubbles

For studying replication on doubly-tethered λ DNA, extracts but not all proteins bound to DNA were removed by flushing the flow cell with ELB++ for at total of 10 minutes at 10 μl min^{-1}. Under these conditions, singly-tethered DNA remained compacted while doubly-tethered DNA remained extended. Next, dig-dUTP incorporation on doubly-tethered λ DNA was stained with anti-digoxigenin-fluorescein Fab fragments (α-Dig) (Roche) and co-stained with SYTOX Orange (Invitrogen). Doubly-tethered DNA was imaged in the absence of flow α-Dig was excited with 488-nm light (2.3 W cm^{-2}, 100 ms) and SYTOX Orange was excited with 568-nm light (3.5 W cm^{-2}, 100 ms).

Replication bubbles were scored in custom MatLab code. The SYTOX image was used to verify continuity and extension (minimum extension analyzed was 70% of contour length) of DNA. Profiles through the α-Dig signals were analyzed for replication bubbles. Stretches that were 2 pixels long and with a signal 50% above background were scored as the smallest bubbles. α-Dig stretches of > 5 pixels were scored as two bubbles if a valley had less than half of the peak signal on either side. The length measurements were assumed to have an uncertainty of ± 1 pixels or ± 166 nm. This uncertainty was assumed to be independent and random. Error bars (error estimate) are the sum of the propagated error and the standard error of the mean (s.e.m).

Tracking bubble growth

For D179A $Fen1^{KikR}$-labeled replication bubbles that eventually reached a length-to-width ratio of at least 3:1 (suggesting they were on doubly-tethered DNA), tracking began when the signal reached a threshold value, 2-4 times above background, and ended at 25 minutes or when the

DNA compacted. Home-built MatLAB code measured the length of the signal above threshold over time and to the nearest pixel. Bubble length in pixels was converted to basepairs by multiplying by a stretching factor 48,502 bp per 80 pixels.

Scoring dynamics initiations

Origins were scored in custom MatLAB code on λ-DNA molecules that showed an end-to-end distance between D179A Fen1^{KikR} signals that was 70 to 90% of λ-DNA contour length, did not interact or shift, and were well-illuminated. Such molecules were analyzed for up to 10 minutes after NPE addition or until the DNA tether compacted. Kymographs were inspected at multiple thresholds, initiation events were scored that fulfilled the following criteria: the new D179A Fen1^{KikR} signal (1) was 50% above background, (2) it grew in intensity or size, (3) it did not disappear for more than one frame. Finally, (4) if the D179A Fen1^{KikR} tract grew by more than 3 pixels (498 nm, ~1,800 bp) within 1 minute (or 3 times the measured rate of bubble growth), a new initiation was scored in its middle. The latter criterion distinguished some origins firing close together such that their diffraction limited images overlapped.

Concentration of D179A Fen1^{KikGR} needed to label replication bubbles

High concentrations of D179A Fen1^{KikGR} were used to label replication bubbles for real-time imaging. We noticed that the pattern of D179A Fen1^{KikGR} binding to λ DNA was concentration and extract dependent. For one pair of extracts, 4 μM D179A Fen1^{KikGR} lead to stable binding of D179A Fen1^{KikR} along the entire length of the replication bubble as long as 15-20 minutes after NPE addition (**Fig. 2c-d** and **Supplementary Fig. 4c,f** and **Supplementary Fig. 5d-e**). Using

three different pairs of HSS and NPE, addition of 2 μM D179A Fen1^{KikGR} was sufficient to achieve the same effect (remaining **Figures**).

Imaging single molecules of Fen1^{KikR} on DNA

λ DNA was replicated in the presence of 125 nM or 250 nM Fen1^{KikGR} and imaged as described. Afterwards, Biotin-mKikGR was introduced to bind the surface at low densities and single molecules were imaged as during replication. DNA was stained with α-Dig and SYTOX. Fen1^{KikR} and Biotin-mKikR images were processed in ImageJ to flatten the illumination and subtract the background (using Rolling Ball plug-in) [44]. In MatLab, the Fen1^{KikR} molecules colocalizing with α-Dig tracts were chosen and their integrated intensity within a 4x4 pixel region of interest (ROI) was measured over time. Events with intensities above a threshold were called "on" after comparing similarly imaged and analyzed Biotin-mKikR molecules. The number of ROIs that contained a signal was reported as "Fen1 foci" (**Fig. 4b-c** and **Supplementary Fig. 6b**). For **Figure 4d-e** and **Supplementary Figure 7b**, ROIs with "on" events in the first frame were isolated, and the number of consecutive "on" frames was measured (e.g., "On time" = 0 if the ROI was only "on" in the first frame) then compared to the on time for Biotin-mKikGR.

Measuring the off-rate of Fen1^{KikGR} from replication bubbles

During replication in the presence of 2 μM WT Fen1^{KikGR}, Fen1^{KikR} was activated and imaged. Extracts were removed; 1 ng ml^{-1} Biotin-mKikGR was introduced into the flow cell to densely coat the streptavidin-coated surface; and, after extensive washing, Biotin-mKikR was activated once and then imaged with 100-ms exposure of 568-nm light (3.5 W cm^{-2}) at 1 Hz for 50 frames

to obtain the rate of photobleaching. The signal loss of Fen1^{KikR} from DNA was then compared to the photobleaching of surface-immobilized Biotin-mKikR. First, the Biotin-mKikR intensity in the region of interest (ROI) defined by the DNA was integrated for each frame and the background value from the preactivation frame was subtracted. After normalization to the first-frame intensity, the vector was plotted against 568-nm exposure time and was well fit by a biexponential decay:

$$y = A1 \cdot e^{\frac{-x}{t1}} + A2 \cdot e^{\frac{-x}{t2}} y \qquad \text{(eq. 1)}$$

where y is the normalized intensity, x is the exposure time, $A1$ and $A2$ are weighting coefficients for the decays and $t1$ and $t2$ are the mean lifetimes of the rapid blinking and slower bleaching decay routes. Similarly, the intensity of Fen1^{KikR} on DNA in the same ROI was integrated over time, a local background was subtracted, and the intensity was normalized to the first frame. Since the majority of mKikR photodarkening during the imaging period was due to the fast blinking decay, we also fit the Fen1^{KikR} decay curves to a biexponential decay (eq. 1) where $t1$ was fixed as the blinking mean lifetime measured from the Biotin-mKikR bleaching curve and $t2$ yielded the mean lifetime of Fen1^{KikR} on DNA.

Online Methods References

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