Centromeric DNA replication reconstitution reveals DNA loops and ATR checkpoint suppression

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Half of the human genome is made up of repetitive DNA. However, mechanisms underlying replication of chromosome regions containing repetitive DNA are poorly understood. We reconstituted replication of defined human chromosome segments using bacterial artificial chromosomes in *Xenopus laevis* egg extract. Using this approach we characterized the chromatin assembly and replication dynamics of centromeric alpha-satellite DNA. Proteomic analysis of centromeric chromatin revealed replication-dependent enrichment of a network of DNA repair factors including the MSH2–6 complex, which was required for efficient centromeric DNA replication. However, contrary to expectations, the ATR-dependent checkpoint monitoring DNA replication fork arrest could not be activated on highly repetitive DNA due to the inability of the single-stranded DNA binding protein RPA to accumulate on chromatin. Electron microscopy of centromeric DNA and supercoil mapping revealed the presence of topoisomerase I-dependent DNA loops embedded in a protein matrix enriched for SMC2–4 proteins. This arrangement suppressed ATR signalling by preventing RPA hyper-loading, facilitating replication of centromeric DNA. These findings have important implications for our understanding of repetitive DNA metabolism and centromere organization under normal and stressful conditions.

Repetitive sequences can impair DNA replication, adopting unusual conformations and predisposing to genome instability. Centromeric DNA in many species contains 171 base pair (bp) repeat arrays, known as alpha-satellite DNA, which contribute to centromere structure formation. To understand repetitive DNA replication dynamics we studied replication of defined human chromosome segments in *Xenopus laevis* egg extract using bacterial artificial chromosomes (BACs). We showed that large circular BACs formed functional nuclei, which were able to import cytoplasmic green fluorescent protein containing a nuclear localization signal (GFP-NLS) (Fig. 1a,b). Such BACs were fully replicated by semiconservative DNA synthesis, and replication together with replication initiation complex assembly was sensitive to geminin, the MCM2–7 loading inhibitor, and to roscovitine, a cyclin-dependent kinase inhibitor, although their replication time was slower than the time commonly required for sperm replication (Fig. 1c–f and Supplementary Fig. 1a–f). For these studies we used BACs containing human centromeric alpha-satellite DNA or repeat-free DNA regions with similar GC base content (Supplementary Fig. 1g–j). For most of the experiments shown we compared non-centromeric RP11-1151L10 (or L10) and centromeric RP11-5B18 (or B18) BACs. Replication efficiencies of L10, B18 and other different BACs, and chromatin binding of replication proteins on L10 and B18, were similar overall (Fig. 1f and Supplementary Fig. 1k–n). However, centromeric B18 replication kinetics was slower than non-centromeric L10 at early time points (Fig. 1g). This was probably due to the repetitive nature of the centromeric DNA impacting on replication fork progression. Replicated L10 and B18 isolated at 3.5 h from replication initiation however did not show detectable differences in the initiation rate and had an average inter-origin distance (IOD) of 16 kilobases (kb), similar to sperm nuclei (Fig. 1h,i and Supplementary Fig. 1o). Interestingly, centromeric DNA selectively recruited CENPA, the histone H3 variant specifically enriched on centromeric chromatin (Fig. 1j).

To identify proteins enriched on centromeric DNA or depleted from it during DNA synthesis we set up quantitative mass spectrometry (MS) of L10 and B18 chromatin isolated from extract using liquid chromatography (LC)–MS–MS analysis and label-free protein quantification (Methods and Supplementary Fig. 2a). Several proteins turned out to be differentially associated with L10 and B18 BACs (Fig. 2a–c and Supplementary Tables 1–3). Geminin-dependent inhibition of many B18-bound proteins suggested their involvement in centromeric DNA replication (Fig. 2b,c). Several B18-enriched and geminin-sensitive factors had a known function in DNA repair, such as the mismatch repair (MMR) proteins MSH2–6, the MRE11–RAD50 complex, HMGB1–3, XRCC1, XRCC5 and PARP1, and formed a

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Figure 1 BAC DNA induced nucleus formation and DNA synthesis in interphase Xenopus egg extract. (a) BACs were incubated in interphase extract for the indicated time. Samples were fixed and stained with 4,6-diamidino-2-phenylindole for DNA and DiOC6 for membranes. (b) Nuclei assembled in interphase extract supplemented with GFP–NLS and Cy3-dCTP (dCTP). (c) BACs replicated for 4 h in egg extract supplemented with buffer or recombinant geminin. (d,e) Chromatin isolated from sperm and BAC nuclei at different times and analysed by western blot with the indicated antibodies. (f) Autoradiography of non-centromeric (L10) and centromeric (B18) BACs replicated in the presence of [32P]dCTP. A representative image is shown. (g) Replication kinetics of non-centromeric L10 and centromeric B18 DNA. The error bars represent \( s.d. \) of the mean. \( n=3 \) experiments; \( P<0.001 \) when comparing L10 and B18 mean values for all the indicated times; unpaired two-tailed \( t \)-test. (h) Scheme of DNA combing experiment and example of DNA fibre visualization by immunofluorescence. BACs were incubated in egg extract, supplemented with digoxigenin–dUTP (dig–dUTP; green) and, at later times, with biotin–dUTP (red). DNA was isolated at 3.5 h from addition to egg extract for combing. Typical combing of L10 BAC is shown. The midpoints of green tracts (dig–dUTP) represent origins of DNA replication (replication eye in the red track). The distance between midpoints of two adjacent replication eyes represents the IOD. (i) Graph showing distribution percentage of IODs measured for control L10 and B18 DNA. At least 100 fibres were scored for each sample. The error bars represent \( s.d. \) of the mean. \( n=3 \) experiments; \( P<0.001 \) when comparing L10 and B18 mean values for all IODs; one-way analysis of variance (ANOVA). (j) Chromatin was isolated at the indicated times of the replication reactions with L10 and B18 DNA and then analysed by western blot using the indicated antibodies. A representative of three experiments is shown. Unprocessed original scans of blots are shown in Supplementary Fig. 6.
Figure 2 Proteomic analysis of replicating centromeric and non-centromeric chromatin using label-free quantitative MS. (a) Centromeric B18 and non-centromeric L10 chromatin were isolated after 150 min incubation of BAC DNA in egg extract and analysed by high-resolution MS–MS. Volcano plots show the mean log₂ protein B18/L10 ratio plotted against the P values of biological replicates. Proteins differentially represented on centromeric and non-centromeric chromatin are shown. Black lines indicate the significance cutoff. (b) Proteins differentially represented on centromeric chromatin in the absence (B18) or in the presence (B18+) of geminin analysed as in a are shown. Experiments shown in a and b were repeated with three different extracts (see Methods and Supplementary Table 2 for statistical significance). (c) Heat map for some non-centromeric (L10), centromeric (B18) and centromeric geminin-sensitive (B18+) proteins. Chromatin-enriched proteins are in red and depleted proteins in green.
Figure 3  Centromeric DNA replication is associated with checkpoint suppression and MMR protein accumulation. (a) Sperm nuclei or L10 or B18 BACs were incubated in egg extract treated with 25 μg ml⁻¹ APH (+) or buffer (−). Cytoplasmic chk1 and its phosphorylated form (p-chk1) were detected using specific antibodies as indicated. Total transferred proteins were stained with ponceau. (b) The average ratio of p-chk1 signal over the total level of chk1 was quantified and plotted in the graph. The error bars represent ±s.d. of the mean. n=5 independent experiments; P<0.05 when comparing sperm, L10 and B18 mean values for APH or buffer-treated conditions; one-way ANOVA. (c) Chromatin from L10 and B18 BACs was isolated after 150 min of incubation in extracts supplemented with APH or buffer. Samples were analysed by western blot using antibodies against the indicated proteins. (d) The average ratio of rpa32 over histone H2B signal was quantified and is shown in the graph. The error bars represent ±s.d. of the mean. n=7 independent experiments; P<0.01 when comparing L10 and B18 mean values for APH or buffer-treated conditions; one-way ANOVA. (e) Western blot of egg cytosol that was mock (M) or msh6 depleted (D) using anti-Xenopus msh6 antibodies. A typical result is shown. (f) L10 and B18 DNA replication in mock and msh6-depleted extracts. The relative average percentage of [³²P]dCTP incorporation is shown. L10 values in mock-depleted extracts were considered as 100%. The error bars represent ±s.d. of the mean. n=3 experiments; P<0.05 when comparing L10 and B18 mean values for mock and msh6-depleted extracts; one-way ANOVA.

RPA binding and a significant reduction in its accumulation induced by APH onto B18 DNA compared with L10 (Fig. 3c,d). Chromatin levels of TopBP1, which requires RPA to load onto DNA, were also lower on centromeric DNA, although decreased levels of RPA were still able to promote normal ATR binding (Supplementary Fig. 2c,d). Intriguingly, APH treatment stimulated further centromeric DNA loading of MSH2–6 proteins (Fig. 3e lanes 2–4 and Fig. 3f), whose values for APH or buffer-treated conditions; one-way ANOVA. (e) Chromatin from L10 and B18 BACs treated as in c was analysed by western blot with antibodies against the indicated proteins. Total transferred proteins were stained with ponceau. The asterisk indicates a nonspecific band. (f) The average ratio of msh6 over orc1 bound to chromatin was quantified and is shown in the graph. The error bars represent ±s.d. of the mean. n=3 independent experiments; P<0.05 when comparing L10 and B18 mean values for APH or buffer-treated conditions; one-way ANOVA. (g) Western blot of egg cytosol that was mock (M) or msh6 depleted (D) using anti-Xenopus msh6 antibodies. A typical result is shown. (h) L10 and B18 DNA replication in mock and msh6-depleted extracts. The relative average percentage of [³²P]dCTP incorporation is shown. L10 values in mock-depleted extracts were considered as 100%. The error bars represent ±s.d. of the mean. n=3 experiments; P<0.05 when comparing L10 and B18 mean values for mock and msh6-depleted extracts; one-way ANOVA.
Overwound centromeric DNA suppresses RPA accumulation and ATR activation. (a) Electron microscopy of B18 DNA isolated after 150 min incubation in interphase extract. Arrows indicate ssDNA bubbles. A scheme is included. Data represent one out of three experiments. (b) Streptavidin–HRP-stained dot blot of L10 and B18 DNA isolated from BAC nuclei replicated with 40 μM TPT or left untreated and treated with biotin–psoralen (bPsoralen) in the presence (+) or absence (−) of ultraviolet light (UV). Data represent one out of three experiments. (c) Total incorporated bPsoralen was measured using a fluorescence-based biotin quantification assay. Values represent fluorescence intensity expressed in arbitrary units. Error bars represent ± s.d. of the mean. n = 3 experiments; P < 0.05 when comparing L10 and B18 mean values for all treatments; one-way ANOVA. (d) Western blot of rpa32 and mcm7 bound to B18 chromatin isolated from nuclei replicated in the presence of 40 μM TPT and 25 μg/ml−1 APH as indicated. (e) rpa32/mcm7 ratio. Error bars represent ± s.d. of the mean. n = 3 experiments; P < 0.01 when comparing B18 mean values for all treatments; one-way ANOVA, which was used to stabilize endogenous DNA structures for the electron microscopy procedure (Supplementary Fig. 4a). Positively supercoiled DNA, which is enriched on centromeric chromatin17,18, is highly refractory to psoralen crosslinking17,18 and might give rise to ssDNA bubbles after melting in the mild denaturing conditions used to spread DNA for electron microscopy (Supplementary Fig. 4b). To verify the presence of positively supercoiled DNA regions we carried out DNA supercoil mapping, a procedure based on the ability of biotinylated psoralen (bPsoralen) to incorporate preferentially in negatively underwound DNA and poorly in positively overwound DNA17,18. Following bPsoralen photocrosslinking of intact L10 and B18 nuclei, bPsoralen incorporation was monitored by dot blot using streptavidin–horseradish peroxidase (HRP) (Fig. 4b) or a fluorescence resonance energy transfer based assay (Fig. 4c). These experiments also Methods). In addition to the expected replication intermediates we noticed large regions of ssDNA (ssDNA bubbles) up to 1,000 ± 200 bp, distributed along centromeric DNA (Fig. 4a) and absent from non-centromeric DNA (Supplementary Fig. 3a) or naked B18 templates (Supplementary Fig. 3b). ssDNA bubbles were not replication intermediates, which are made of double stranded DNA (dsDNA; Supplementary Fig. 3c) and not of ssDNA (Supplementary Fig. 3d). ssDNA bubbles were abolished by geminin (Supplementary Fig. 3e), were more frequent than replication intermediates (Fig. 4a), were rarely associated with centromeric replication forks (Supplementary Fig. 3f) and were unlikely to be the product of degraded DNA replication intermediates, as overall efficiencies of B18 and L10 replication were similar. We hypothesized that ssDNA bubbles arose from DNA regions resistant to psoralen crosslinking,
revealed low levels of bPsoralen incorporation in centromeric B18 DNA, suggesting the presence of overwound DNA.

We then investigated the mechanisms responsible for this topological arrangement. Condensins promote positive supercoiling of DNA in vitro in the presence of topoisomerase I\(^{10}\). Centromeric chromatin accumulation of condensins revealed by MS and confirmed by WB (Supplementary Fig. 5a,b) prompted us to test whether overwound DNA was actively formed in the presence of condensins and topoisomerase I. To this end we used the topoisomerase I inhibitor topotecan (TPT)\(^{19}\). TPT prevented accumulation of positively supercoiled DNA on centromeric DNA (Fig. 4b,c). bPsoralen incorporation in TPT treated DNA was not due to DNA breakage (Supplementary Fig. 5c). Also, TPT did not affect centromeric DNA-bound condensins (Supplementary Fig. 5d). These observations suggested that topoisomerase I, possibly together with condensins, was involved in the formation of overwound DNA regions on centromeric DNA.

Since positive supercoiling limits DNA unwinding\(^{20}\) we asked whether this topological arrangement was responsible for RPA chromatin binding inhibition. Remarkably, TPT restored normal RPA loading and accumulation on centromeric chromatin (Fig. 4d,e).

To verify whether interference with centromeric DNA topology had an effect on the activation of the ATR-dependent checkpoint we monitored ATR activity using a previously established more sensitive assay\(^{21}\), which is based on histone H2AX phosphorylation. This assay confirmed that ATR was not activated by stalled forks arising on centromeric DNA (Fig. 4f), also showing lower basal levels of checkpoint signalling on untreated centromeric DNA. Consistent with the effects on RPA chromatin binding, TPT treatment was able to restore normal ATR-dependent checkpoint activation on centromeric DNA in the presence of APH (Fig. 4f).

To understand the physiological significance of ATR checkpoint suppression we tested the effect of restoring ATR signalling using TPT and APH at concentrations that did not inhibit the bulk of DNA polymerization but that were able to induce limiting amounts of ssDNA\(^{22}\) and ATR activity (Fig. 4f). Significantly, centromeric DNA replication was inhibited by low levels of ATR activity and could be restored by an ATR-specific inhibitor (Fig. 4g). Taken together, these data suggested that suppression of ATR signalling facilitates centromeric DNA replication.

During early stages of mitosis, condensins and topoisomerases initiate a process that leads to chromosome condensation, in which DNA is organized in large DNA loops held together by a protein matrix\(^{10}\). Condensins play important but poorly understood roles in the general organization of chromosomes and condensers\(^{19}\). Overwound DNA regions in the centromere might underlie the presence of DNA loops linked to condensins. To uncover the presence of these structures and to visualize them, we isolated chromatin under limiting protein digestion conditions that preserved sufficient amounts of DNA-bound proteins able to maintain centromeric chromatin structure (Supplementary Fig. 5e,f and Methods). Using this approach we observed dsDNA loops that were distributed on either side of centromeric DNA (Fig. 5a). We could not distinctively visualize the matrix holding the loops, probably due to electron microscopy shadowing interference caused by the DNA, although granules and other structures with different electron density were observed (Fig. 5a). To verify that DNA loops were held by a protein matrix, milder extraction conditions were followed by complete protease K mediated digestion (Supplementary Fig. 5e,f and Methods). This led to DNA loop disappearance and revealed the presence of ssDNA bubbles similar to the ones described earlier (Fig. 5b). We counted about 20–25 loops every 50 kb of DNA with an average size for each loop from base to apex of 1,000 ± 200 bp. DNA loops were absent from geminin-treated samples (Fig. 5c), suggesting that they were actively formed and stabilized by proteins loaded on centromeric DNA during or after DNA replication initiation. Loops were stably maintained over time (Fig. 5c), and inhibition of topoisomerase I prevented loop formation, although it did not perturb loops already formed (Fig. 5d), suggesting that superhelical tension, which could be easily lost during DNA isolation, was involved in DNA loop formation but not maintenance.

Here we successfully reconstituted replication of defined chromosome regions in Xenopus laevis egg extract. We established the proteome of a specific human chromosome segment during its replication, revealing the structure, the replication dynamics and the replication stress response of centromeric DNA. We showed that efficient centromeric DNA replication requires MMR proteins, which can bind mismatched hairpins and other DNA secondary structures\(^{23,24}\), and are probably involved in their resolution, facilitating replication fork progression (Fig. 5c). Such structures can form on alpha-satellite DNA repetitive units, which harbor conserved palindromic regions of homopurine and homopyrimidine elements that can pair following DNA unwinding\(^{25,26}\), potentially altering fork progression. In the presence of replication stress caused by APH, extensive ssDNA regions generated by helicase uncoupling\(^{27}\) might generate even more hairpins\(^{28}\), causing further MMR protein accumulation on centromeric DNA (Fig. 5e, panel ii).

Using electron microscopy we directly observed the ultrastructural organization of centromeric DNA, uncovering the presence of dsDNA loops, which might be held by a condensin-enriched protein matrix. Condensins could promote formation of DNA loops by an extrusion-based (loop extrusion)\(^{29,30}\) or a topoisomerase-dependent mechanism (chiral loops)\(^{10}\). Positively supercoiled DNA and condensin complex II enrichment have previously been observed at centromeres\(^{10,16}\). The dependence of DNA loops on replication and topoisomerase II suggests that they are actively formed as replication progresses. Topoisomerase II participation in this process cannot be ruled out. Inefficient centromere replication in the absence of MSH2–6 might also affect their formation.

The presence of dsDNA loops might reflect centromeric DNA organization in the interphase nucleus during early chromosome condensation processes\(^{10}\), which might start from the centromere in interphase and spread to other regions of the chromosomes in mitosis. DNA loop formation might be facilitated by topoisomerase-induced superhelical tension in the context of centromeric nucleosome fibres, and might require both chiral and extrusion-based mechanisms. Interestingly, condensins promote CENP-A chromatin binding\(^{5}\). However, CENP-A loading kinetics are different on centromeric BAC from somatic cells, in which CENP-A chromatin recruitment takes place between telophase and early G1 (ref. 31), and might not entirely reflect new CENP-A molecules loading onto pre-existing centromeres. Selective accumulation mechanisms of CENP-A and condensins onto centromeric BAC, respective roles of condensin I and II complexes in
Figure 5 Centromeric BACs generate stable dsDNA loops during DNA replication. (a) Electron microscopy of centromeric B18 chromatin isolated after partial proteinase K digestion (see Methods). (b) Electron microscopy analysis of samples isolated as in (a) and subjected to complete protein digestion. (c) Graph showing the frequency of DNA loops in L10 or B18 DNA incubated in extracts that were untreated or treated with geminin (GEM) for the indicated times. Experiments were repeated three times, scoring the equivalent of about one megabase of DNA in total. Error bars represent ±s.d. of the mean. n = 3 experiments; *P < 0.001 when comparing L10, B18 and B18 + GEM mean values for the indicated times; one-way ANOVA. (d) Frequency of loops on B18 DNA incubated in extracts supplemented with buffer or treated with TPT at the start of reaction (TPT early) or after its completion at 300 min (TPT late) and collected 1 h later. Experiments were repeated three times, scoring the equivalent of about one megabase of total DNA. Error bars represent ±s.d. of the mean. n = 3 experiments; *P < 0.001 when comparing B18 mean values for all treatments; one-way ANOVA. (e) Model illustrating the arrangement and response to replication stress of centromeric chromatin, which is organized in loops of dsDNA formed in the presence of condensins (SMC2–4) and active topoisomerase I (Top I). Loops accumulate most probably behind replication forks. Individual centromeric repeats might form hairpins or other secondary structures on unwound ssDNA at replication forks, attracting MMR proteins, which might be required to resolve them (a). This topological arrangement limits the formation of extensive ssDNA regions and RPA hyper-loading onto chromatin triggered by the uncoupling of helicase and polymerase induced by APH or other fork stalling events. Inhibition of RPA binding to chromatin prevents activation of ATR. ssDNA generated during fork uncoupling folds into several hairpins, leading to MMR protein accumulation (b). Interference with centromeric DNA topology by the topoisomerase I inhibitor TPT restores RPA loading and ATR activity, which inhibits centromeric DNA replication (c), suggesting that topology-dependent suppression of ATR normally facilitates replication of hard-to-replicate repetitive DNA sequences present at the centromere.
loop formation and their involvement in CENP-A loading remain to be clarified.

Accumulation of DNA loops must be coordinated with DNA replication to allow replication fork progression\(^{20}\), and it is likely to take place behind forks (Fig. 5e, panel i). The observed dsDNA loops resemble previously proposed centromeric loops organized in spring-like structures linked to centromere and kinetochore function in mitosis\(^{4}\). However, we uncovered another function for this structural organization, showing that this arrangement prevents the exposure of extended regions of ssDNA and consequent RPA hyper-loading in conditions of replication stress (Fig. 5e, panel ii). Physically constrained DNA loops in the nucleus accelerating behind forks could limit the extent of normal fork movements and rotation\(^{22}\), restraining the helicase-mediated DNA unwinding needed for RPA chromat binding\(^{18}\) (Fig. 5e, panel ii). As RPA hyper-loading is required to promote chromatin binding of ATR-activating cofactors, including TopBP1 (refs 11,13), restraining RPA binding to chromatin prevents full activation of the ATR-dependent checkpoint, which normally limits DNA replication origin firing under stress\(^{11}\) (Fig. 5e, panel ii). Interference with centromeric DNA topology restores normal RPA loading and promotes activation of ATR, which inhibits DNA replication (Fig. 5e, panel iii). Suppression of ATR activity facilitates replication of repetitive centromere DNA, whose natural tendency to form DNA secondary structures can slow down replication fork progression. Therefore, the ATR-dependent checkpoint triggered by secondary DNA structures arising in the context of repetitive DNA could be locally suppressed by DNA loops that form as replication progresses, facilitating completion of DNA replication.

Checkpoint suppression is common to other chromosome regions containing repetitive DNA, such as telomeres\(^{33}\), which can form DNA loops and can be organized in positively supercoiled domains by telomeric proteins\(^{34}\). The experimental approach described here will be useful to dissect the role of checkpoint, DNA repair and structural proteins at repetitive chromosome loci, whose aberrant metabolism can predispose to cancer and other human diseases.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note: Supplementary Information is available in the online version of the paper.**

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**AUTHOR CONTRIBUTIONS**

A.A. and V.S. prepared the materials and carried out the experiments. P.S. and A.B. carried out MS analysis. A.A. and V.C. designed, analysed and wrote the manuscript. A.A. and V.S. prepared the materials and carried out the experiments. P.S. and A.B. provided reagents and resources. M. G. obtained funding. J.G. and M. L. provided funding. A.A. and V.C. provided oversight and project administration. J.J. and Y.H. provided supervision. M. G. and A.A. wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

BACs. Non-centromeric BAC (RP11-1151L10, RP11-332P22, RP11-927N14) and centromeric BAC DNA (RP11-5B18, RP11-449A3 and RP11-643N23) were purchased from http://bacpac.chori.org/home.htm. The sizes of the individual BACs and the corresponding genomic inserts are indicated in Supplementary Fig. 1g. RP11 BACs derive from the pBAE3.6 vector. The size of the vector alone without the genomic insert is 7.6 kb.

The BACs were amplified and isolated from bacteria using a QIAGEN Plasmid Maxi Kit. The DNA was resuspended in 50% CsCl supplemented with 12.5 g ml−1 ethidium bromide and centrifuged for 20 h at 60,000 r.p.m. in a 70.1 Ti rotor (Beckman). Formation of a continuous CsCl gradient allowed the precise recovery of supercoiled DNA from nicked, linear and fragmented DNA. Removal of ethidium bromide from DNA was carried out using butanol, and the DNA was finally dialysed overnight in TE buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA). BAC integrity was checked by electron microscopy (Supplementary Fig. 2b). Only BAC preparations containing a significant fraction of intact circular BACs supported efficient nucleus formation and replication reactions.

Microscopy analysis. 60 ng BAC DNA was incubated for 2 h in 20 μl egg extract supplemented with Cy-3dCTP and/or recombinant GFP-NLS. 2 μl reaction in a fixed solution (45 mM PIPES at pH 7.2, 45 mM NaCl, 240 mM KCl, 10% formalin, 50% glycerol, 2 μg ml−1 Hoechst and 3.3-dihexylxloxacarbocyanine (DioCe6)) was then mounted between a slide and coverslip. Slides were analysed by fluorescence microscopy. Recombinant GFP-NLS was obtained from J. Gannon, The Francis Crick Institute, UK.

Replication of BAC DNA in interphase extract. Xenopus interphase egg extracts were prepared as previously described35. BAC DNA (10 ng μl−1) was added to 20 μl interphase egg extract supplemented with [γ-32P]dCTP and incubated at 23 °C. Reactions were stopped with Stop buffer (1% SDS, 8 mM EDTA, 80 mM Tris-HCl at pH 8 and 1 mg ml−1 proteinase K). The mixture was then incubated at 50 °C for 2 h. 5 μl replication reactions were resolved by electrophoresis on a 0.8% agarose gel and further analysed by autoradiography, visualized with Storm Imager and quantified using ImageJ.

For replication efficiency quantification, replication reactions were precipitated with 5% trichloroacetic acid, 2% pyrophosphate solution and spotted on a Whatman GF-C glass filter. After ethanol washes, filters were dried and the incorporated trichloroacetic acid precipitable radioactivity was counted in a scintillation counter and quantified as previously shown36.

For density substitution, replication reactions of sperm DNA or BACs were carried out in interphase egg extracts supplemented with 400 μM BrdUTP and 1 μCi [α-32P]dCTP. The reaction was stopped with Stop buffer (20 mM Tris-HCl at pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.5% SDS, 1 mg ml−1 RNase) and incubated for 10 min at 37 °C. Proteinase K (1 mg ml−1) was added to the mixture for 2 h at 37 °C. Finally, the DNA was extracted with phenol–chloroform and separated from free-labelled nucleotides by gel filtration on a 20% Cysl column (Amersham). DNA samples were diluted in 3 ml TE containing 0.91 g ml−1 CsCl and layered over TE containing 1.13 g ml−1 CsCl. Gradients were spun at 36,000 r.p.m. for 40 h in a 50Ti rotor (Beckman). The density from 30 collected fractions was determined using a refractometer and the incorporated radioactivity was assessed by agarose gel electrophoresis. Aphidicolin, roscovitine and geminin were used at a concentration of 25 μg ml−1 (high APH) or 1 μg ml−1 (low APH). 0.5 mM and 60 nM respectively. Recombinant geminin and roscovitine from J. Gannon (The Francis Crick Institute, London, UK). Anti-IFOM antibody service or previously described14,35,36 were used at a concentration of 25 μg ml−1.

Chromatin preparation for mass spectrometry analysis. 300 μl of interphase extract containing 10 ng μl−1 of BAC DNA was incubated for 150 min at 23 °C. The reaction was stopped by adding one volume of ice-cold EB supplemented with 2 mM dithiothreitol, 0.5 mM spermine, 0.15 mM spermine, 0.1% Triton X-100 and protease inhibitor cocktail (Calbiochem). The chromatin was pelleted through a 500 mM sucrose cushion at 6,000g for 15 min at 4 °C, then washed with EB and sonicated using a Bioruptor with 60 ON–OFF cycles of 3 s at high amplitude.

Protein digestion and peptide preparation. Proteins isolated from chromatin were digested in solution with Lys-C and trypsin with spin ultrafiltration units of nominal molecular mass cutoff 10,000 Da. Briefly, approximately 65 μg of protein from each different sample was transferred to YM-10 Microcon filters (catalogue no. MRFC0R010, Millipore) and centrifuged at 14,000g for 20 min. 60 μl of 8 M urea in 0.1 M Tris-HCl at pH 8.5 (UT) was added and the sample was reduced by adding 10 μl of 100 mM dithiothreitl for 30 min at room temperature. Samples were centrifuged at 14,000g for 20 min. Then, 60 μl of UT and 10 μl of 55 mM iodoacetamide were added to the filters and incubated in the dark for 20 min. Filters were washed twice with 100 μl of UT. 1.2 μg of Lys-C (Wako) was added and incubated overnight at room temperature. Subsequently, 100 μl of 0.1 M Tris-HCl at pH 8.5 were added to dilute the urea concentration and 0.6 μg of trypsin was added in 100 μl 0.1 M Tris-HCl at pH 8.5. Samples were incubated at room temperature for 4 h and centrifuged at 14,000g for 20 min, and finally 50 μl of 0.5 M NaCl was added to the filters and the released peptides were collected by centrifugation. The resulting peptides were dried on a home-made C18 StageTip. Peptides were reconstituted in 60 μl of Solvent A (2% acetonitrile, 0.1% formic acid) and 1 μl was injected for each technical replicate.

Mass spectrometry analysis and proteins quantification. Mass spectrometry analysis was carried out by LC–MS–MS on a quadrupole Orbitrap Q Exactive mass spectrometer (Thermo Scientific). Peptide separation was achieved on a linear gradient from 95% Solvent A to 40% Solvent B (80% acetonitrile, 0.1% formic acid) over 50 min and from 40% to 100% Solvent B in 2 min at a constant flow rate of 0.25 μl min−1 on a HUPLC Easy-nLC 1000 (Thermo Scientific), where the LC system was connected to a 25 cm fused-silica emitter of 75 μm inner diameter (New Objectieve), packed in house with ReproSil-Pur C18–AQ 1.9 μm beads (Maisch) using a high-pressure bomb loader (Proxeon).

MS data were acquired using a data-dependent top10 method for HCD fragmentation.

Survey full scan MS spectra (300–1750 Th) were acquired in the Orbitrap with 70,000 resolution, AGC target 1e6, IT 120 ms. For HCD spectra the resolution was set to 35,000, AGC target 1e5, IT 120 ms; normalized collision energy 25% and isolation width 3.0 m/z.

Three technical replicates of each sample were carried out. Raw data were processed with MaxQuant version 1.5.1.2. Peptides were identified from the MS–MS spectra searched against the uniprotKB_Xenopus_150218 database (48106 entries canonical + isoforms) using the Andromeda search engine, in which trypsin specificity was used with up to two missed cleavages allowed.

Cysteine carbamidomethylation was used as a fixed modification, methionine oxidation and protein amino-terminal acetylation as variable modifications. The maximum number of MS and MS–MS peaks was set at 5 and 20 ppm respectively. The peptide and protein false discovery rates (FDRs) were set to 0.01; the minimal length required for a peptide was six amino acids; a minimum of two peptides and at least one unique peptide were required for high-confidence protein identification. The lists of identified proteins were filtered to eliminate reverse hits and known contaminants.

Label-free analysis was carried out, including a ‘match between runs’ option (time window of 5 min). A minimum ratio count of 2 was considered and the ‘1 LFQ intensities’, which are the intensity values normalized across the entire data set, were used. Non-centromeric L10 chromatin, centromeric B18 chromatin and centromeric B18 chromatin in the presence of geminin was obtained from three different extracts, to be considered as biological replicates.

Statistical t-test analyses were done using the Perseus program (Version 1.5.1.6) in MaxQuant environment. Missing values were replaced by random numbers drawn from a normal distribution by the function ‘imputation’ (width 0.3, down shift 1.8, separately for each column). For all the statistical analysis an FDR 0.05 was applied using a permutation test (500 randomizations).

Gene ontology annotation for biological process and molecular function was added with Perseus (Mainannot.xenopus.txt.gz 2015). 119 proteins involved in DNA repair and replication, chromatin structure and dynamics, RNA processing and modification, intracellular trafficking, secretion and vesicular transport, and translation, ribosomal structure and biogenesis were selected and subjected to hierarchical clustering (median LFQ intensity from the three biological replicates was calculated for each protein. Hierarchical clustering was set with the following
parameters: distance, Euclidean; linkage, average; number of clusters, 300, for both row and column tree dendrograms generated by clustering in the Perseus environment).

Identification of DNA replication factors by label-free quantitative MS analysis. Proteins associated with the different L10 and B18 BACs, in three independent biological replicates, were characterized via label-free quantification and LC–MS–MS analysis. Samples were analysed in technical triplicate to make the data reliable.

Moreover, 33,124 and 214 proteins in the three replicates have a fold change higher than two with an FDR of 0.05. 11, 53 and 74 proteins respectively were coherently downregulated in B18 versus L10 BACs. On the other hand, 109, 66 and 117 proteins turned out to be coherently upregulated in B18 versus L10 BACs (Supplementary Table 2). An identical approach was applied to analyse the proteins associated with B18 BAC in the presence or absence of geminin (B18+) +, 198, 115 and 214 proteins turned out to be significant in the one-way ANOVA of the three biological replicates respectively. Of these 89, 49 and 67 proteins have a fold change higher than two with an FDR of 0.05 and are coherently downregulated in B18 versus B18+.

Moreover, 33,124 and 214 proteins in the three replicates have a fold change higher than two with an FDR of 0.05 and are coherently downregulated in B18 versus B18+. On the other hand, 109, 66 and 117 proteins turn out to be coherently upregulated in B18 versus B18+ (Supplementary Table 2). After gene ontology enrichment analysis we found that biological processes and molecular functions enriched for proteins differentially regulated in B18 versus B18+ or B18 versus L10 can be divided into the following: DNA repair and replication; chromatin structure and dynamics; RNA processing and modification; intracellular trafficking, secretion and vesicular transport; carbohydrate transport and metabolism; translation, ribosomal structure and biogenesis; general function prediction only; signal transduction mechanisms; energy production and conversion; cell cycle control, cell division, chromosome partitioning and post-translational modification, protein turnover and chaperones; amino-acid transport and metabolism.

DNA combing. BACs were incubated at 7 ng ml−1 in interphase egg extracts supplemented with dig-dUTP (Roche). Where indicated, biotin–dUTP (Roche) was added at 110 min and the DNA replication reaction was stopped at 3.5 h by diluting the extract with an equal volume of ice-cold PBS buffer. Nuclei were then pelleted on a sucrose cushion by centrifugation. Nuclei pellet were resuspended in 1% low-melting-point agarose (Lonza) and transferred to a casting mould to prepare the plugs. Plugs were then treated with 2 mg ml−1 proteinase K (Roche) at 30 °C overnight. Treatment was repeated with freshly prepared proteinase K. Subsequently, plugs were washed several times in TE. The TE buffer was replaced with 50 mM MES buffer at pH 5.7 (3 ml for each plug) and the plugs were incubated at 65 °C for 15 min. Once melted, the plugs were treated with β-agarase (3 units per plug; New England Biolabs) at 42 °C overnight. The resulting solution was used for stretching DNA fibres on silanized slides (Genomic Vision) at a constant speed of 18 mm min−1. Slides were then dried at 65 °C for 30 min and stained as previously described. DNA was scored for at least 100 fibres for each sample and the average percentage of the different classes of IODs was then calculated. Statistical analysis of DNA combing data was carried out using Prism 5 software.

H2AX kinase assay. H2AX phosphorylation was assessed by isolating nuclear proteins, which were extracted from nuclei assembled with BACs incubated in interphase egg extracts for 150 min, diluted with 5 ml of EB, layered onto 2 ml EB plus 30% (w/v) sucrose and centrifuged at 3,000 g for 10 min at 4 °C. Nuclei were washed in EB, resuspended in three volumes of osmotic lysis solution (pH 7.2, 0.32 M sucrose, 1 mM NaHCO3), and then pelleted in 1.5 ml tubes at 14,000 r.p.m. using an FA-45 rotor in an Eppendorf benchtop refrigerated centrifuge for 25 min. 5 ml nuclear extracts were mixed with 20 µl of EB kinase buffer (20 mM HEPES at pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM NaF, 1 mM Na3VO4, and 10 mM MnCl2) supplemented with 0.5 µg ml−1 histone H2AX peptide (Sigma-Genersys), 50 µM ATP and 1 µl of [γ-32P]ATP (10 Ci mmol−1) in the absence or in the presence of 10 µM VE-821 (Merck). Samples were incubated at 30 °C for 20 min; reactions were stopped by adding 20 µl 50% acetic acid and spotted on p81 phosphocellulose filter paper (Upstate Biotechnology). Filters were air-dried and washed three times in 10% acetic acid. Radioactivity was quantified in a scintillation counter.

TUNEL assay. The TUNEL assay on chromatin has been previously described.

Electron microscopy. DNA for electron microscopy analysis was processed as previously described with some modifications. Briefly, for ssDNA bubbles and replication intermediate visualization 10 ng µl−1 BAC DNA was incubated at 23 °C in 300 µl egg extracts for 150 min, diluted with 5 ml of EB, layered onto 2 ml of EB plus 30% (w/v) sucrose and centrifuged at 3,000 g for 10 min at 4 °C. Pellets were resuspended in 600 µl EB and transferred to a 96-well plate (100 µl well). 4.5–8-Trimethylpsoralen (TMP) was added at 10 µg ml−1 to each well. Samples were incubated on ice for 5 min in the dark and irradiated with 365 nm ultraviolet light for 7 min on a precooled metal block. The procedure from TMP addition to irradiation with ultraviolet light was repeated three more times. For complete protein digestion, psoralen–crosslinked chromatin was incubated with proteinase K (1 mg ml−1) and RNase A (167 µg ml−1) for 2 h at 37 °C in EB. Genomic DNA was extracted with 25:25 (v/v) phenol–chloroform, precipitated with isopropanol and processed for electron microscopy analysis. For DNA loop analysis milder extraction conditions were used. Briefly, samples were incubated with proteinase K (1 mg ml−1) and RNase A (167 µg ml−1) for 5 min at 30 °C and genomic extraction was carried out with a 25:5 (v/v) phenol–chloroform mix. To assess the role of the protein matrix in DNA loops partially digested chromatin used for electron microscopy was further digested for 2 h with proteinase K (1 mg ml−1) at 37 °C. DNA was extracted as described above and subjected to electron microscopy procedures for visualization. Electron microscopy grid shadowing with a Leica MED200, and image acquisition with an FEI Tecnai 20 EM microscope equipped with a GATAN high-resolution camera, were carried out at the IFOM electron microscopy facility.

DNA supercoil mapping. DNA supercoil–psoralen mapping was carried out by adapting a protocol previously described. Briefly, 10 ng µl−1 BAC DNA was incubated at 23 °C in 300 µl egg extracts treated with or without 40 µM TPT, diluted with 5 ml of EB, layered onto 2 ml of EB plus 30% (w/v) sucrose and centrifuged at 3,000 g for 10 min at 4 °C. The pellets were resuspended in 600 µl EB and transferred to a 96-well plate (100 µl per well). Psoralen–biotin (EZ-link psoralen, Thermo Fisher) was added at 10 µg ml−1 to each well. Samples were incubated on ice for 5 min in the dark and irradiated with 365 nm ultraviolet light for 7 min on a precooled metal block or left untreated. The procedure from addition of psoralen to irradiation with ultraviolet light was repeated three more times. Psoralen–crosslinked chromatin was incubated with proteinase K (1 mg ml−1) for 2 h at 37 °C and RNase A (167 µg ml−1) in EB. Genomic DNA was extracted with 25:25 (v/v) phenol–chloroform mix and precipitated with isopropanol. DNA was fragmented by sonication (ten times for 20 s). Biotin incorporation into DNA was detected by dot blotting using a custom-built dot blot apparatus and streptavidin–HRP as a probe. For quantitative analysis of psoralen–biotin incorporation biotinylated fragmented DNA was further digested with benzozene and subsequently processed for Sensolite fluorescence resonance energy transfer based quantification assay (AnaSpec) according to the manufacturer’s instructions. Fluorescence was monitored using a Cary Eclipse fluorescence spectrophotometer.

Statistics and reproducibility. All experiments processed for quantification were repeated using at least three different egg extracts to be considered as biological replicates. An unpaired two-tailed t-test was used to determine the significance of means between two groups of samples. One-way ANOVA was used to determine the significance of means of three or more groups of samples. Two-way ANOVA was used to compare DNA replication of six independent bacmid templates. Microscopic and immunoblot images were taken from one representative result of three or more independent experiments.

Proteomic data deposition. Proteomic data as raw files, total proteins and peptides identified with relative intensities and search parameters have been loaded on the Peptide Atlas repository (http://www.peptideatlas.org/PASS/PASS00755).

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Supplementary Figure 1 BACs replication in egg extract (a) L10 DNA BAC or Sperm chromatin incubated in Xenopus egg extract with $^{32}$PdCTP. DNA synthesis was monitored by measuring the percentage of radiolabelled nucleotide incorporation relative to the input DNA. Error bars represent $\pm$ sd of the mean. $n=3$ independent experiments; $p<0.05$ when comparing L10 and B18 mean values; unpaired two-tailed t-test. (b) L10 BAC was replicated in egg extract in the presence of geminin or roscovitine and then analyzed by WB as indicated. (c) Replicating L10 DNA BAC was pulse labelled with $^{32}$PdCTP for 20 minutes and samples were fixed and analyzed at the indicated time by autoradiography. Quantification of one representative experiment is shown. (d) Chromatin was isolated at different times using L10 BAC DNA incubated in egg extracts treated with geminin or roscovitine and then analyzed by WB as indicated. (e) Graph showing incorporation of $^{32}$PdCTP in fractions derived from CsCl gradient centrifugation of sperm and L10 BAC DNA replicated in egg extract in the presence of $^{32}$PdCTP and BrdUTP. Heavy-Light (HL) and Heavy-Heavy (HH) DNA fractions are indicated. Quantification performed as in (b) from one representative experiment is shown. (f) Molecular combing of RP11-1051L10 (L10) and RP11-5B18 (B18) BACs fully replicated in the presence of digoxigenin-dUTP (green). (g) Table representing the nomenclature, the size and the chromosome regions of the BACs used in the present study. (h) Chromosome mapping of the centromere BACs. (i) Graph showing the percentage of alpha-satellite sequences in each centromeric BAC used. (j) Graph showing the GC base content in the BAC DNA sequences. (k) Replication of B18 and L10 DNA BACs incubated for five hours in Xenopus egg extracts supplemented with $^{32}$PdCTP. DNA synthesis was monitored by measuring the percentage of radiolabelled nucleotides incorporation relative to the input DNA. Error bars represent $\pm$ sd of the mean. $n=3$ experiments; $p<0.001$ when comparing L10 and B18 mean values; unpaired two-tailed t-test. (l) DNA replication of the different BACs DNA described in (g). Average percentage of $^{32}$PdCTP incorporation. L10 values were considered as 100%. Error bars represent $\pm$ sd of the mean. $n=3$ experiments; $p<0.001$ when comparing DNA replication mean values for all the indicated BACs. Two-way Anova. (m) (n) Control and centromeric chromatin was isolated at the indicated times and then analyzed by WB using the indicated antibodies. (o) Analysis of the average inter-origin distance (IODs) relative to at least hundred fibres of DNA in replicated L10 and B18 BACs. Error bars represent $\pm$ sd of the mean. $n=3$ experiments; $p<0.001$ when comparing L10 and B18 mean values; unpaired two tailed t-test.
**Supplementary Figure 2** Proteomic analysis and validation of control and centromeric chromatin. (a) Scheme of MS experiments (see text). (b) STRING analysis of DNA repair factors enriched on centromeric chromatin. (c) WB analysis of L10 and B18 chromatin isolated at 150 minutes from DNA addition to egg extract and probed with the indicated antibodies. MSH2 antibodies used here were different from the ones used in Fig 3. Graphs showing TopBP1 (d) and MSH2 (e) relative abundance on control L10 and centromeric B18 chromatin compared to ATR. Error bars represent ± sd of the mean. n=3 experiments; p<0.05 when comparing L10 and B10 mean values for TopBp1-ATR and MSH2-ATR ratios; One-way Anova.
Supplementary Figure 3 Visualization by EM of L10 and B18 DNA intermediates. (a) EM of a segment of L10 DNA isolated after 150 minutes incubation in interphase egg extract. (b) EM of intact circular centromeric B18 DNA molecule not incubated in egg extract. (c) A typical replication bubble made of doubled stranded DNA (dsDNA). (d) Typical ssDNA bubble observed on centromeric b18 DNA. Differences in ssDNA and dsDNA fiber thickness can be appreciated. (e) Graph showing the frequency of ssDNA bubbles for each mega base of non-centromeric DNA (L10) or centromeric B18 DNA incubated in egg extracts that were untreated (B18) or treated with geminin (B18 + Geminin). Experiments were repeated three times and one mega base of total DNA was scored. Error bars represent ± sd of the mean. n=3 experiments; p<0.001 when comparing L10, B10 and B10 + Geminin mean values; One-way Anova. (f) Typical replication fork intermediate present on B18 DNA.
**Supplementary Figure 4** DNA isolation procedure for EM. (a) Nuclei were isolated at different times from addition of L10 or B18 BAC DNA to egg extract and subjected to psoralen photo-crosslinking with 365 nm UV light. Chromatin was completely digested with 1 mg ml⁻¹ Proteinase K for 2 hours at 37°C. DNA was isolated by phenol:chloroform extraction (25:25 v/v) and spread in the presence of 1.5 % formamide on carbon coated EM grids, which were subjected to rotary shadowing. (b) Scheme showing DNA positive supercoil (+) mediated inhibition of psoralen crosslinking, resulting in ssDNA bubbles after melting in mild denaturing conditions used for the EM preparation.
Supplementary Figure 5 Condensins accumulation and structural arrangement of centromeric chromatin. (a) WB analysis showing SMC2 loading on L10 and B18 chromatin isolated at 150 and 300 minutes from BAC DNA addition to egg extract. MCM7 was used as loading control. (b) Graphs showing SMC2 relative abundance on control L10 and centromeric B18 chromatin compared to MCM7. Error bars represent ± sd of the mean. n=3 experiments; p<0.01 when comparing L10 and B18 mean values for the indicated times; One-way Anova. (c) Tunel assay showing 32PdCTP incorporation catalysed by Terminal Transferase (TdT) on DNA derived from L10 and B18 nuclei isolated from egg extracts that were untreated, treated with topotecan (TPT) (+) or left untreated (-). MCM7 was used as loading control. A representative result is shown. (e) Modified EM protocol used to analyse BAC chromatin. Nuclei were isolated at different times from addition of L10 or B18 DNA to egg extracts and subjected to psoralen photocrosslinking with 365 nm UV light. Chromatin was treated with 1 mg ml⁻¹ Proteinase K for 5 minutes at 30°C. DNA was isolated by phenol:chloroform extraction (25:5 v/v) and spread in the presence of 1.5 % formamide on carbon coated EM grids, which were subjected to rotary shadowing. (f) WB showing levels of SMC2 protein present on B18 derived centromeric chromatin following procedures for EM staining performed at low (5 minutes) and high (2 hours) proteinase K treatment (See Methods). A typical result of is shown.

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Supplementary Figure 6 Whole scans relative to cropped immunoblot images. Red boxes indicate the cropped regions used to assemble images in the present study. Black bars indicate position of size markers (kDa). Some panels include samples not used.
Supplementary Figure 6 continued
Supplementary Figure 6 continued

Fig 3g
Mock
MSH6 dep

Fig 4b
L10 B18 B18+TPT No DNA
-UV +UV
Psoralen biotin

Fig 4d
TPT TPT TPT APH APH
MCM7
RPA 32
Supplementary Figure 6 continued
Supplementary Figure 6 continued
Table Titles and legend

**Table S1 Total proteins identified by MS.** The table contains raw data for proteins and peptides identified by MS.

**Table S2 Proteins differentially represented on centromeric and non-centromeric chromatin in biological replicates.** The table contains proteins differentially represented on centromeric and non-centromeric DNA (B18_L10) and on centromeric DNA in the absence or in the presence of geminin (B18_B18+) in three biological replicates (Bio1, Bio2, and Bio3).

**Table S3 Proteins differentially represented on centromeric and non-centromeric chromatin considered for heatmap.** The table contains protein differentially represented on centromeric (B18) and non centromeric (L10) DNA and on centromeric DNA in the presence of geminin (B18+) considered for the heatmap analysis shown in Fig 2c. Color code is described in Fig 2.