Cell Cycle 13:10, 1607–1616; May 15, 2014; © 2014 Landes Bioscience

Establishment of a replication fork barrier following induction of DNA binding in mammalian cells

Paolo Beuzer, Jean-Pierre Quivy, and Geneviève Almouzni*

Institut Curie; Centre de Recherche; Paris, France; CNRS; UMR3664; Paris, France; Equipe Labellisée Ligue contre le Cancer; UMR3664; Paris, France; UPMC; UMR3664; Paris, France; Sorbonne University; PSL; Paris, France

Keywords: LacO-LacI, TetO-TetR, replication fork block, DNA replication, DNA damage repair, genome instability.

Abbreviations: 53BP1, p53 binding protein 1; DDR, DNA damage repair; DSB, double-strand break; EBNA1, Epstein–Barr nuclear antigen 1; EBV, Epstein–Barr virus; Fob1, fork blocking protein 1; G_1, gap 1; G_2, gap 2; G4, G-quadruplexes; HR, homologous recombination; HU, hydroxyurea; Kb, kilobase; LacI, Lac repressor; LacO, Lac operator; M, mitosis; NHEJ, non-homologous end joining; PCNA, proliferating cell nuclear antigen; RFB, replication fork block; RF, replication fork; RPA, replication protein A; Sap1, switch-activating protein 1; S phase, synthesis phase; SSA, single-strand annealing; ssDNA, single-strand DNA; TetO, Tet operator; TetR, Tet repressor; TOPBP1, topoisomerase II binding protein

Understanding the mechanisms that lead to replication fork blocks (RFB) and the means to bypass them is important given the threat that they represent for genome stability if inappropriately handled. Here, to study this issue in mammals, we use integrated arrays of the LacO and/or TetO as a tractable system to follow in time a process in an individual cell and at a single locus. Importantly, we show that induction of the binding by LacI and TetR proteins, and not the presence of the repeats, is key to form the RFB. We find that the binding of the proteins to the arrays during replication causes a prolonged persistence of replication foci at the site. This, in turn, induces a local DNA damage repair (DDR) response, with the recruitment of proteins involved in double-strand break (DSB) repair such as TOPBP1 and 53BP1, and the phosphorylation of H2AX. Furthermore, the appearance of micronuclei and DNA bridges after mitosis is consistent with an incomplete replication. We discuss how the many DNA binding proteins encountered during replication can be dealt with and the consequences of incomplete replication. Future studies exploiting this type of system should help analyze how an RFB, along with bypass mechanisms, are controlled in order to maintain genome integrity.

Introduction

At every cell division, each daughter cell must inherit only one copy of the completely duplicated genome to maintain genome stability (see ref. 1 for review). Importantly, obstacles such as replication fork blocks (RFBs) can impede its correct execution by hindering replication fork passage. When improperly handled, RFBs can lead to incomplete replication and genomic instability, consequences previously associated with several hereditary disorders and cancer.[2-5] To date, in mammals, our knowledge on these issues has relied on studies on global fork arrest using drugs that interfere with replication.[6-10] Indeed, chemicals such as aphidicolin and hydroxyurea have been successfully exploited to induce a global arrest of replication fork progression in mammalian cells.[11,12] Interestingly, DNA binding proteins that block RF progression, exemplified by Fob1, Sap1, and Rtf1[13-15] in yeast and the viral protein Epstein–Barr nuclear antigen 1 (EBNA1) in mammalian cells,[16-19] have provided examples of specific barriers involving protein–DNA interactions. Secondary DNA structures such as hairpins, cruciforms, triplex DNA, and G4s have also been described as potential RFBs, yet whether RFB formation depends directly on the DNA structure or on the DNA binding protein that recognize these structures is still unclear;[15,20-25] reviewed in references 4, 8, and 26–29. Importantly, the existence of single RFB site has been proposed to explain the occurrence of specific fragile sites that can cause genome instability and cancer[30] or hereditary disorders.[26] Thus, to date, while the existence of genomic regions or situations that lead to replication fork arrest have been identified in mammals, our progress in understanding the mechanism that drives their formation has been hampered by the lack of a tractable system enabling the induction and visually following the process at a single locus in time.

In this paper, we sought out a model system that features: (1) an ability to synchronously induce an RFB; (2) a single and

*Correspondence to: Geneviève Almouzni; Email: genevieve.almouzni@curie.fr
Submitted: 11/19/2013; Revised: 03/18/2014; Accepted: 03/23/2014; Published Online: 03/25/2014
http://dx.doi.org/10.4161/cc.28627

identical RFB site in each cell; and (3) a site that is traceable. For this purpose, inserting the LacO and the TetO repeat arrays in the genome was particularly attractive. Indeed, the LacO/TetO systems have been extensively exploited in mammalian cells for a variety of applications, including tracking a single DNA locus,[31] inducing the binding of proteins to specific DNA loci,[32,33] and monitoring the DDR response at single sites.[10,34] Moreover, fork stalling at repeats of the LacO sites in mammalian cells has been proposed.[35] This view is supported by the appearance of fragile sites at the LacO array bound by LacI, indicated by the presence of γH2AX, chromosomal breaks, and segregation defects.[36] However, whether this was due to the presence of the repeats alone, or whether the binding of the repressor was necessary (as seen for natural RFB with the binding of Fob1, Sap1, Rtf1 in yeast[13-15] and EBNA1 in mammalian cells[17]) had not been deciphered, and a replication analysis has been missing. In *E. coli*, the binding of the LacI or TetR repressor proteins to their respective arrays has been shown to hinder replication fork progression,[36,37] which then resumes upon release of the repressor,[38] suggesting that the binding is leading to RFB. In *S. pombe*, the presence of the LacI protein at LacO arrays generates an RFB that causes genome instability, DNA bridges, and DSBs during mitosis or cytokinesis. Yet, the contribution of the LacI binding or the presence of the LacO repeats was not systematically distinguished, since most data were obtained with the LacI repressor continuously expressed.[39] These data thus prompted us to revisit the LacO and TetO arrays in mammalian cells to determine how the RFB is formed in a system enabling the analysis of events at a single RFB site and at a single-cell level to achieve: (1) a spatiotemporal control of RFB induction; and (2) a means to follow the cellular response.

In this paper, we characterize the behavior of LacO and TetO arrays integrated at single loci in human and murine cell lines after inducing the specific binding of the LacI and TetR repressor proteins. For this we selected clones with single integration sites in mammalian cell lines. To assess replication at these loci, we monitored PCNA localization after induction of LacI and TetR binding. Next, to visualize the extent of DSBs and stalled replication forks, we analyzed the accumulation of γH2AX at the arrays.[40,41] Finally, we characterized the activation of the DDR response by following the DNA repair proteins TOPBP1[42,43] and 53BP1.[44-50] Finally, we show that following binding of TetR for a short time, a DDR response is detected as a function of cell progression in S phase. Taken together our results reveal that it is the binding of LacI/TetR at LacO/TetO arrays and S-phase progression that is key for RFB formation, rather than the repeats, and, thus, we provide a system that can be exploited to further analyze the establishment and bypass mechanisms of an RFB that enable the maintenance of genome integrity.

Results

We first characterized cell lines that stably integrated the LacO and TetO repeats. Given that the nature, number, and spacing between repeats, combined with the genome insertion site could impact DNA replication and repair, we exploited several cell lines that stably integrated LacO repeats, TetO repeats, and a combination of LacO–TetO repeats (**Fig. 1A**; **Fig. S1**): HeLa38, (human cervical cancer) with a LacO system, U2OS-19, (human osteosarcoma) with a TetO system, and U2OS-D2 (human osteosarcoma) and 3T3 NIH2/4 (murine fibroblast) both with a LacO–TetO system. The LacO system (U2OS-D2, HeLa38, NIH2/4) comprises 256 LacO repeats spaced by a 15-bp intervening sequence flanked by a unique I-ISceI cleavage site. The TetO system (U2OS19) comprises 200 repeats spaced by ISceI cleaving sites. The LacO–TetO system (U2OS-D2 and 3T3 NIH2/4) comprises the LacO system as above juxtaposed with 96 TetO repeats spaced by a 23-bp intervening DNA sequence (**Fig. S1**). We verified that we could detect by FISH a single locus of the LacO and TetO arrays in each cell line (**Fig. 1A**) in the absence of the LacI or TetR proteins. Next we verified that we could detect by immunofluorescence (IF) the binding of GFP-LacI and mCherry-TetR at their corresponding arrays following transfection of the cells with a GFP-LacI- or mCherry-TetR-expressing plasmid (**Fig. 1B**). FISH and IF in combination enabled the simultaneous detection of the repeats and bound factors. Indeed, we observed a colocalization of the IF and FISH signals, indicating that repressor binding does not impair the detection of the repeat by FISH (**Fig. 1B**). To examine the LacO arrays at a chromosomal level, we prepared metaphase spreads and revealed the arrays by the binding of the GFP-LacI or mCherry-TetR. We confirmed that insertion of a LacO–TetO array in the murine NIH2/4 cell line took place in the arm of chromosome 3 as previously reported.[10] We found that the LacO–TetO array for the U2OS-D2 cell line inserted into the telomeric region, and that the LacO in the HeLa 38 cell line and the TetO in U2OS19 cell line also integrated into the telomeric region (**Fig. 1C**). These distinct cell lines thus provided us with various types of repeats integrated in different chromosomal regions, a convenient tool to evaluate whether the binding of the repressors to their corresponding sequences could induce an RFB in different contexts.

We next asked whether the binding of the LacI or TetR repressor proteins to their respective LacO and TetO repeats represent RFB sites. We first verified that we could monitor ongoing replication at the LacO and TetO arrays with the repressor bound. By using FISH to visualize the repeats and IF to visualize: (1) the bound repressor (GFP-LacI) following transient transfection; and (2) PCNA as a marker of DNA replication, we could identify single foci where the PCNA, LacO, and GFP-LacI signals co-localized, indicating that replication is ongoing at or in close vicinity to the array (**Fig. 2A**). Quantification of this co-localization in each cell line shows an increase in the percentage of cells undergoing replication at the array when the repressor is expressed, where the increase correlates with the number of repeats (**Fig. 2B**). Given that we did not detect any change in the cell cycle profile after the binding of LacI and TetR (**Fig. S2A**), these results indicate that PCNA persists at the arrays upon repressor binding, potentially due to replication slowing down at the arrays and/or that the replication timing is altered. To consider each scenario, we investigated the replication timing of the arrays upon repressor binding.

We performed immunostaining of PCNA or incorporated BrdU, which can be exploited to visualize replication patterns reflecting early, mid, and late S phase[51] (**Fig. S2B**) and quantified the number of cells showing PCNA localization at the LacO or TetO sites in each sub-S phase (**Fig. 2C** for U2OS19). We found that the colocalization occurs between mid and late S phase in U2OS-D2, NIH2/4, and U2OS19 cells, while in HeLa38 cells, it takes place in early to mid S phase (**Fig. 2C and D**), indicating that the replication timing of the arrays could depend on the cell type rather than being imposed by the array.

We next investigated whether repressor binding would impact the timing of replication onset at the arrays. We found that LacI/TetR binding did not affect when we could initially detect PCNA localization at LacO or TetO sites compared with controls, suggesting that replication onset at LacO and TetO sites is not severely delayed. However, we found in each cell line that PCNA localization persisted at the arrays following replication onset (**Fig. 2D**). Notably, in HeLa38 cells, we observed colocalization for a similar proportion of cells in early and mid S phase in the absence and presence of LacI, suggesting a similar replication timing, yet colocalization persisted in late S specifically when LacI was present. This suggests that the increase of PCNA localization at the arrays reveals a slowdown of replication at these sites, rather than a perturbed replication timing. We then exploited 2 strategies to further investigate the replication timing of the LacO and TetO repeats in the different S subphases. First, using a molecular approach, we used anti-BrdU antibodies to immunoprecipitate replicated DNA from HeLa38 cells that had been treated with BrdU for 15 min and sorted by FACS into G_1, early S, late S, and G_2 phases, and we analyzed the presence of the arrays by PCR[52] (**Fig. S3A**, left). Importantly, the control genes for early replicating (HBA1) and late replicating (HBB) were detected only in G_1 and early S phase, and in late S and G_2, respectively, in the absence and presence of the GFP-LacI repressor, thus validating our experimental conditions (**Fig. S3A**, right). In agreement with our previous observation, we found that similar amounts of LacO are detected in early S phase in the presence and absence of the repressor, but higher amounts are detected in the late S and G_2 fraction, when the repressor is expressed (**Fig. S3A and B**). This supports the idea that the replication timing of the LacO array is prolonged during S phase upon repressor binding. Then, using live cell imaging, we directly monitored PCNA dynamics at LacO and TetO sites. Following transfection with plasmids expressing PCNA fused to mCherry or GFP (mCherry-PCNA with the GFP-LacI repressor in U2OS-D2, and GFP-PCNA with mCherry-TetR in U2OS19), we monitored the colocalization of PCNA with the repressor. Time-lapse movies confirmed that colocalization starts in mid S phase, and that PCNA localization at LacO or TetO arrays is prolonged until very late S phase, when all other PCNA foci have disappeared (**Fig. S4**). This confirms that the binding of repressor proteins increases the persistence time of PCNA and replication foci at the arrays, but does not

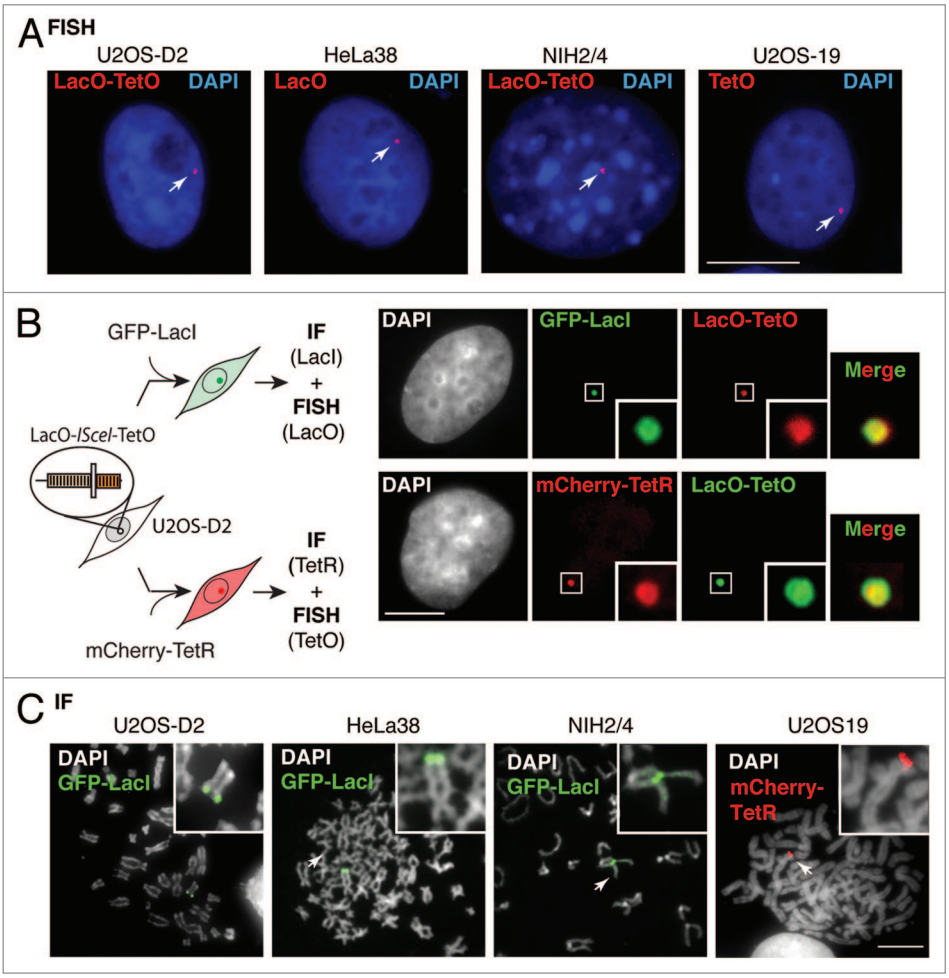

Figure 1. Localization of LacO–TetO, LacO and TetO arrays in different cell lines by FISH. (**A**) Visualization of LacO and TetO arrays by fluorescent nick-translated FISH probes (arrows, red) in the 4 cell lines U2OS-D2, NIH2/4, U2OS-19, and HeLa38. DAPI (blue) stains nuclei. Scale, 10 μm. (**B**) Visualization of the LacO–TetO arrays by FISH and GFP-LacI and mCherry-TetR by IF in transiently transfected U2OS-D2 cells. Left: scheme of the experiment. Right: Images taken 48 h after transfection. We revealed GFP and mCherry by immunofluorescence, while the LacO–TetO site was labeled with fluorescent nick-translated probes. Scale, 10 μm. Insets show a 4× magnification of the boxed area with a merged image on the right. (**C**) Chromosomal location of LacO and TetO arrays insertion in the different cell lines. LacO arrays are visualized by GFP-LacI immunodetection (green) on metaphase spreads in U2OS-D2, HeLa38, and NIH2/4. TetO arrays are visualized by mCherry-TetR immunodetection (red) on metaphase spreads in U2OS19 cells. Scale bar is 10 μm. (Top-right panels) DAPI stains nuclei. Scale, 10 μm. Insets show a 4× magnification of the boxed area corresponding to the chromosome where the array is integrated.

delay the timing of initial PCNA recruitment. Taken together, our data indicate that the binding of the repressor is consistent with the formation of an RFB.

Since RFBs can lead to DNA damage as a consequence of incomplete DNA replication, we next analyzed whether a DDR response was activated as an attempt to bypass the block by monitoring γH2AX, TOPBP1, and 53BP1 accumulation at the array. We performed an analysis with the LacO–TetO system in U2OS-D2 cells after transfection of either GFP-LacI or mCherry-TetR (**Fig. 3A**) and the TetO system in U2OS-19 cells after transfection of mCherry-TetR (**Fig. 3B**). In the absence of repressors, we did not observe localization of γH2AX, TOPBP1, or 53BP1 at LacO and TetO arrays within our detection limits. In contrast, when LacI and TetR were bound to LacO and TetO arrays, γH2AX, TOPBP1, and 53BP1 were detected at the LacO and TetO arrays (**Fig. 3A and B**). Interestingly, we observed a similar percentage of cells with γH2AX at the LacO array to that observed by Jacome and Fernandez-Capetillo.[53] These results indicate that the LacO and TetO arrays do not lead to DDR activation on their own; rather, the binding of repressors at these arrays elicits a DDR response. In our experimental conditions, we detect DDR activation with GFP or mCherry fused to the repressor. This suggests that DDR activation can result from the tethering at a LacO or TetO array of factors that are distinct from a repair factor[33]. Interestingly, we observed that the percentage of cells where we detect the recruitment of 53BP1 and γH2AX at the arrays increases with the number of repeats (**Fig. 3A**, compare 256 LacO repeats and 96 TetO repeats in U2OS; **Fig. 3B**, compare 200 and 96 TetO repeats in U2OS19),

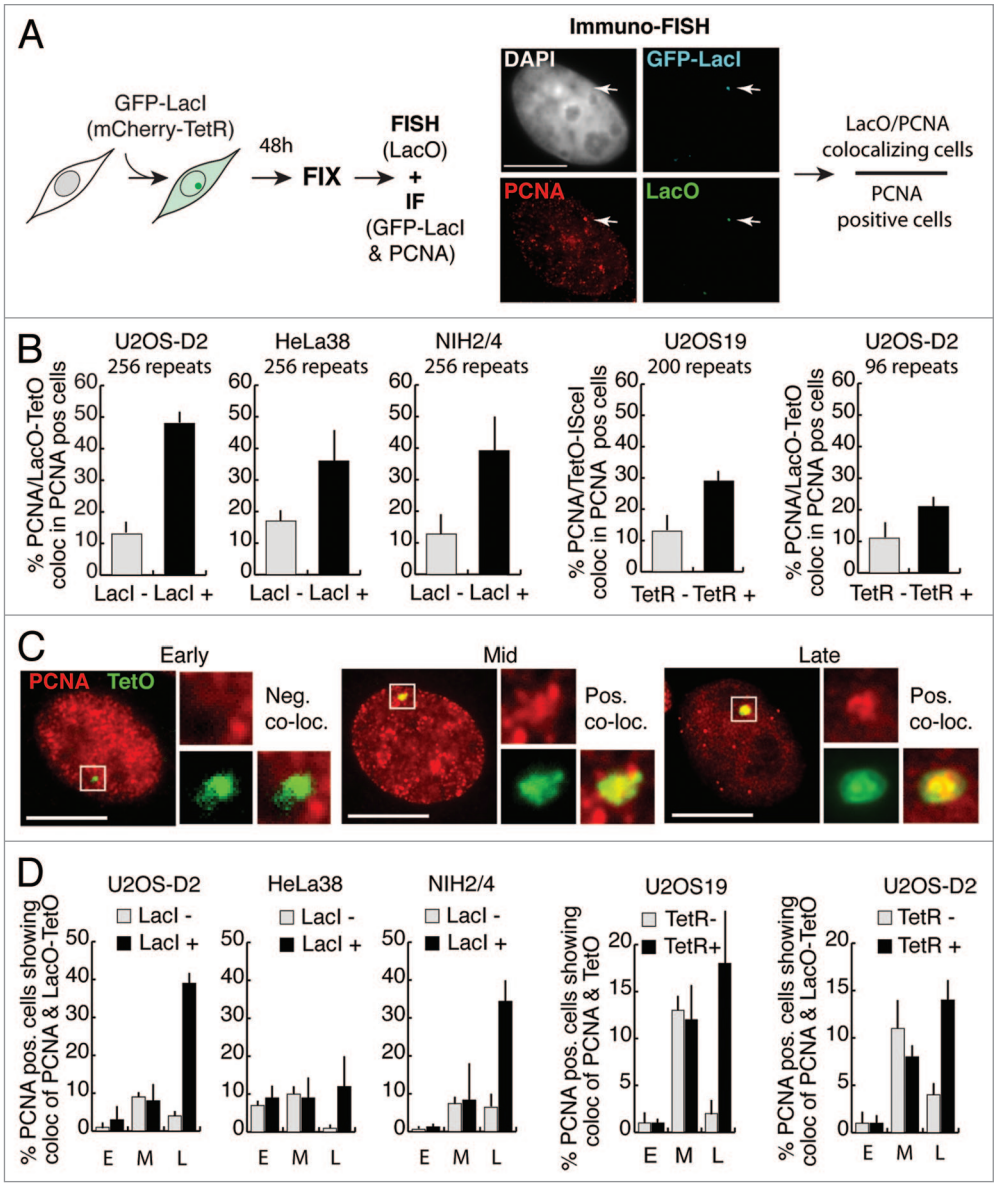

Figure 2. PCNA localization at LacO and TetO sites. (**A**) Left: experimental scheme for the analysis of PCNA localization at LacO/TetO arrays; we performed immuno-FISH 48 h after transfection of either GFP-LacI or mCherry TetR selecting cells, which have been transfected and which were fixed in S phase as indicated by the PCNA signal. Right: Immuno-FISH analysis of PCNA (red) co-localization with the LacO (green) and the GFP-LacI repressor (blue). The arrow indicates the LacO array. Scale 10 μm. We revealed mCherry (or GFP) and PCNA by IF. TetO and LacO arrays were visualized by FISH. Ratio between the number of cells showing PCNA localized at LacO (TetO) arrays and the number of PCNA-positive cells determines the percentage of cells showing replication foci at the arrays during S phase. (**B**) Quantitative analysis of the percentage of PCNA-positive cells with PCNA localized at the LacO or TetO arrays with or without the DNA binding proteins. Immuno-labeling and FISH were performed as described in (**A**), 48 h after mCherry-TetR or GFP-LacI transfection. We calculated mean values and standard deviation from 3 independent experiments in which at least 100 cells were analyzed. (**C**) PCNA colocalization at the TetO arrays in early, mid, and late S sub-phases of U2OS-19 cells 48 h after mCherry-TetR transfection. We revealed PCNA by IF (red), while the TetO array was visualized by FISH (green). Merge signal is shown and the array is boxed. Scale, 10 μm. Insets show a 4× magnification of the array. Colocalization is detected by a yellow signal in the merge. (**D**) Quantitative analysis of the percentage of PCNA-positive cells with PCNA localized at the LacO or TetO arrays for each S sub-phase with or without the indicated DNA-binding protein in the 4 cell lines. We calculated mean values and standard deviation from 3 independent experiments in which at least 100 cells were analyzed.

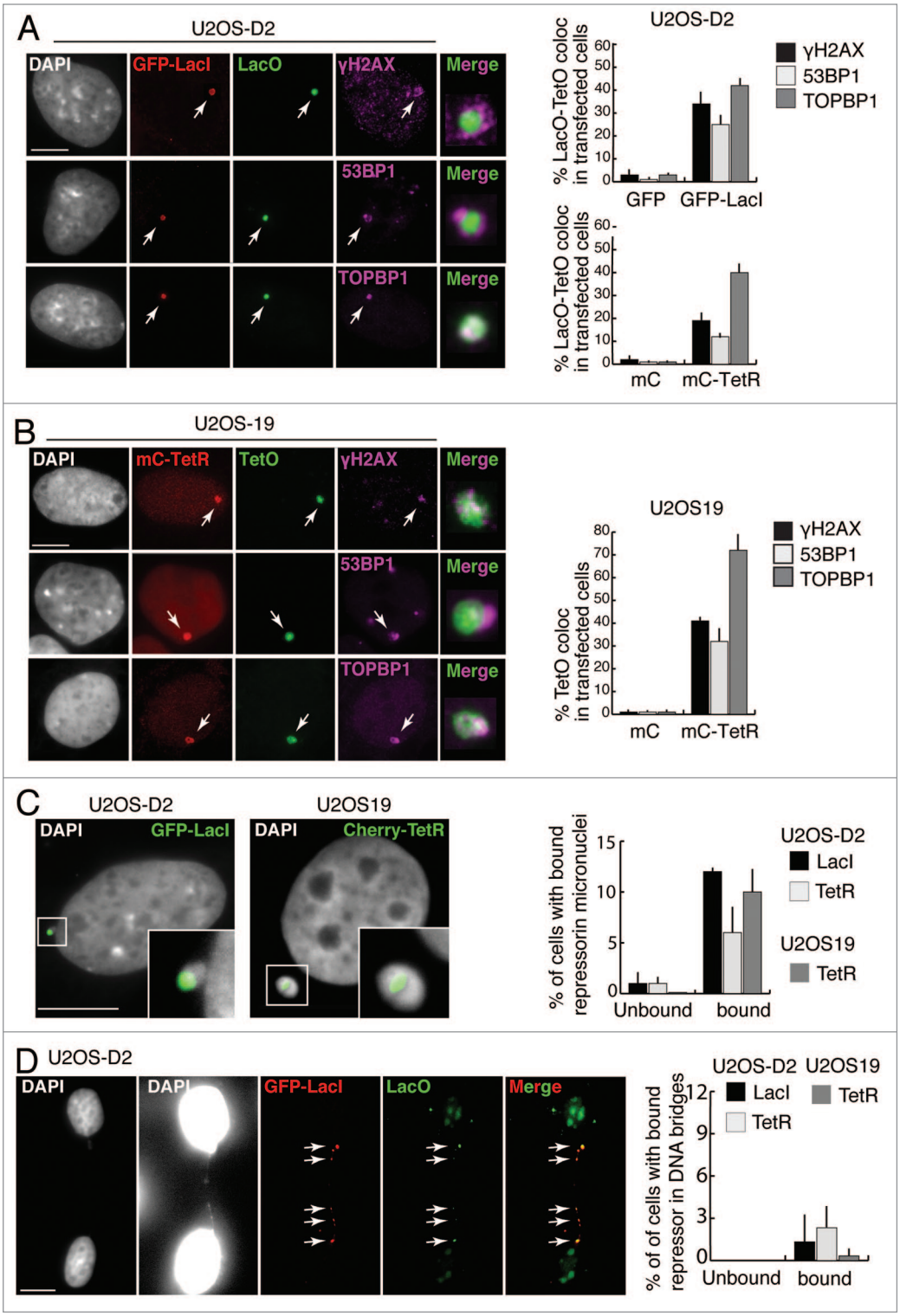

Figure 3. For figure legend, see page 1612.

a pattern observed previously with PCNA accumulation at the array (**Fig. 2B**). Taken together, our results indicate that the binding of repressors at the arrays could activate DDR. This thus suggests that an RFB generated by the binding of the repressors might lead to replication stress and potentially unresolved DNA replication. Given that DNA bridges, micronuclei, and uneven segregation have been documented at unreplicated regions after the completion of mitosis,[39,54,55] we monitored for such phenotypes. Indeed, when GFP-LacI or mCherry-TetR was bound for 48 h, we consistently detected cells with micronuclei containing the LacO and TetO site (**Fig. 3C**), DNA bridges comprising the LacO and TetO arrays (**Fig. 3D**), and uneven segregation of the LacO-TetO array. However, we did not detect any defects in the absence of repressor binding (**Fig. S5**). Notably, in U2OS-D2 cells that integrated a LacO–TetO array comprising 256 LacO repeats, the fraction of cells displaying micronuclei and DNA bridges when GFP-LacI was bound was twice that of mCherry-TetR bound to 96 repeats (**Fig. 3C**). This thus indicates that, as previously observed for PCNA colocalization (**Fig. 2B**) and DDR protein recruitment (**Fig. 3A and B**), the length of the array correlates with the amount of cells displaying abnormal chromosome segregation (in the presence of the bound repressor). Taken together these data suggest that the binding of the repressor impedes replication fork passage, which elicits DDR and leads to an increased occurrence of abnormal mitosis phenotypes.

Finally, we asked whether the DDR response at the array elicited by repressor binding requires passage of the replication fork. We used γH2AX as a marker of DDR activation, a modification previously shown to be one of the earliest responses in DDR and stalled replication forks.[9,40,41] First, we transfected asynchronous U2OS-19 cells with an mCherry-TetR-ER-expressing plasmid to promote binding of the repressor at the array and cultured

Figure 3 (See previous page). DDR activation and segregation defects at LacO-TetO/TetO 48 h after GFP-LacI and mC-TetR binding. (**A**) Left: Immuno-FISH of DDR proteins γH2AX, 53BP1, and TOPBP1, and the LacO and TetO arrays in U2OS-D2 cells which integrated a LacO–TetO array. LacO was visualized by FISH (red). We visualized GFP-LacI (red) and DDR proteins (purple) γH2AX, 53BP1, and TOPBP1 by IF. DAPI stains nuclei. The arrow indicates the array. Scale, 10 μm. The insets show merged images of LacO and DDR proteins at a 4× magnification. Right: quantitative analysis of γH2AX, 53BP1, and TOPBP1 localization at LacO (top) and TetO (bottom) array revealed by immuno-FISH as described above. Mean values and standard deviation from 3 independent experiments. At least 100 cells were analyzed for each transfection. (**B**) As above but with U2OS19 cell line and TetO/mCherry-TerR repressor. The insets show merged images of TetO and DDR proteins at a 4× magnification. (**C**) Left: visualization of micronuclei containing TetO and LacO detected by FISH (green and boxed) after 48 h binding of TetR and LacI in U2OS-D2 and U2OS19 cells. DAPI stains the nuclei. Scale, 10 μm. The inset shows a 4× magnification. Right: Quantitative analysis of the % of cells showing micronuclei with or without 48 h binding of LacI and TetR repressors in both cell lines. Mean values and standard deviation were calculated from 3 independent experiments in which at least 150 cells were analyzed for each transfection. (**D**) Left: visualization of micronuclei containing LacO detected by FISH (green) after 48 h binding of GFP-LacI detected by immunofluorescence (red) in U2OS-D2. DAPI stains the nuclei. Overexposure of DAPI shows the DNA bridge. Merged GFP-LacI and LacO is shown. Arrows point to accumulation of GFP-LacI colocalizing with LacO. Scale, 10 μm. Right: quantitative analysis of LacO and TetO arrays observed with DNA bridges with or without 48 h binding of LacI and TetR repressors in U2OS-D2 and U2OS19 cell lines. Mean values and standard deviation were calculated from 3 independent experiments in which at least 150 cells were analyzed for each transfection.

cells for 14 h with EdU to be able to monitor cells that passed through S phase. We detected γH2AX at the TetO array only in EdU-positive cells, indicating that in addition to repressor binding, passage through S phase is required to elicit DDR activation (**Fig. S6A**). To strengthen this result, we used cells expressing mCherry-TetR-ER synchronized at the G_1/S border by double thymidine treatment (**Fig. S6B**). We promoted the binding of mCherry-TetR-ER with 4-OHT treatment (nuclear translocation) during the second thymidine release, allowing cells to enter and progress through S phase and the subsequent G_2 phase with the mCherry-TetR-ER repressor bound to the TetO array (**Fig. 4A**). We detected a 5-fold increase of cells showing γH2AX accumulation at the TetO array in cells that were released and progressed through S-phase compared with non-released cells (**Fig. 4C**, 54% vs. 11%). Assuming that the 11% of cells showing γH2AX at the TetO array in non-released cells might reflect cells that escaped synchronization by the double thymidine block, this thus indicates that progression through S phase, in addition to the binding of the repressor, is necessary for DDR activation. Thus, we next monitored DDR activation in response to repressor binding during S-phase progression. Following the binding of the repressor and the release into S phase, we collected cells at various time points after the release (**Fig. 4A**) and verified the progression of cells through S phase by FACS (**Fig. S6B**). We then analyzed the proportion of cells displaying γH2AX localization at the TetO array by mCherry immunodetection (**Fig. 4A**). We detected γH2AX localization at the TetO array after 3 h of release in a low percentage of cells. A major increase occurred between 4.5 and 7.5 h, during the progression through mid–late S phase (**Fig. 4C**; **Fig. S6B**), which corresponds to the replication time of the array in this cell line (**Fig. 2C and B**). We detected the highest percentage of cells showing γH2AX at the TetO array at 10.5 h in G_2 (55% of cells), which started decreasing after 12 h, when cells began to enter into G_1 phase (**Fig. 4C**). These data thus indicate that phosphorylation of H2AX at the TetO array with the repressor bound requires progression through S phase and occurs during S phase at the time when the array is replicated. Taken together, these results show that the DDR elicited by the binding of TetO repressor proteins is dependent on the replication of the array. This supports the hypothesis that bound repressor proteins hinder fork progression, representing an RFB.

Discussion

In this work, using an artificial cellular system we could induce and follow RFB formation in mammalian cells. This system exploits the binding of proteins at specific repeat sequences to study DNA replication and DNA repair. Here, with a combination of several LacO and TetO repeat arrays inserted at several distinct genomic loci in different human and mouse cell lines, we could delineate the respective contribution of repeat sequences vs. the binding of defined DNA-binding factors to act as a replication barrier in mammalian cells.

Importantly, we could show that the binding of distinct repressor proteins LacI and TetR to LacO and TetO repeats, respectively, led to: (1) a persistence of replication foci at the repeats; (2) a local DDR response after replication of the sequences; and (3) the appearance of defects after mitosis usually associated with incomplete replication. Based on these data we propose that in the absence of the repressor proteins, a replication fork can progress through the arrays to properly replicate them and permit segregation during mitosis. However, upon binding of the repressor on the array, an RFB occurs that leads to DDR activation and subsequent attempts to bypass it. If this response leads to a proper removal of the RFB, replication can be completed for normal mitosis. If the RFB persists, replication is not completed, and abnormal mitosis ensues (**Fig. 4D**).

The consistent observation of mitotic defects for both LacI and TetR proteins and the corresponding binding sequences in mammalian cells is in line with previous work in *S. pombe*.[39] Thus, based on our data, we can now propose a mechanism to explain the fragility of the LacO site bound by the LacI protein observed in previous studies in mammals.[53] Indeed, we provide evidence that it is the binding of the repressor protein rather than the presence of repeat sequence that cause the defects. Our study also highlights how the nature of the binding of repressor proteins can elicit a typical RFB. Indeed, we found that when repressors bind the longest sequences, replication foci persisted longer (**Fig. 2A**), as a higher percentage of cells showed a DDR response at the array (**Fig. 3A**) and mitotic defects (**Fig. 3D**). This could relate to the establishment of a structure that blocks replication fork at once, or to contiguous successive RF barriers corresponding to successive repeats bound by the repressor. In the latter case, the risk to fail bypassing all of them would increase with the

number of repeats and, hence, lead to increased DDR activation and mitotic defects. Thus, in agreement with previous observations in other model organisms,[39,56] our data support the principle in which the efficiency of the block depends on the length of the bound sequence through which the replication fork must pass.

Taken together our results indicate that hindering replication fork progression via the binding of repressor proteins to the LacO and TetO arrays is conserved in mammals.

The question that arises then is how does the cell bypass an RFB. Given that the major repair pathway reported to be activated to bypass several endogenous natural single site RFBs in *S. cerevisiae*[13,27,57,58] and *S. pombe*[25,58-62] is homologous recombination (HR), it is tempting to speculate that it could also be at work in mammals. However, different reported bypass mechanisms of the RFB imposed by LacI binding to LacO repeats in bacteria[36,38] and *S. pombe*[39] offer alternative views. In bacteria, the HR pathway is not elicited,[38] while in *S. pombe*, the main pathway activated to bypass the RFB is single-strand annealing (SSA).[39] This latter mechanism involves recombination, however, among sequences on the same DNA single strand. Based on our results in mammalian cells, the accumulation of 53BP1 could indicate a role for the NHEJ pathway. Thus, it is also possible that a combination of repair mechanisms could be at work to best ensure the maintenance of the genome. Future work should evaluate in detail how the inducible RFB at a single site that we characterize here elicits the contribution of HR and NHEJ vs. SSA pathways and how usage of these distinct pathways is coordinated to bypass the RFB. As illustrated by the presence of DNA bridges and micronuclei, an RFB activating DDR in S phase may leave unreplicated DNA that impacts the next steps in the cell cycle. Recent work suggests that HR could be activated in S and G_2 phase as a rescue mechanism to try and replicate non-replicated sequences. The non-replicated DNA stretches would then

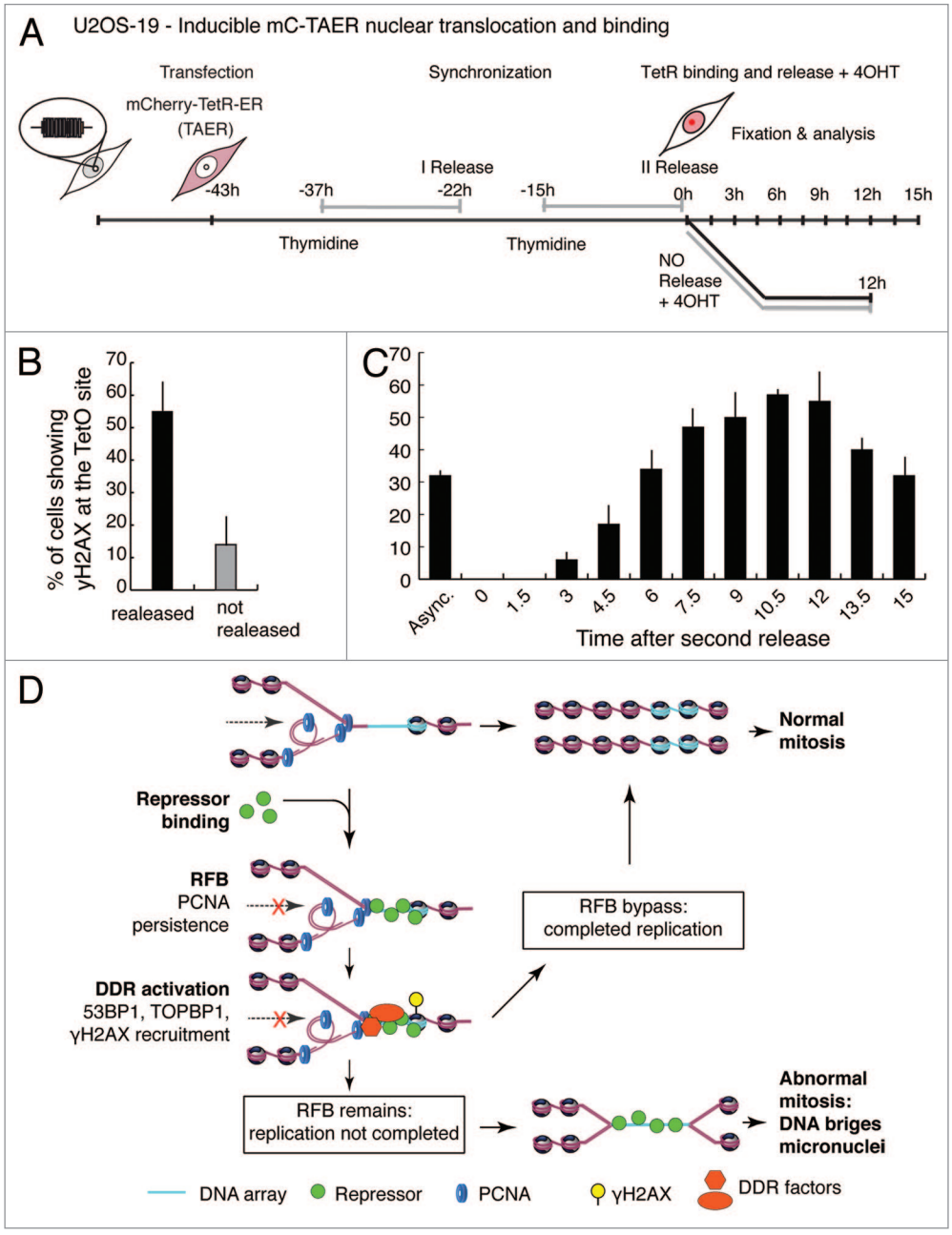

Figure 4. Replication and repressor binding are both necessary for γH2AX accumulation at the array. (**A**) Experimental scheme: We transfected U2OS19 cells with mCherry-TetR-ER and synchronized them in G_1/S with double thymidine treatment. After the second release, we treated them with 4OHT to promote nuclear translocation of mCherry-TetR-ER and binding to DNA. Cells were treated with 4OHT and either kept blocked in thymidine for 12 h or released for 12 h (see **B**) or treated with 4-OHT and released from the block and taken for analysis every 1.5 h. For the analysis, we scored the percentage of cells with γH2AX at the TetO array (bound by mCherry-TetR-ER) by IF. (**B**) Quantitative analysis of the cells released or non-released 12 h after 4-OFT treatment. Mean values and standard deviation are calculated from 3 independent experiments in which at least 100 cells were analyzed for each time point. (**C**) As in (**B**) but for cells released after 4-OHT treatment and analyzed every 1.5 h during S-phase progression. Mean values and standard deviation are calculated from 3 independent experiments in which at least 100 cells were analyzed for each time point. (**D**) Model based on our results for the formation of RFB as a consequence of the binding of repressors to the array and a progressing fork and the cellular outcomes. See text for details.

be protected by 53BP1 during G_1 until the following S phase.[47,48] In favor of this hypothesis, we found that the accumulation of 53BP1 occurred predominantly in G_1. A possible hypothesis is that γH2AX and TOPBP1 could be recruited at the LacO array early in S phase as soon as the RFB is imposed, and 53BP1 would only be recruited later, when cells pass through G_1 after mitosis. Indeed, γH2AX, TOPBP1, and 53BP1 localize at the LacO and TetO arrays in a distinct manner (**Fig. 3A and B**). We show here that the induction of an RFB at a single site can be controlled during S-phase progression using synchronized cells and 4-OHT treatment with the mCherry-TetR-ER (**Fig. 4A**), and that the cellular response at this site can be monitored in time for distinct factors (**Fig. 4C**). Thus, our work opens the possibility for further studies to investigate at a single locus and single-cell level which pathways are involved to bypass an RFB, and which parameters are critical to maintain genome integrity.

To date, it is not entirely clear whether the DDR response is activated at stalled forks as a mechanism to bypass the RFB in response to fork collapse and DSB, or both. Thus, given the possibility to induce DSBs in the proximity of the repeat sequences with the ISceI restriction enzyme, the system we characterize here represents a powerful tool to compare DDR pathways activated upon DSB induction and RFB and the interrelation between both. In particular, it will enable the investigation of whether and to what extent the DDR pathway activated by a DSB plays a role in the resolution of RFBs imposed by a physical block. Importantly, genome integrity not only comprises genetic but also chromatin organization and epigenetic features, which are both maintained during replication and repair.[29,63-65] RFBs and mechanisms involved in their bypass thus are likely to challenge chromatin organization and epigenetic maintenance as reported by changes in the chromatin marks after putative G4s and RFB[29,66,67] and should be taken into account. The possibility to program an RFB at a single site with the system we describe here will certainly be most useful to explore which mechanisms control histone dynamics, their marks, and the inheritance of chromatin/epigenetic patterns at the RFB.

Materials and Methods

Cell culture and transfections

We cultured the cells in Dulbecco modified Eagle medium (DMEM; GIBCO), containing 10% fetal calf serum (FCS, Eurobio), 10 mg/ml penicillin and streptomycin (GIBCO). U2OS, 3T3-NIH2/4, and HeLa-38 cells with stable integration of the LacO-ISceI-TetO and LacO-ISceI array were a kind gift from Dr E Soutoglou (IGBMC). Integration of the construct was performed by the Soutoglou lab as previously described for 3T3 NIH2/4 cells.[10] U2OS-19 cells with stable integration of the TetO-ISceI array were a kind gift from professor Akira Yasui (Tohoku University).

We performed transient transfection of cells using Effectene Transfection Reagent (Qiagen), 0.2 μg/mL of plasmid DNA according to the manufacturer's instructions. We used peGFP-N1 and pCherry-N1 (Clontech) for control GFP and mCherry expression. eGFP–LacI plasmid was a kind gift from Evi Soutoglou[10], mCherry-TetR from Edith Heard (Institut Curie), and mCherry-TetR-ER from Akira Yasui (Tohoku University).[68]

Immunofluorescence (IF) and fluorescence in situ hybridization (FISH)

We grew cells on coverslips and performed IF as described.[69,70] We used primary antibodies described in **Table 3** and secondary antibodies Alexa488, Alexa594, or Alexa647-conjugated goat anti-rabbit or anti-mouse IgG (Invitrogen) for visualization. We used 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co) for DNA staining and mounted coverslips with ProLong mounting medium (Invitrogen). We quantified percentages of colocalization by counting at least 100 cells in each case.

For FISH, we grew cells on coverslips pre-coated with collagen and fibronectin and fixed them for 20 min in 4% PFA (Electron Microscopy Sciences). We generated the LacO probes by nick translation of plasmids containing the LacO and TetO sequences (kindly provided by M Dubarry and Dr A Taddei, Institut Curie, and from Dr Akira Yasui, Tohoku University) with the Nick Translation Mix (Roche) and Spectrum Red-dUTP or Spectrum Green-dUTP (Vysis). We performed FISH and immuno-FISH experiments as previously described.[71] For combination of FISH experiments with immunolabeling, we performed the immunofluorescence first, followed by PFA post-fixation and DNA-FISH.

Antibodies

Primary antibodies used during immunofluorescence experiments are listed below. Rabbit polyclonal anti-TOPBP1 (1:500; Ab2402; ABCAM); rabbit polyclonal anti-53BP1 (1:500; AB2402; NOVUS BIO); mouse monoclonal anti-phospho-H2A.X (1/500; CAT05-636; Millipore); rabbit polyclonal anti-phospho-H2A.X (1:250; 2577S; Cell Signaling); mouse monoclonal anti-GFP (1/500; 1184144600; Roche); mouse monoclonal anti-mCherry (1/250; AB125096; ABCAM); rabbit polyclonal anti-mCherry (1:250; 632496; Clontech); secondary antibodies used for detection were Alexa antibodies (In Vitrogen). The antibodies used during immunoprecipitation experiments are listed below. Mouse monoclonal anti-BrdU (1:40; 555627; BD); rabbit polyclonal anti-mouse (3.45 μl/sample; M-7023; Sigma).

Image acquisitions and deconvolution

We acquired images on a Delta Vision imaging station (Applied Precision) with an Olympus IX70 inverted microscope equipped with 100×, 1.35 NA oil-immersion objective, and a Photometrics HQ2 camera. We acquired z-stacks of images with a z-step of 0.2 μm, and subjected the 3-D stacks to constrain iterative deconvolution when indicated. Images were analyzed using the DeltaVision/Soft WoRx software package (Applied Precision) and ImageJ to evaluate colocalization of the different proteins.

For live cell imaging we collected 3-dimensional image stacks (0.5 μm in z) every 20 min for 24–48 h with DeltaVision and Soft WoRx software package (Applied Precision). Exposure time was 100–200 ms.

TetR-ER binding on synchronized cells

We synchronized U2OS-19 cells at the G_1–S boundary by double-thymidine block[72] with 2 mM thymidine for 16 h and a release for 8 h with 24 μM 2'-deoxycytidine. After the second

release we added 250 nM 4-OHT to promote nuclear translocation and DNA binding of mCherry-TetR-ER protein. We fixed and analyzed cells by IF at the indicated time points. We confirmed cell cycle arrest by FACS.

Metaphase spreads

We incubated cells for 6 h in 200 ng/mL nocodazol (SIGMA) at 37 °C, we harvested them and performed a hypotonic shock with 8 g/L sodium citrate for 10 min at 37 °C. We collected cells on coverslips for 5 min at 90 G with a cytospin 3 (SHANDON) and immediately performed IF 24 h after transfection with GFP-LacI or mCherry-TetR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Sarah Lambert, Zachary Gurard-Levin, and members of the "Chromatin Dynamics" team for critical reading of the manuscript, and Angela Taddei for helpful discussions. This work was supported by the European Commission ITN (FP7-PEOPLE-2007-215148 "Image DDR", FP7-PEOPLE-2008-238176 "Nucleosome 4D"), la Ligue Nationale contre le Cancer (Equipe labellisée Ligue 2010 to G.A.), PIC Programs, the European Commission Network of Excellence EpiGeneSys (HEALTH-F42010-257082), ERC Advanced Grant (2009-AdG_20090506 "Eccentric"), the European Commission large-scale integrating project (FP7_HEALTH-2010-259743 "MODHEP"), ANR "ECenS" (ANR-09-BLAN-0257-01), ANR "ChromaTin" (ANR-10-BLAN-1326-03), INCa "GepiG", ANR-11-LABX-0044_DEEP, and ANR-10-IDEX-0001-02 PSL*, ANR "CHAPINHIB" ANR-12-BSV5-0022-02, and Aviesan-ITMO cancer project "Epigenomics of breast cancer".

Supplemental Materials

Supplemental materials may be found here:
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