



DNA mediated chromatin pull-down for the study of chromatin replication

SUBJECT AREAS:

DNA REPLICATION

PROTEOMICS

CHROMATIN STRUCTURE

EPIGENETICS

Anna E. Kliszczak^{1,2}, Michael D. Rainey^{1,2}, Brendan Harhen¹, Francois M. Boisvert³
& Corrado Santocanale^{1,2}

¹National Centre of Biomedical Engineering and Science, National University of Ireland Galway, Galway, Ireland, ²Centre for Chromosome Biology, National University of Ireland Galway, Galway, Ireland, ³Department of Anatomy and Cell Biology, Université de Sherbrooke, Sherbrooke, Canada.

Received

20 July 2011

Accepted

2 September 2011

Published

19 September 2011

Correspondence and requests for materials should be addressed to

C.S. (corrado.santocanale@nuigalway.ie)

Chromatin replication involves duplicating DNA while maintaining epigenetic information. These processes are critical for genome stability and for preserving cell-type identity. Here we describe a simple experimental approach that allows chromatin to be captured and its content analysed after *in vivo* replication and labeling of DNA by cellular DNA polymerases. We show that this technique is highly specific and that proteins bound to the replicated DNA can be analyzed by both immunological techniques and large scale mass spectrometry. As proof of concept we have used this novel procedure to begin investigating the relationship between chromatin protein composition and the temporal programme of DNA replication in human cells. It is expected that this technique will become a widely used tool to address how chromatin proteins assemble onto newly replicated DNA after passage of a replication fork and how chromatin maturation is coupled to DNA synthesis.

It has long been recognized that the fidelity of DNA replication is crucial for the maintenance of genome stability¹. More recently it has also been discovered that the proteins coating the DNA, such as histones and transcription factors also carry important information that specifies cell function and identity². As the DNA is replicated, histones and DNA binding proteins are displaced from the DNA in front of a replication fork and reoccupy their binding sites after its passage. Since twice as many binding sites exist after DNA duplication, previously resident DNA binding proteins are supplemented from a pool of soluble proteins. Therefore the duplication of DNA imposes a source of stress for the maintenance of the epigenetic information and for the regulation of gene expression³. How cells reassemble chromatin and duplicate epigenetic marks is poorly understood due to the lack of techniques that allow recovery of proteins that are associated with newly synthesized DNA.

Chromatin immunoprecipitation (ChIP), a technique developed in the early 90s, has revolutionized our experimental approaches to studying transcription, replication and DNA repair⁴. It allows the association occurring *in vivo* between defined proteins at specified loci to be investigated. For example it has been pivotal in probing promoter occupancy by transcription and chromatin remodeling factors^{5,6}, in assessing the recruitment of DNA repair proteins at double strand breaks⁷ and in demonstrating the recruitment of replication proteins both at replication origins and at replication forks⁸. Briefly, proteins are cross-linked to DNA, cells are lysed, chromatin sheared into small fragments and protein-DNA complexes are immunoprecipitated using antibodies against specific proteins. DNA fragments that co-immunoprecipitate with target proteins are purified after reversal of the cross-link. As the final product of ChIP procedure is a DNA molecule, techniques such as semi-quantitative or quantitative PCR are used to assess the enrichment of specific sequences over the input DNA. Alternatively, more global approaches such as microarray hybridization (ChIP on Chip) or next generation sequencing (ChIP-Seq) are used to reveal the distribution of a given DNA interacting protein across the genome.

Halogenated nucleosides, such as 5-bromo-2'-deoxyuridine (BrdU), have been exploited for the detection of cellular DNA synthesis in a variety of organisms in both cell-based assays or *in vivo* models⁹. These molecules are cell permeable and upon phosphorylation are incorporated into the nascent DNA by the cellular DNA polymerases. The labeled DNA is then detected by using specific antibodies raised against halogenated nucleosides¹⁰. Because of its simplicity the use of BrdU has essentially replaced [³H] thymidine in proliferation assays, and it has been applied to multiple technological platforms including flow cytometry, immunofluorescence microscopy (IF) and immunohistochemistry (IHC). A key limiting factor for BrdU-based DNA replication assays is the need to use harsh conditions such as extreme pH or temperatures to denature the double stranded DNA to allow epitope exposure and antibody recognition. These conditions cause protein degradation, thus preventing efficient



immunostaining and in particular impeding the efficient recovery of BrdU labeled chromatin using immunoaffinity procedures. In IF and flow cytometry applications, these problems have been resolved by using 5-ethynyl-2'-deoxyuridine (EdU) to label DNA^{11,12}. EdU, like BrdU, is incorporated into the nascent DNA, but its detection is normally achieved by covalent linkage of a fluorochrome through a very specific azide-alkyne Huisgen cycloaddition (1, 3-dipolar cycloaddition) reaction also known as Click chemistry. As this reaction occurs under mild conditions and the detection step does not require DNA denaturation, the proteins bound to DNA are not obviously adversely affected¹³.

A classical feature of DNA replication in post-embryonic eukaryotic cells is the asynchronous firing of DNA replication origins that occurs according to a well defined pattern¹⁴. Thus, the timing of replication of any given DNA tract is dependent on the timing of the firing of the replication origin giving rise to the replication fork that duplicates that track, the distance that separates it from the origin and the speed at which the replication fork moves. All these parameters are highly variable throughout S-phase and can be subjected to regulation (reviewed in¹⁵).

Correlations between the replication timing, genomic location and chromatin features such as histone modifications and transcriptional activity have been identified (reviewed in¹⁶). DNA positioning into

nuclear sub-domains also appears to have an important role in determining the timing of DNA replication and indeed replication factories have different spatial distribution during the progression of S-phase¹⁷⁻¹⁹.

In this work we have devised a novel strategy to label and capture newly replicated chromatin and to analyze its protein content. Using this technique in conjunction with cell cycle synchronized cells, we demonstrate that specific proteins can be enriched on DNA replicating at different times during S-phase, thus providing a novel approach to elucidate the molecular correlation between chromatin features and the spatial/temporal programme of DNA replication.

Results

Development of a DNA mediated chromatin pull-down technique. In order to capture newly assembled chromatin, we exploited the versatile azide-alkyne Huisgen cycloaddition reaction, normally used to link fluorochromes to EdU for the detection of DNA synthesis, to couple biotin to EdU containing chromatin and allow it to be selectively captured and analyzed. Using this novel strategy, which we have termed DNA mediated chromatin pull-down (Dm-ChP), the enrichment of newly replicated chromatin is achieved by simply combining EdU labeling of nascent DNA with biotin mediated capturing (Fig. 1) using the well established experimental conditions for

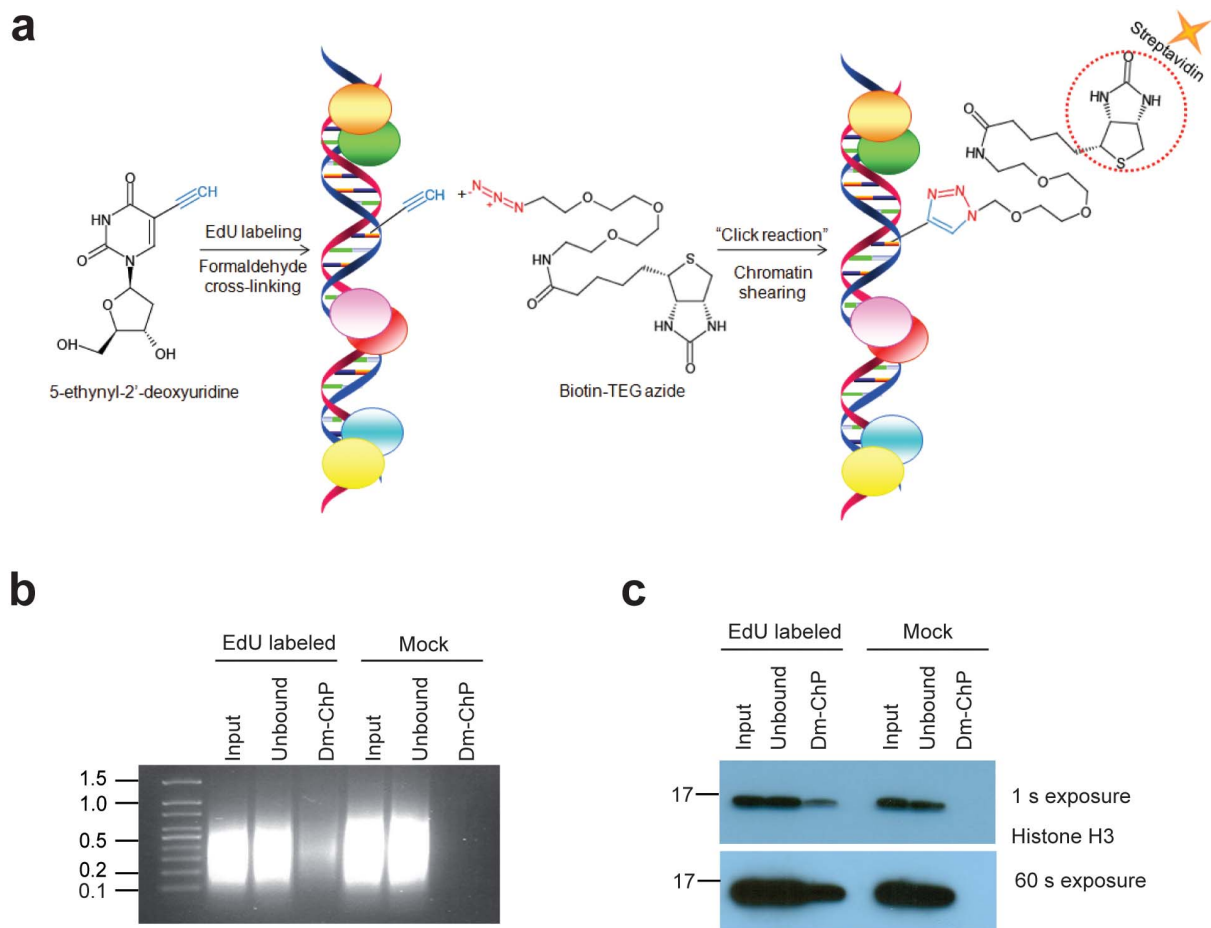


Figure 1 | DNA mediated chromatin pull-down technology. (A) Strategy for tagging and capturing newly synthesized chromatin. 5-ethynyl-2'-deoxyuridine (EdU) is used to label newly replicating DNA. After protein-DNA cross-linking, biotin-TEG azide is selectively linked to the reactive alkyne group of EdU containing DNA. After DNA shearing small fragments of chromatin are captured on streptavidin beads. (B) Agarose gel electrophoresis of DNA prepared from either EdU labeled or mock treated cells from input material (Input), material that did not bind to streptavidin beads (Unbound) or DNA mediated chromatin pull-down (Dm-ChP). (C) Detection of histone H3 by western blotting in protein fractions prepared from either EdU labeled or mock treated cells from input (Input), material that did not bind to streptavidin beads (Unbound) or eluted from streptavidin beads (Dm-ChP); immunoreactive bands were visualised with HRP-labeled secondary antibodies and chemoluminescence reaction. Two exposures of the same film are shown. Short exposure is 1 second, long exposure 1 minute.



protein-DNA cross-linking and cellular fractionation⁴. After EdU labeling and formaldehyde protein-DNA cross-linking, cells are permeabilized and upon Click reaction the EdU incorporated into the DNA is coupled to a biotin-TEG azide molecule. Cells are lysed in isotonic buffer, nuclei are resuspended in RIPA buffer and DNA is sheared by sonication into fragments ranging from 150 to 600 nucleotides with an average size of 300 nucleotides. Finally, labeled DNA together with bound proteins is recovered using streptavidin beads (Fig. 1a). Chromatin fragments are then eluted from beads by incubation in *Laemmli* buffer at a high temperature, a step that also reverses the formaldehyde cross-link⁴, thus obtaining DNA and protein containing fractions that can be subsequently analyzed.

In the first set of experiments aimed at assessing the feasibility and specificity of Dm-ChP, logarithmically growing HeLa cells were either mock treated or incubated with EdU for 24 hours prior to collection; both samples were then identically processed for Dm-ChP. DNA purified from input, unbound and streptavidin captured material was analyzed on a 1.5 % agarose gel. Figure 1b shows that fragments of DNA are recovered in the streptavidin pull-down only when the cells were incubated with EdU. The presence of biotinylated residues on this DNA was further confirmed by dot blotting (Supplementary Fig. 1). In order to assess if chromatin proteins could be detected after these treatments, we analyzed the eluted material by SDS-PAGE and western blotting using anti-histone H3 antibody as a probe. Histone H3 was detected in the input and unbound fractions from both EdU labeled and mock treated cells but recovered only in the pull-down of the former; histone H3 was not detected in the negative control even after a prolonged exposure (Fig. 1c). Together these data indicate that capture of DNA and histone H3 is dependent on EdU labeling of the DNA and it is not due to non-specific binding to beads or aggregation/precipitation during the pull-down. Using this general strategy in our experiments we were able to specifically detect histone H3 in the pull-down material using EdU pulses as short as 5 minutes.

Specificity of Dm-ChP. In order to validate this methodology we performed a series of control experiments where either EdU, biotin-TEG azide or copper sulfate as a catalyst of the Click reaction were not included. In all cases the omission of one of these critical reagents prevented the capture of chromatin as assessed by a lack of histone H3 detection (Fig. 2a).

It is important to point out that by using a non-synchronized cell population and/or short labeling times, only a fraction of the cells are

in S-phase and in these cells only a proportion of the cellular DNA is labeled with EdU (Supplementary Fig. 2 as an example). Therefore a chromatin extract prepared from these cells contains both labeled and non-labeled DNA. To further demonstrate that Dm-ChP specifically captures EdU labeled chromatin from a mixture, we combined an equal amount of extract prepared from HeLa cells that, together with normal histones, also expresses a functional GFP-histone H3 fusion (Supplementary Fig. 3), and extracts from unmodified HeLa cells, either EdU labeled or not. This strategy allows the origin of the material recovered in the pull-down to be distinguished. As expected with Dm-ChP, we recovered GFP-histone H3 when HeLa GFP-histone H3 cells were labeled with EdU, and unmodified histone H3 and histone H4 in every EdU labeled sample. Importantly, we did not detect GFP-histone H3 when extracts from EdU labeled HeLa cells were mixed with extracts of unlabeled chromatin that was marked with GFP-histone H3, indicating that intermolecular aggregation between biotinylated and untagged chromatin fragments does not occur (Fig. 2b). Together these results support the notion that chromatin can be specifically pulled-down by EdU labeling and biotin tagging of nascent DNA.

Characterization of proteins associated with EdU labeled DNA.

When EdU labeling was performed for one hour and proteins were analyzed by silver staining, we observed a great number of bands that co-purified with newly synthesized DNA as well as the predominant streptavidin and histone derived bands (Fig. 3a). Using a range of available antibodies we were able to detect the presence of non-histone proteins such as SMC1 and SMC3, involved in sister chromatid cohesion, Mcm7 and PCNA, key components of the DNA replication machinery and the nuclear chaperone nucleophosmin, specifically in the pull-downs from EdU labeled cells (Fig. 3b).

To assess if this material was also amenable to proteomic analysis, after elution from the streptavidin beads, we prepared tryptic peptides by adapting the Filter Aided Sample Preparation (FASP) method described recently²⁰. Using an Agilent Q-TOF in MS/MS mode, 277 proteins were recognized. As expected, proteins known to interact with nucleic acids, either because they are involved in chromosome function or because they are highly positively charged and show adventitious binding to chromosomes such as ribosomal proteins as well as chromosomal components, were found (Fig. 3c and Supplementary Tables 1–3).

Dm-ChP studies of DNA replicating at different times during S-phase.

Having developed the DNA mediated chromatin pull-down,

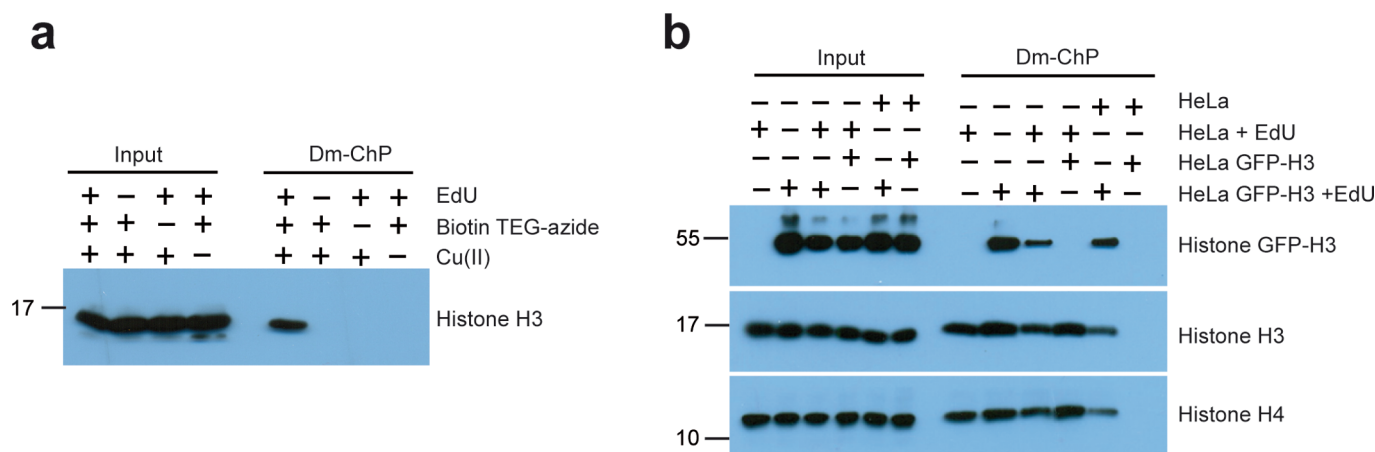


Figure 2 | Specificity of Dm-ChP procedure. (A) Dm-ChP was performed in the presence (+) or absence (-) of the indicated components. Input and Dm-ChP material was analyzed by western blotting using anti-histone H3 antibodies. (B) Dm-ChP was performed with (+ EdU) or without EdU labeling in cells either expressing GFP-histone H3 (HeLa GFP-H3) fusion protein or parental HeLa cells (HeLa). 600 μ g of extract from the indicated samples (+) was either directly used for Dm-ChP or mixed together before streptavidin pull-down. Input (25 μ g) and Dm-ChP material was analysed by western blotting using anti-GFP antibody (top panel), anti-histone H3 (middle panel) or anti-histone H4 (bottom panel) antibodies.

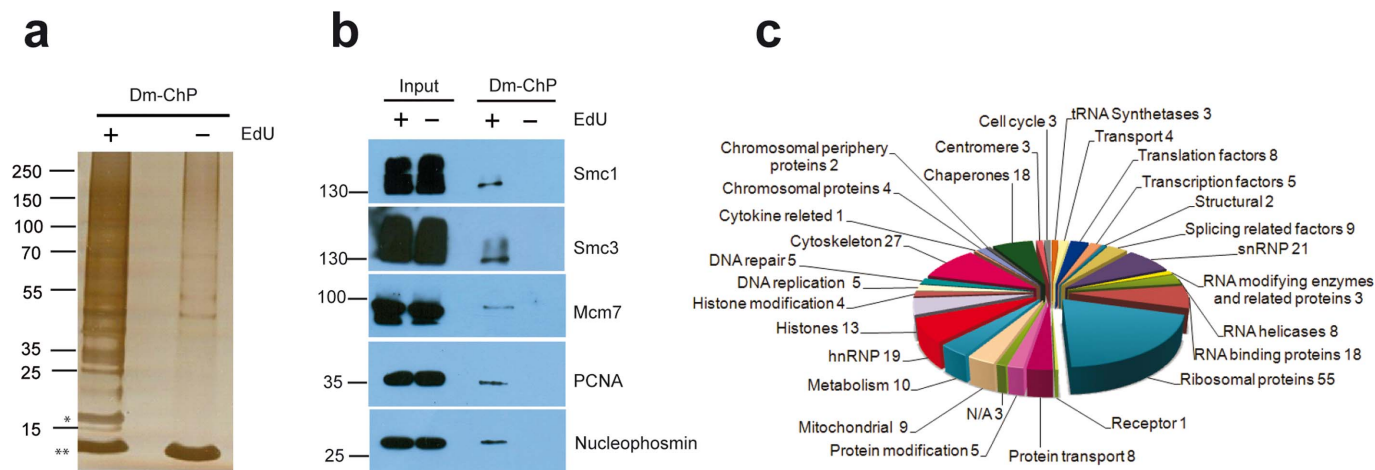


Figure 3 | Analysis of protein associated with EdU labeled DNA. (A) Dm-ChP material performed from EdU labeled (+) or mock treated (-) cells was separated on SDS-acrylamide gel and stained with silver. Histones (*) and streptavidin (**) bands are indicated. (B) Detection of non-histone proteins after Dm-ChP. Dm-ChP material performed from EdU labeled (+) or mock treated (-) cells was separated on SDS-PAGE and probed with antibodies recognising the indicated proteins. (C) Functional classification of the proteins identified by mass spectrometry in Dm-ChP material. 277 proteins that were identified with a $\log(e) \leq -3$, where the $\log(e)$ corresponds to the base-10 log of the expectation that any particular protein assignment was made at random (E-value) from both XTandem! and XHunter! searches. Proteins are listed in Supplementary Table 3 and were classified manually into 29 different classes using information from UniProt (Universal Protein Resource) protein Knowledgebase (<http://www.uniprot.org/>). The number of proteins in each class is indicated.

we started to address the qualitative and quantitative differences in the protein component associated with DNA that replicates at different times during S-phase.

Isotopically labeled HeLa cells were arrested at the G_1/S boundary by double thymidine block and released into a synchronous S-phase. At 1 hour 30 minutes and 5 hours 30 minutes after release from the double thymidine block, EdU was added to the medium for 30 minutes; cells were collected at 2 and 6 hours post-release. Flow cytometry analysis indicated that at 2 hours cells had just reentered S-phase and initiated DNA synthesis, while cells taken after 6 hours from the release were either in mid or late S-phase (Supplementary Fig. 4). After Dm-ChP and quantitative MS analysis by SILAC (Supplementary Table 4) we found that the amount of several factors involved in DNA synthesis such as Replication Factor C, Flap Endonuclease 1 and Topoisomerases 1 and 2, over the amount of all captured proteins was greater when the Dm-ChP was performed at 2 hours compared to 6 hours after the release from the double thymidine block. Intriguingly, we observed that most of the ribosomal proteins were instead enriched in the 6 hour time point (Supplementary Table 4).

To extend our analysis to regions of the genome replicating very late in S-phase, a similar experimental design was used. G_1/S arrested cells were released into S-phase for 2 hours (early S), 6 hours (mid/late S) and 8 hours (very late S/ G_2). In each case, EdU labeling was performed for 15 minutes prior to harvesting the cells. Samples were then analyzed by Dm-ChP followed by western blotting, for DNA content by flow cytometry and by fluorescence microscopy to determine the pattern of replication foci. We verified that $> 97\%$ of the cells taken at the 2 hour time point were labeled with EdU at the beginning of S-phase and showed a classical dispersed foci pattern¹⁷ (Fig. 4b, 2h time point). Cells taken at 6 hours were mostly in mid/late S-phase with perinuclear and nucleolar staining (Fig. 4b, 6h time point) and cells taken at 8 hours post-release were only 40% EdU positive, typically with a very late-S replication foci staining pattern (Fig. 4a, Fig. 4b 8h time point and Fig 4c). By analyzing Dm-ChP material by western blotting we further confirmed the specificity of the Dm-ChP for several proteins across multiple samples (Fig. 4d, compare 2, 6, 8 hour time points +/- EdU). Although western blotting is only semiquantitative, we observed that the levels of a number of replication proteins including Mcm7, Fen1 and PCNA, were

decreased in the 8 hour sample while the levels of histone H4 and the histone chaperone CAF-1 remained fairly constant in all the samples from EdU labeled cells. Intriguingly, the binding of NONO, a DNA and RNA binding protein involved in several nuclear processes including pre-mRNA splicing and non-homologous end joining repair of double stranded DNA breaks^{21,22}, showed a marked preference for DNA replicating at 6 hours after the release from thymidine block (Fig. 4d).

Discussion

In this work we show that Dm-ChP is a technique that allows the study of chromatin proteins that are associated with DNA that has been labeled *in vivo* by cellular polymerases. Once cells are collected, Dm-ChP involves multiple steps including formaldehyde cross-linking, biotin conjugation to DNA and formaldehyde cross-link reversal of protein-DNA adducts. After all these steps proteins appeared to be largely unaffected and were further analyzed by immunoblotting and by mass spectrometry. In a study aimed at assessing the chemical modification of formaldehyde on peptides, formaldehyde was shown to react with the amino group of the N-terminal amino acid residue and the side-chains of arginine, cysteine, histidine, and lysine residues. Depending on the peptide sequence, methylol groups, Schiff-bases, and methylene bridges were formed²³. However with the short incubation times and low concentrations of formaldehyde used in our study and more generally in classical chromatin immunoprecipitation protocols, the major reactive sites are in fact largely limited to lysine, tryptophan side chains and the amino termini of peptides²⁴. The protein-protein and protein-DNA cross-links are reversible by heating, but if there are any adducts left on the epsilon amino groups of lysine, it could generate a skipped cleavage by trypsin. This would not be a problem in studies aimed only at determining the identity of proteins cross-linked to nascent DNA as most of the softwares used for this purpose allow for one or more missed cleavage. However, it might interfere with detection of post-translational modifications unless potential adducts on lysine residues are searched for in the analysis of the MS data. This will need to be further investigated in the future. Lysine residues contain charge-bearing polar side chains that are known to contribute significantly to the affinity of antibodies, and residual adducts may mask the epitopes of proteins during immunodetection. Thus the complete reversal of the formaldehyde cross-linking step appears to be an important parameter

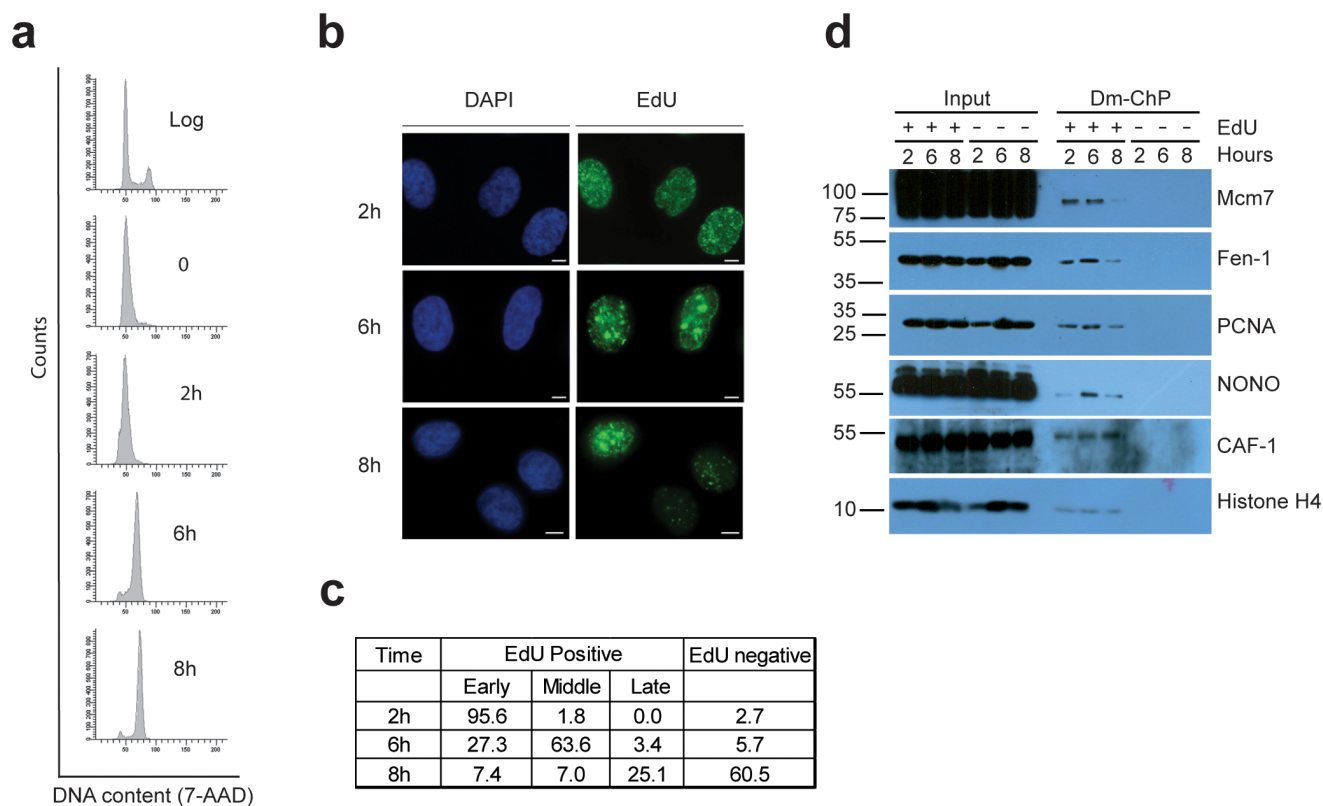


Figure 4 | Analysis of chromatin replicating at different times of the S-phase. HeLa cells arrested at the G_1/S transition by double thymidine cell cycle block were released and allowed to progress into a synchronous S-phase. (A) DNA content analysis of cell populations logarithmically growing (Log) and at different times after release. (B) Representative images of DNA replication foci identified by EdU incorporation in cells taken at the indicated times from the release into S-phase. Scale bars correspond to 10 μm . (C) Quantification of cells showing EdU incorporation pattern consistent with early, mid and late S-phase in the samples taken at the indicated times. Approximately 300 cells were scored or each time point. (D) Dm-ChP analysis of cells taken at the indicated times following the release and either labeled with EdU for 15 minutes before collection (+) or not labeled (-). Proteins associated with newly synthesized DNA were analysed by western blotting with the indicated antibodies.

to keep under consideration. From a practical perspective, in our experiments the incubation for 5 minutes at 95°C either in 1 x *Laemmli* sample buffer or in 1% SDS appears to be sufficient to avoid these potential problems.

Under the experimental conditions of EdU labeling and biotin DNA tagging here described, we cannot exclude that unreacted EdU is left in the DNA making some of the EdU labeled DNA unavailable for capture. However, we have observed that after Click reaction not all of the biotinylated chromatin binds to the streptavidin beads. Titration experiments suggest that one of the limiting factors for the quantitative capture of biotinylated chromatin is the amount of beads used in the pull-down. It is possible that the amount of biotin residues linked to EdU labeled DNA exceeds the available streptavidin moieties. Alternatively, because of the large molecular weight of the captured chromatin fragments, steric hindrance may reduce the binding capacity of the resin. A careful quantification of the amount of biotin that is linked to DNA would be essential to address this question in future work.

While completing this study, Sirbu and co-workers reported the use of EdU labeling coupled with biotin capturing for the study of protein dynamics at active and stalled replication forks and they named this method iPond (isolation of proteins on nascent DNA)²⁵. However once EdU is incorporated, it is maintained and can be detected in the DNA for a long period of time, even after a cell division. Therefore biotin capturing following Click chemistry reaction also allows identification of proteins that are bound to mature DNA and not just immediately after DNA synthesis. We therefore suggest that DNA mediated chromatin pull-down (Dm-ChP) is a more versatile name for this technique.

Combining western blotting and mass spectrometry approaches, we have identified possibly the most abundant proteins that are recovered by Dm-ChP. Among these are proteins involved in chromatin structure, DNA replication and repair, as well as RNA binding proteins. Similar to other proteomics studies where the composition of chromosomes was examined²⁶, ribosomal proteins were also retrieved in the Dm-ChP. It has been suggested that these ribosomal proteins can bind adventitiously to DNA possibly during extract preparation because they are highly positively charged and have been defined as chromosome hijacker proteins²⁶. Since our procedure exploits *in vivo* protein-DNA cross-linking followed by stringent washes, coupled with the observation that ribosomal proteins are enriched in samples taken at the time when replication of rDNA occurs and considering that ribosomal DNA transcription and ribosome assembly are closely coupled²⁷, we suggest that ribosomal proteins may interact with the chromatin *in vivo* in a localized manner.

The careful quantification of the levels of any given protein in different samples is a key technical hurdle for the use of this technique in future studies. In our study single point immunoblotting detection did not fully recapitulate the small quantitative differences in the 2 hours versus the 6 hours time points as observed by SILAC. This may be related to intrinsic experimental variability, slightly different experimental design (15 minutes versus 30 minutes EdU labeling time), but is more likely due to the low dynamic range of chemoluminescence western blotting. A reasonable solution to this issue would be the use of infrared fluorescence imaging systems capable of quantitative immunoblotting as performed by Sirbu and colleagues²⁵.

Using Dm-ChP we provide preliminary evidence that DNA replicating at different times during S-phase can be found together with



different relative amount of proteins involved in DNA synthesis. The decrease in the level of proteins known to be at the replication fork in the samples taken at the 8 hour time point (late S-phase) is intriguing. At present we speculate that this observation is possibly consistent with the idea that in mammalian cells in late S-phase, replication forks move at a faster rate^{15,28}, thus the ratio between EdU labeled DNA in close proximity to the replication machinery and EdU labeled DNA away from it and not cross-linkable to replication factors is lower. As a consequence the chances of capturing replication proteins by Dm-ChP are worse in late rather than earlier in S-phase. Using Dm-ChP in combination with larger scale experiments and the use of multiple cellular models will determine if specific proteins and/or protein signatures marking early and late replicating DNA can be identified.

In summary, because of the specificity and flexibility of the DNA mediated chromatin pull-down procedure, together with the possibility of manipulating the cellular systems, labeling times and conditions of extract preparation, it is very likely that Dm-ChP will become a leading method, not only for the study of the relationships between chromatin proteins and the temporal regulation of DNA synthesis, but more generally in studies on chromatin remodeling and maturation and for investigating how these processes are linked to the duplication of its basic constituent the DNA.

Methods

Reagents. 5-ethynyl-2'-deoxyuridine (EdU) (Cat. n. PY 7562), 6-Carboxyfluorescein-TEG azide (Cat. n. FF 6110) and biotin-TEG azide (Cat. n. BT 1085) were from Berry & Associates, Inc. (Dexter, USA). High capacity streptavidin Agarose (Cat. n. 20357) was purchased from Pierce, Thermo Scientific (Runcorn, UK). All other chemicals used in this work were from Sigma-Aldrich (Arklow, Ireland) unless otherwise stated.

Antibodies. Antibodies anti-histone H3 (ab-1791), anti-histone H4 (ab-10158) and anti-nucleophosmin (ab-24412) were from Abcam (Cambridge, UK), anti-biotin HRP conjugated antibody (Clone BN-34) was from Sigma (Cat. n. A-0185). Mouse monoclonal antibody anti-GFP was from Roche (Mannheim, Germany) (Cat. n. 11814460001) anti-PCNA (sc-56) and anti-SMC3 (sc-8198) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, USA); anti-Mcm7 (clone 47DC141) was from Thermo Scientific (Runcorn, UK). Anti-Smc1 (Cat. n. A300-055A) antibody was from Bethyl (Montgomery, USA).

Cell culture and cell cycle synchronization. HeLa CCL-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat inactivated 10% fetal bovine serum (FBS), 1 × non-essential amino acids, 2 mM L-glutamine and 1% penicillin-streptomycin all from Sigma-Aldrich (Arklow, Ireland). Cells were grown in 5% CO₂ at 37°C. For synchronization studies cells were exposed to 2 mM thymidine for 18 hours, thymidine was removed by rinsing cells with PBS and the culture was released into fresh medium for 9 hours. Cells were blocked for a second time by adding 2 mM thymidine for a further 17 hours. Synchronized cells were washed with PBS and released into fresh medium.

For stable isotope labeling with amino acids in cell culture (SILAC), cells were grown for six cell divisions in DMEM SILAC media (Dundee Cell Products, Dundee, UK). Light (unlabeled arginine and lysine - R0K0), medium (labeled 13C labeled arginine and deuterium labeled lysine - R6K4) and heavy (labeled 13C and 15N labeled arginine, and 13C and 15N labeled lysine - R10K8) supplemented with filtered FCS (Dundee Cell Products, Dundee, UK).

Analysis of DNA replication by fluorescence microscopy. For detection of DNA synthesis by fluorescence microscopy, cells growing on coverslips were incubated with 10 μM EdU for 30 minutes and fixed with 1% (v/v) formaldehyde for 10 minutes at room temperature. After PBS wash cells were permeabilized with 0.3% (v/v) Triton X-100 in cold PBS for 15 minutes at 4°C and subsequently washed three times with 1% (w/v) BSA in PBS. Click reaction was performed in PBS, 10 mM (+)-sodium-L-ascorbate, 0.1 mM 6-Carboxyfluorescein-TEG azide and 2 mM copper (II) sulfate. Coverslips were then washed three times in 0.5% (v/v) Tween 20 in PBS, nuclei stained with 1 μg/ml DAPI and after three further washes, mounted onto slides and dried at 37°C. Images were taken using an Olympus BX-51 microscope with 60 × (NA 1.4) or 100 × (NA 1.35) and driven by OpenLab software (version 5, Improvision, Emeryville, USA).

DNA mediated chromatin pull-down procedure. Typically 2 × 10⁶ cells were incubated for 1 or more hours with 10 μM EdU (for shorter labeling times 2 × 10⁷ were used) and then cross-linked for 10 minutes at 4°C with 1% (v/v) formaldehyde. To quench unreacted formaldehyde 0.125 M glycine was added and cells were incubated for additional 10 minutes at 4°C. Cells were harvested and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 minutes on ice and washed with PBS. To perform the Click reaction, the following components were added sequentially,

10 mM (+)-sodium-L-ascorbate, 0.1 mM biotin-TEG azide and 2 mM copper (II) sulfate and cells were incubated in the dark for 30 minutes at room temperature followed by addition 10 volumes of 1% (w/v) BSA, 0.5% (v/v) Tween 20 in PBS (PBST) and incubated for a further 10 minutes. After three washes in PBS, soluble proteins were extracted in 500 μl of CL lysis buffer (50 mM HEPES pH 7.8, 150 mM NaCl, 0.5% (v/v) NP-40, 0.25% (v/v) Triton X-100, 10% (v/v) glycerol) containing protease inhibitors (Protease inhibitor cocktail III, Fisher Scientific BPE 9709-1) by incubation at 4°C with end-over-end mixing for 10 minutes followed by slow speed centrifugation (1300 rpm /150 × g). The residual pellet was then washed for 10 minutes at 4°C by end-over-end mixing in 500 μl of wash buffer (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.5 mM DTT). The pellet was then resuspended in 500 μl of RIPA buffer (10 mM Tris-HCl pH 8.0, 140 mM NaCl, 1% (v/v) Triton X-100, 0.1% (v/v) Na-Deoxycholate, 0.1% (w/v) SDS) containing protease inhibitor cocktail. To shear the chromatin, lysates were sonicated on ice at 40% amplitude for six rounds of 10 seconds with 2 minutes interval between rounds using a Digital Sonifier (Branson, UK). The extract was clarified by centrifugation at 16,100 × g for 10 minutes at 4°C. Protein content was quantified using the Pierce BCA kit (Thermo Scientific, Runcorn, UK) and 25 μg of the supernatant was saved as an input for western blotting analysis. Typically 1 mg of this extract was used for pull-down with 50 μl of wet streptavidin beads. Before use, beads were washed twice with 500 μl wash buffer, equilibrated in RIPA buffer and blocked overnight at 4°C with 0.5 mg/ml BSA and 0.4 mg/ml pre-sheared salmon sperm DNA to minimize non-specific binding. On the next day, beads were washed three times and transferred to a new tube. Chromatin extracts were incubated for 2 to 16 hours at 4°C with pre-blocked streptavidin beads. After binding, unbound material was collected and beads were washed three times with 500 μl of wash buffer. To reverse protein-DNA cross-linking and elute proteins from streptavidin beads, samples were incubated for 5 minutes at 95°C either in 1 × Laemmli sample buffer before SDS-PAGE or in 1% (v/v) SDS for proteomics analysis.

DNA purification. DNA was purified from chromatin by treatment with 0.1 mg/ml RNase A at 37°C for 30 minutes followed by 2 hour incubation at 45°C with 0.1 mg/ml Proteinase K and phenol: chloroform: isoamyl alcohol (25:24:1) extraction. DNA was recovered by ethanol precipitation.

1. Sclafani, R. A. & Holzen, T. M. Cell cycle regulation of DNA replication. *Annu Rev Genet* **41**, 237–280 (2007).
2. Kouzarides, T. Chromatin modifications and their function. *Cell* **128**, 693–705 (2007).
3. Jasencakova, Z. & Groth, A. Restoring chromatin after replication: how new and old histone marks come together. *Semin Cell Dev Biol* **21**, 231–237 (2010).
4. Orlando, V., Strutt, H. & Paro, R. Analysis of Chromatin Structure by in Vivo Formaldehyde Cross-Linking. *Methods* **11**, 205–214 (1997).
5. Ren, B. & Dynlacht, B. D. Use of chromatin immunoprecipitation assays in genome-wide location analysis of mammalian transcription factors. *Methods Enzymol.* **376**, 304–315 (2004).
6. Ren, B. *et al.* E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. *Genes Dev.* **16**, 245–256 (2002).
7. Berkovich, E., Monnat, R. J., Jr. & Kastan, M. B. Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. *Nat. Cell Biol.* **9**, 683–690 (2007).
8. Katou, Y. *et al.* S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature* **424**, 1078–1083 (2003).
9. Morstyn, G. *et al.* Bromodeoxyuridine in tumors and chromosomes detected with a monoclonal antibody. *J. Clin. Invest.* **72**, 1844–1850 (1983).
10. Gratzner, H. G. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* **218**, 474–475 (1982).
11. Salic, A. & Mitchison, T. J. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci. U S A* **105**, 2415–2420 (2008).
12. Cappella, P., Gasparri, F., Pulici, M. & Moll, J. A novel method based on click chemistry, which overcomes limitations of cell cycle analysis by classical determination of BrdU incorporation, allowing multiplex antibody staining. *Cytometry A* **73**, 626–636 (2008).
13. Buck, S. B. *et al.* Detection of S-phase cell cycle progression using 5-ethynyl-2'-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2'-deoxyuridine antibodies. *Biotechniques* **44**, 927–929 (2008).
14. Fangman, W. L. & Brewer, B. J. A question of time: replication origins of eukaryotic chromosomes. *Cell* **71**, 363–366 (1992).
15. Herrick, J. & Bensimon, A. Global regulation of genome duplication in eukaryotes: an overview from the epifluorescence microscope. *Chromosoma* **117**, 243–260 (2008).
16. Aladjem, M. I. Replication in context: dynamic regulation of DNA replication patterns in metazoans. *Nat. Rev. Genet.* **8**, 588–600 (2007).
17. Dimitrova, D. S. & Gilbert, D. M. The spatial position and replication timing of chromosomal domains are both established in early G1 phase. *Mol. Cell.* **4**, 983–993 (1999).
18. Dimitrova, D. S. & Berezney, R. The spatio-temporal organization of DNA replication sites is identical in primary, immortalized and transformed mammalian cells. *J. Cell. Sci.* **115**, 4037–4051 (2002).
19. Leonhardt, H. *et al.* Dynamics of DNA replication factories in living cells. *J. Cell Biol.* **149**, 271–280 (2000).



20. Wisniewski, J. R., Zougman, A., Nagaraj, N. & Mann, M. Universal sample preparation method for proteome analysis. *Nat. Meth.* **6**, 359–362 (2009).
21. Zhang, Z. & Carmichael, G. G. The fate of dsRNA in the nucleus: a p54(nrb)-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs. *Cell* **106**, 465–475 (2001).
22. Li, S. *et al.* Involvement of p54(nrb), a PSF partner protein, in DNA double-strand break repair and radioresistance. *Nucleic Acids Res.* **37**, 6746–6753 (2009).
23. Metz, B. *et al.* Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J. Biol. Chem.* **279**, 6235–6243 (2004).
24. Sutherland, B. W., Toews, J. & Kast, J. Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. *J. Mass Spectrom.* **43**, 699–715 (2008).
25. Sirbu, B. M. *et al.* Analysis of protein dynamics at active, stalled, and collapsed replication forks. *Genes Dev.* **25**, 1320–1327 (2011).
26. Ohta, S. *et al.* The protein composition of mitotic chromosomes determined using multiclassifier combinatorial proteomics. *Cell* **142**, 810–821 (2010).
27. Boisvert, F. M., van Koningsbruggen, S., Navascues, J. & Lamond, A. I. The multifunctional nucleolus. *Nat. Rev. Mol. Cell. Biol.* **8**, 574–585 (2007).
28. Frum, R. A., Khondker, Z. S. & Kaufman, D. G. Temporal differences in DNA replication during the S phase using single fiber analysis of normal human fibroblasts and glioblastoma T98G cells. *Cell Cycle* **8**, 3133–3148 (2009).

Acknowledgements

We thank Paolo Cappella (Nerviano Medical Sciences) for discussion and sharing of reagents, David Pinto and Andrew Flaus for the GFP-histone H3 stable cell line. We thank Maciej Kliszczak and Ciaran Morrison for assistance with fluorescence microscopy, Alessandro Natoni and Mark Coyne for assistance with flow cytometry analysis. We thank

Hienz Peter Nasheaur, Bob Lahue, Steve Rea, Noel Lowndes and the members of the Centre for Chromosome Biology at NUIG for sharing antibodies and discussions. We thank Paul Ajuh (Dundee Cell Products Ltd, UK) for advice on SILAC, Gemma O'Brien and the researchers in the lab for continuous support and Sandra Healy for critical reading of this manuscript.

This work was supported by Science Foundation Ireland grants 08/IN.1/B2064 and 08/RFP/NSCI235 to CS and by a Thomas Crawford Hayes Research & Travel Fund Scheme award to AEK.

Author contributions

A.E.K. performed and designed experiments, B.H. performed Q-TOF Mass spectrometry analysis, M.D.R. implemented FASP method for mass spectrometry sample preparation, F.M.B. performed data analysis, C.S. designed and supervised experiments, wrote the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

License: This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>

How to cite this article: Kliszczak, A.E., Rainey, M.D., Harhen, B., Boisvert, F.M. & Santocanale, C. DNA mediated chromatin pull-down for the study of chromatin replication. *Sci. Rep.* **1**, 95; DOI:10.1038/srep00095 (2011).