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Genome-wide and sister chromatid-resolved profiling of protein occupancy in replicated chromatin with ChOR-seq and SCAR-seq

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Elucidating the mechanisms underlying chromatin maintenance upon genome replication is critical for the understanding of how gene expression programs and cell identity are preserved across cell divisions. Here, we describe two recently developed techniques, chromatin occupancy after replication (ChOR)-seq and sister chromatids after replication (SCAR)-seq, that profile chromatin occupancy on newly replicated DNA in mammalian cells in 5 d of bench work. Both techniques share a common strategy that includes pulse labeling of newly synthesized DNA and chromatin immunoprecipitation (ChIP), followed by purification and high-throughput sequencing. Whereas ChOR-seq quantitatively profiles the post-replicative abundance of histone modifications and chromatin-associated proteins, SCAR-seq distinguishes chromatin occupancy between nascent sister chromatids. Together, these two complementary techniques have unraveled key mechanisms controlling the inheritance of modified histones during replication and revealed locus-specific dynamics of histone modifications across the cell cycle. Here, we provide the experimental protocols and bioinformatic pipelines for these methods.

Introduction

Chromatin organizes and regulates the eukaryotic genome. The basic units of chromatin are the nucleosomes, which are interspaced with DNA linkers. The nucleosomal core particle is composed of 145–147 bp of DNA wrapped around a histone core, composed of a central H3-H4 tetramer flanked by two H2A-H2B dimers. Histones carry a large variety of histone post-translational modifications (PTMs) and come in many types, including canonical histones showing replication-dependent incorporation and various replacement variants. Together, histone PTMs and histone variants create locus-specific chromatin environments that regulate genome function and reflect cell identity.

Chromatin, being a barrier for the replication machinery, is transiently disrupted during DNA replication. Nucleosomes are disassembled ahead of the replication forks and reassembled immediately after fork passage on the newly replicated DNA strands. The parental H3-H4 tetramers are recycled with their modifications and chaperoned to the leading and the lagging strands by distinct core replisome components. On the leading strand, H3-H4 recycling is mediated by subunits of DNA polymerase epsilon, while H3-H4 recycling to the lagging strand is mediated by MCM2, a subunit of the replicative helicase. Histone recycling is faithfully coordinated with asymmetrical DNA synthesis such that both sister chromatids inherit nearly equal amounts of old modified histones in a largely symmetric fashion. Concomitantly, new histones largely lacking locus-specific modifications are incorporated during DNA replication. After reassembly, chromatin matures, and the nucleosome positioning and histone modification landscape are progressively restored to the pre-replicative state. Histone recycling and post-replicative chromatin maturation ensure the faithful inheritance of chromatin organization in dividing cells. To uncover the...
mechanisms underlying these essential processes, we developed two next-generation sequencing techniques, chromatin occupancy after replication (ChOR)-seq\textsuperscript{15,16} and sister chromatids after replication (SCAR)-seq\textsuperscript{11}. Whereas ChOR-seq allows characterizing locus-specific dynamics of histone PTMs and chromatin-associated factors across the cell cycle, SCAR-seq enables tracking of histone recycling and de novo deposition processes on replicated sister chromatids, thereby facilitating the dissection of the mechanisms underlying histone inheritance.

Development and overview of the protocols

ChIP, in combination with genomic and more recently proteomic read-outs, has been fundamental for revealing the global and locus-specific composition of chromatin in steady-state cell populations. However, these technologies cannot resolve the dynamics and mechanisms of chromatin inheritance across DNA replication. Breakthroughs have been achieved with proteomic analysis of replicated chromatin through immunoprecipitation of proteins on nascent DNA (iPOND)\textsuperscript{27,28} and nascent chromatin capture (NCC)\textsuperscript{19}. These techniques have been pivotal in understanding the impact of DNA replication on the protein composition of chromatin. iPOND and NCC have identified an extensive catalog of DNA replication and chromatin maintenance factors enriched in nascent and mature chromatin\textsuperscript{18,19} and characterized the global dynamics of modifications on new and old histones after DNA replication\textsuperscript{9}. However, proteomic data do not provide locus-specific information. We developed two genomic approaches, SCAR-seq and ChOR-seq, to address the locus-specific composition of replicated chromatin. These methods allowed us to get a better understanding of the mechanisms governing histone inheritance and measure the locus-specific kinetics of restoration of chromatin landscapes after DNA replication\textsuperscript{7}.

ChOR-seq and SCAR-seq share some common principles. Both protocols entail labeling of replicated DNA in cell culture with 5-ethyl-2′-deoxyuridine (EdU)\textsuperscript{20} followed by ChIP with antibodies against histone modifications. Next, the EdU-labeled DNA fragments are biotinylated by click chemistry\textsuperscript{21-23}, captured with streptavidin beads and analyzed by next-generation sequencing (Fig. 1).

However, some key steps are specific for each protocol. In ChOR-seq (Fig. 1a), the nascent sample is fixed and collected immediately after pulse-labeling followed by chromatin fragmentation by sonication\textsuperscript{24}. The nascent sample is compared with mature samples in a pulse-chase EdU-labeling time course. To allow quantitative comparison between different time points, an equal amount of exogenous spike-in (Drosophila melanogaster) EdU-labeled chromatin is added to all samples before the ChIP step. After sequencing, results are normalized to the total number of D. melanogaster reads\textsuperscript{25} (Fig. 2a). This normalization allows the quantitative assessment of the restoration kinetics of individual histone PTMs/non-histone proteins to pre-replication levels. More recently, an alternative strategy for spike-in normalization has been described\textsuperscript{26}, which may also be considered when performing ChOR-seq experiments. This strategy additionally corrects for minor variations in added spike-in chromatin in different samples by using the ratio of D. melanogaster to mammalian total read counts in the corresponding input samples.

In SCAR-seq (Fig. 1b), a native ChIP is performed on chromatin fragmented to mononucleosomes with micrococcal nuclease (MNase)\textsuperscript{27-29}. EdU-labeled replicated DNA is biotinylated, captured with streptavidin beads and subsequently denatured to remove the unlabeled parental strands. The newly synthesized EdU-containing strands, still physically bound to streptavidin beads, are amplified and sequenced, preserving strand-specific information. Sister chromatid partitioning is computed as a proportion of forward and reverse reads in non-overlapping bins across the genome. The partitioning value indicates the relative difference in histone modification abundance between forward- and reverse-replicated sister genome copies in a cell population (Fig. 2b). Partitioning can be analyzed in various fashions depending on the specific biological questions. It can be averaged across genomic features or correlated with other genomic measurements, such as replication, transcription\textsuperscript{30-32} or chromatin self-interaction directionality\textsuperscript{33}. For example, to assess histone inheritance to the leading and lagging strands during replication, we correlated histone partitioning with DNA replication fork directionality (RFD)\textsuperscript{34}. RFD was measured by strand-specific sequencing of Okazaki fragments (OK-seq), nascent intermediates of the lagging strands\textsuperscript{30}. RFD was computed as a proportion of reverse and forward reads in non-overlapping bins across the genome and thus reflected the proportion of leading strand replication of forward and reverse genomic strands in the cell population.
**Fig. 1 | Experimental workflow of ChOR-seq and SCAR-seq.** a and b, Illustration of the key experimental steps in ChOR-seq (a) and SCAR-seq (b). Common steps are in black, and specific steps for ChOR-seq and SCAR-seq are in purple and orange, respectively. In the ChOR-seq protocol, libraries for the different time points (‘nascent’ and ‘mature’ samples) are combined after adapter ligation so that the click reaction and streptavidin pull-down are performed in one single tube. In the SCAR-seq protocol, strand separation is performed after streptavidin capture. The expected output of each technology is listed in boxes at the bottom. Lagging strand (blue), leading strand (pink), nucleosomes (blue and gray beads), EdU (green), biotin (red), streptavidin magnetic beads (black) and Illumina TruSeq adapters (gray (P5) and yellow (P7), respectively). X-ChIP, ChIP on crosslinked cells.
Applications and outlook

ChOR-seq and SCAR-seq technologies complement the panel of existing biochemical, structural and proteomic approaches for studying chromatin replication and epigenome maintenance in mammalian cells. We used ChOR-seq in human cancer cells (HeLa)\(^{15}\) to reveal that old parental histones H3-H4 maintain their genomic position across replication in both eu- and heterochromatin domains\(^{15}\). We profiled chromatin occupancy after replication of several functionally distinct histone PTMs and revealed that restoration of histone PTM levels follows mark- and locus-specific kinetics\(^{15}\). Using ChOR-seq in mouse embryonic stem cells (mESCs), we profiled the occupancy of serine 5-phosphorylated RNA polymerase II (RNApolII S5phos)\(^{16}\), a hallmark of transcription initiation\(^{33}\). We demonstrated that nascent chromatin was depleted of RNApolII S5phos, indicating that transcription is shut down upon fork passage. We revealed that RNApolII S5phos occupancy progressively increased during the first 2 h of chromatin maturation, identifying the time frame for
transcription restart after replication. In conclusion, ChOR-seq is a powerful quantitative approach for profiling chromatin occupancy of histone PTMs and non-histone proteins after replication and across the cell cycle until a given locus replicates again. Using SCAR-seq in mESCs, we revealed that histone recycling is not entirely symmetrical between the leading and lagging strands\(^{11}\). Furthermore, we demonstrated that the inheritance of histone PTMs is directly coupled to DNA replication by showing that MCM2, part of the CMG helicase, is responsible for recycling histones to the lagging strand. SCAR-seq thus revealed a mechanism of histone recycling that could not be detected with classical biochemistry approaches\(^{11}\). In perspective, we envisage that using SCAR-seq in loss-of-function mutants of distinct DNA replication and chromatin factors will resolve the complex connections between genome replication and chromatin assembly from new and old histones.

Comparison with other methods
Other laboratories have developed similar types of next-generation sequencing (NGS)-based technologies for profiling replicating chromatin (reviewed in ref. \(^{7}\)). These methods share a conceptual framework with ChOR-seq and SCAR-seq while presenting noteworthy differences. ChIP on nascent chromatin (NAS-ChIP), similar to ChOR-seq, measures the occupancy of histone and non-histone proteins on replicated DNA in mammalian cells, using ChIP followed by purification and sequencing of replicated DNA\(^{34,35}\). NAS-ChIP does not include the spike-in of exogenous EdU-labeled chromatin and therefore cannot be used to quantitatively compare different samples. The same authors developed a variation of this method, NasChIP-BS-seq, that preserves strand-specific information and detects 5′-cytosine DNA methylation. NasChIP-BS-seq involves ChIP, followed by purification and separation of new and parental strands of replicated DNA, and includes bisulfite treatment of replicated strands. NasChIP-BS-Seq was designed for the simultaneous analysis of protein enrichment and DNA methylation and is not directly comparable to ChOR-seq or SCAR-seq\(^{35}\).

Another method for sister chromatid-resolved chromatin profiling in mammalian cells has become available recently\(^{13}\). Enrichment and sequencing of protein-associated nascent DNA (eSPAN) was initially developed for the analysis of strand-specific enrichment of proteins\(^{36}\) and histone modifications\(^{36}\) around replication origins in synchronized cultures of budding yeast (reviewed in ref. \(^{7}\)). Recently, eSPAN was adapted for genome-wide sister chromatid profiling in mESCs\(^{13}\), largely mimicking the strategy of SCAR-seq for tracking old and new histones\(^{11}\) and the analytical approach of combining SCAR-seq with OK-seq for genome-wide profiling of sister chromatid asymmetry and replication directionality\(^{11,30}\). eSPAN confirmed the results from SCAR-seq in mESCs and demonstrated that PolE3/E4 is responsible for histone recycling to the leading strand in mammalian cells as originally reported in yeast\(^{10}\). Although sharing similar principles, the experimental workflows of SCAR-seq and eSPAN differ substantially at key steps. In eSPAN the newly synthesized DNA is labeled with BrdU. In yeast, the chromatin targets were immunoprecipitated from cross-linked chromatin, fragmented by sonication\(^{36}\) or MNase digestion\(^{10}\). Sequencing adapters were ligated to single-stranded immunopurified BrdU-labeled fragments. In contrast, SCAR-seq uses a commonly used strategy of library preparation from double-stranded DNA, with the additional advantage that the parental strands can be analyzed. Click chemistry and biotin pull-down have a higher sensitivity than BrdU immunoprecipitation, and longer labeling times are used for eSPAN.

The adaptation of eSPAN for mammalian cells\(^{13}\) replaced the ChIP step with antibody-guided chromatin tagmentation by protein A-transposase Tn5 fusion preloaded with mosaic adapters, similarly to the novel chromatin profiling techniques cleavage under targets and tagmentation (CUT&TAG)\(^{37,38}\), antibody-guided chromatin tagmentation (ACT-seq)\(^{39}\), chromatin integration labeling (ChIL-seq)\(^{40}\), and combinatorial barcoding and targeted chromatin release (coBATCH)\(^{41}\). To allow strand separation and strand-specific sequencing, the adapters need to be attached to both genomic strands, which is achieved in additional steps by oligonucleotide replacement and gap repair\(^{13,42,43}\). The newest version of eSPAN is an important advance in terms of reducing input cell numbers, but longer labeling times because of the use of BrdU may limit the analysis of fork proximal events in nascent chromatin\(^{13}\).

Limitations
Similar to other ChIP-based approaches, ChOR-seq and SCAR-seq rely on the availability of specific high-quality antibodies and therefore permit study only of known modifications and proteins. Furthermore, as in many NGS-based techniques, these methods provide an averaged read-out of a
cell population and do not provide unique signatures of single cells. ChOR-seq and SCAR-seq require a significant amount of starting material, because only a minor fraction of the genome is replicated during a short EdU pulse, and both purification and library preparation require multiple steps. Importantly, even though ChOR-seq can quantitatively assess the difference in occupancy of chromatin-associated proteins, this quantification is relative, and the datasets can be directly compared only within the same time course and for the same target protein. ChOR-seq is a ChIP-based technology, and it is possible to compare the occupancy patterns between bulk ChIP-seq and ChOR-seq. It is not feasible to quantitatively compare signal between bulk ChIP-seq and ChOR-seq using spike-in normalization, because these two techniques involve different procedures. However, a good proxy for the pre-replication histone PTM levels can be obtained by including a late time point in ChOR-seq time courses, where labeling is chased until the next round of replication, or cells are arrested at the G1/S transition to avoid re-replication.

Expertise needed to implement the protocols
ChOR-seq and SCAR-seq require strong skills in molecular biology. The protocols are accessible to most molecular biology laboratories and rely on common laboratory equipment. Basic computational biology skills are necessary to perform ChOR-seq analysis using Galaxy, and SCAR-seq analysis via pre-built pipelines requires experience in R.

Experimental design
Here, we present some critical considerations and key steps for the experimental workflows of ChOR-seq and SCAR-seq.

Considerations for distinct histone modifications
To track the old and the new histones by ChOR-seq and SCAR-seq, we take advantage of distinct modifications carried by old and new histones H3 and H4 in nascent chromatin, as identified by mass spectrometry assays. As a proxy for new histones, we have used acetylation of lysine 5 on histone H4 (H4K5ac), because new histone H4 is uniformly acetylated at lysines 5 (K5) and 12 (K12) during nucleosome assembly. Therefore, all newly deposited histone H4 in nascent chromatin contains this modification immediately after replication. H4K5ac and H4K12ac are deacetylated 10–20 min after the replication fork passage. Because H4K5ac and H4K12ac are transient modifications, they allow tracking of new histone H4 only immediately after replication.

As a proxy for old histones, we have used H4K20me2 present on ~80% of old histones in cultured human and mouse cells and nearly homogeneously enriched genome-wide. Importantly, methylation of H4K20 is not imposed on new histones until the G2 phase of the cell cycle, after completion of DNA replication, making this mark the ideal candidate to follow the inheritance of old histones through DNA replication. Other histone modifications such as H3K4me3, H3K79me3, H3K27me3 or H3K36me3 can be used as proxies for old histone H3 in nascent chromatin, because these modifications are not found on new histone H3. However, unlike in stable isotope labeling by amino acids in cell culture (SILAC)-based proteomics, old and new histone H3 cannot be distinguished over a maturation time course in NGS approaches, because the new histones become progressively modified after replication. Furthermore, different modifications are acquired with distinct kinetics. Some modifications (e.g., H3K4me3, associated with active transcription) are restored within a few hours after replication, whereas others and mostly those associated with silencing (e.g., H3K27me3 and H3K9me3) are slow recovering and continue to accumulate after mitosis in daughter cells.

Besides, it is critical to take into consideration the genomic distribution of the analyzed modifications. This will affect the yield of the ChIP experiment as well as the coverage in the sequencing results and the strategy for data analysis. Some histone PTMs, such as H3K4me3, H3K27me3 and H3K36me3, are enriched across specific genomic regions and can be analyzed using standard peak-calling pipelines for ChIP-seq analysis. However, histone PTMs like H4K20me2 or H4K5ac are broadly distributed across the genome, without a specific pattern, and the enrichment of these modifications can only be controlled by comparison with the input samples (see Procedure 2).

In sum, the inherent histone mark-specific post-replicative restoration dynamics and genomic occupancy must be considered when planning and analyzing an experiment.
Cell culture and labeling of newly synthesized DNA

We anticipate that both protocols can be used in any proliferating mammalian cell lines in culture and probably other model organisms that allow efficient uptake and incorporation of EdU (e.g., EdU labeling was used to map post-replication nucleosome occupancy in budding yeast54–56 and Drosophila cells57 (reviewed in ref.7)). It is critical to maintain optimal cell proliferation rates and identical culture conditions across experiments. We recommend controlling for cell density and passage number and regularly supplying cultures with fresh media. Special attention should be paid to cell lines forming larger colonies (e.g., mESCs), because they may need to be passaged a day before the experiment to maintain smaller colonies and ensure uniform labeling.

To label newly synthesized DNA at genomic sites of ongoing replication, EdU is added to the culture media. The labeling time needs to be carefully considered depending on the question to be addressed. Short labeling times (10–15 min) will increase the temporal resolution for the analysis of

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**Box 1 | Quality control for EdU incorporation efficiency**

This step aims to control the EdU incorporation and to determine the fraction of EdU-positive cells in the population. FACS provides information about cell cycle distribution, the number of EdU-positive cells (S-phase) and the efficiency of EdU incorporation. In a pulse-chase setup, the progression of EdU-labeled cells can be followed through the cell cycle.

**Procedure**

**EdU FACS**

**Timing** 2 h plus analysis on flow cytometer

1. Analyze the percentage of EdU-positive cells and cell cycle distribution by following the manufacturer’s instructions for Click-iT EdU flow cytometry cell proliferation assay. Remember to include an unlabeled control.

*Quality control for EdU incorporation efficiency.* Labeling control using FACS analysis in E14 mESCs. A representative FACS image is used to identify EdU-positive cells. EdU was stained with Alexa Fluor 647 azide, and total DNA was stained with propidium iodide (PI). Cells were pulse-labeled with 10 µM EdU for 10 min. Data were analyzed using FlowJo software 10.7.1. Dots represent cells, the square represents gating of EdU-positive cells in S-phase and black arrowheads mark EdU-negative cells in G1- and G2-phases.
chromatin assembled shortly after replication fork passage. This is critical for the analysis of very dynamic histone modifications like H3K4me3, rapidly imposed on newly synthesized histones after replication, and transient PTMs such as H4K5ac. We have applied labeling times up to 30 min for the analysis of slow-restoring histone modifications like H3K27me3 and histone modifications not acquired until the G2 phase such as H4K20me2. Extending the labeling pulse will increase the labeled fraction of the genome, which can be an option to increase the amount of purified material in experiments with limited cell numbers (e.g., FACS-sorted cells). EdU incorporation needs to be controlled by EdU FACS (Box 1) or by immunofluorescence microscopy.

Starting cell number

In ChOR-seq experiments, we use ~1–2 × 10^7 cells per condition (i.e., each modification and/or each time point, if performing a time-course analysis). For each SCAR-seq experiment, we use ~2–3 × 10^7 cells per condition. However, for both protocols, we recommend collecting enough cells for several ChIP reactions, which conveniently provides material for optimization and a technical repeat if needed. These starting cell numbers are indicative for mESCs in asynchronous cultures and may need to be empirically adjusted for other cell types depending on the fraction of cells in S-phase under the specific culturing conditions, duration of the EdU pulse, the abundance of the histone modification analyzed and the quality of the antibody used for ChIP. For cell lines with a low fraction of cells in S-phase in asynchronous populations, a synchronization set-up might be advisable (see the procedure for synchronization of HeLa S3 cells in the Supplementary Information). In that case, it is critical to consider the replication timing of the loci enriched in the PTM of interest\textsuperscript{15,58}. This will ensure that the labeling is performed at an optimal time during S-phase to recover a representative number of loci after streptavidin pull-down.

Chromatin preparation and fragmentation

In ChOR-seq, we prepare chromatin from formaldehyde-crosslinked cells by cell lysis and shearing by sonication. We fix cells with 1% (wt/vol) formaldehyde for 5 min for histone ChOR-seq and 10 min for RNApol II ChOR-seq. The fixation and sonication conditions will need to be optimized for individual cell types, baits and antibodies. However, we do not recommend exceeding 10 min of fixation, because it may result in over-crosslinked, difficult-to-sonicate chromatin. Sonication is a critical step and needs to be controlled to achieve reproducible shearing of DNA to an average fragment length of 250–300 bp (Box 2). We have used Covaris M220 and Bioruptor Plus sonicators, but other equivalent sonication platforms may be applied according to the manufacturer’s recommendations.

In SCAR-seq, we isolate nuclei and fragment native chromatin with MNase. Because different cell types present different sensitivity to MNase digestion, we recommend empirically determining the optimal MNase concentration both for individual cell types and each new lot of MNase enzyme. For the initial titration, we recommend using two to three different concentrations ranging up to five

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**Box 2 | Quality control for chromatin fragmentation**

**Procedure**

(A) Quantification of genomic DNA and quality control of chromatin shearing (ChOR-seq) ★ Timing 18–20 h including overnight incubation

(i) Add 1 μl of RNase A (10 mg/ml) to 25 μl of sheared chromatin from Step 39 (Procedure 1) and incubate for 30 min at 37 °C in a thermocycler.

(ii) Add 1 μl of proteinase K (10 mg/ml) and incubate for 10 h at 37 °C followed by a 6-h incubation at 65 °C to reverse crosslinking.

(iii) Purify DNA using a commercial column-based kit (e.g., Qiagen QIAquick PCR purification kit, cat. no. 28104).

(iv) Elute in 50 μl of Qiagen EB buffer.

(v) Use 1 μl to measure DNA concentration using a Qubit fluorometer and following the manufacturer’s instructions for the Qubit dsDNA BR assay kit.

(vi) Check the fragment size distribution by running 1 μl on an Agilent Bioanalyzer using a high-sensitivity DNA kit.

★ CRITICAL STEP Chromatin shearing must be optimized for each cell line and sonicator. Once the optimal parameters have been established, it is not required to check fragment size distribution for every experiment before proceeding to ChIP.

(B) Quality control of MNase-digested chromatin (SCAR-seq) ★ Timing 1.5 h

(i) Resuspend the pellet from Step 27 (Procedure 2) in 100 μl of TE 1× and add 2.5 μl of 20% (wt/vol) SDS. Incubate in a ThermoMixer for 15 min at 37 °C and 300 rpm.

(ii) Transfer 10 μl of the supernatant from Step 27 (Procedure 2) to a new 1.5-ml tube and add 90 μl of TE 1× and 2.5 μl of 20% (wt/vol) SDS. Incubate in a ThermoMixer for 15 min at 37 °C and 300 rpm.

(iii) Use 1 μl to measure DNA concentration from the pellet and the supernatant fractions (Step 27) using a Qubit fluorometer and following the manufacturer’s instructions for the Qubit dsDNA BR assay kit.

★ CRITICAL STEP Typically, ~90% (mass/mass) of chromatin (measured as the amount of DNA) is released into the supernatant, whereas ~10% (mass/mass) will remain in the pellet.

(iv) Purify the DNA from step ii on the Qiagen MinElute reaction cleanup column following the manufacturer’s instructions, except performing the final elution in 50 μl of MinElute EB buffer.
(v) Check fragment size distribution by running 1 μl of purified DNA on an Agilent Bioanalyzer, an equivalent fragment analyzer or a 1.8% (wt/vol) agarose gel in 1× TBE buffer. Store the remaining purified DNA at −20 °C for the input preparation (proceed directly to Procedure 2, Step 53).

**Critical step**  The MNase digestion should result in predominantly mononucleosome-sized fragments. MNase digestion conditions must be calibrated for each cell type and each batch of MNase as discussed in ‘Experimental design’.

**Troubleshooting**

**Quality control for chromatin fragmentation.**

(a) Representative profile of crosslinked chromatin sheared using a Covaris M220 sonicator obtained by Agilent Bioanalyzer. In ChOR-seq, chromatin fragments ranging from 100 to 500 bp are desired, with an average fragment size around 250 bp (calculated in the Bioanalyzer electropherogram from the area under the curve).

(b) Representative profile of DNA purified from native chromatin digested with MNase obtained by Agilent Bioanalyzer. For SCAR-seq, a chromatin preparation containing >50% mononucleosome fragments (147–150 bp) is desired (calculated in the Bioanalyzer electropherogram from the area under the curve).

FU, fluorescence units.

Times below and above the concentration listed in Procedure 2. The goal of this titration is to obtain mostly mononucleosomes while avoiding overdigestion to subnucleosomal fragments (Box 2). Chromatin fragmentation should be controlled by analysis of purified and, for ChOR-seq samples, decrosslinked material by using an Agilent Bioanalyzer or an equivalent fragment analyzer or by electrophoresis on an agarose gel (Box 2).
## ChIP

A critical step in both ChOR-seq and SCAR-seq is ChIP. We recommend using high-quality ChIP-grade antibodies whenever possible. It is essential to validate antibody specificity. Every antibody needs to be tested in ChIP-seq or ChIP-qPCR or by immunofluorescence microscopy preferably by comparison to knock-out cell lines lacking the target epitope to ensure that the enrichment is specific. The specificity of an antibody against histone modifications can be additionally validated in histone peptide arrays or dot-blot assays or by sample normalization and antibody profiling (SNAP)-ChIP\textsuperscript{59}. For commercial ChIP-grade antibodies, data for antibody validation and experimental conditions can often be retrieved from manufacturers. A list of antibodies successfully applied in ChOR-seq and/or SCAR-seq is included in Table 1.

We believe that ChIP steps can, in principle, be replaced with other ChIP approaches optimized for specific chromatin targets, including new technologies for profiling of chromatin occupancy that have been developed recently (reviewed in refs. \textsuperscript{60,61}). The prospect of combining these novel approaches with quantitative (ChOR-seq) and strand-specific (SCAR-seq) analysis of replicated chromatin is very promising. However, this would require further method development and validation.

As in standard ChIP experiments, it is critical to perform all pipetting steps on ice and to use protease inhibitors, as well as the relevant phosphatase and deacetylase inhibitors when necessary. We recommend controlling for the size of purified immunoprecipitated DNA and comparing it with input material by electrophoresis using an Agilent Bioanalyzer or similar. Some antibodies preferentially capture larger chromatin fragments, which may influence downstream library preparation and compromise reproducibility between replicates.

In SCAR-seq, it is particularly important to control the size distribution of material obtained from the ChIP, because strictly mononucleosome-sized fragments need to be recovered. To ensure this, we apply a dual-size selection using AMPure XP beads just after ChIP, which removes DNA fragments longer than ~400 bp and a second dual-size selection on the final amplified library for a stringent selection of mononucleosome-sized inserts (Box 3). The ChOR-seq protocol includes a dual-size selection step after library preparation to remove fragments longer than 700 bp (Box 3). However, it may be advisable to perform an initial dual-size selection on the immunoprecipitated DNA if using antibodies with a strong preference for large DNA fragments. In some situations, when the yield of the ChIP experiment is too low, it may be necessary to scale up the experiment. We also advise combining the immunoprecipitated DNA from several ChIP reactions before proceeding with library preparation and EdU pull-down if the yield is severely reduced after the initial size-selection step. See the recommendations below for starting material in library preparation.

### Library preparation

For library preparation in ChOR-seq, we recommend pooling all samples from the same time-course experiment after ligation of Illumina TruSeq adapters and performing all subsequent steps on the pooled sample. This reduces tube-to-tube variation and improves spike-in normalization. Note that this strategy is recommended only when using indexed adapters during library preparation. In other

### Table 1 | Antibodies successfully used in ChOR-seq and/or SCAR-seq

<table>
<thead>
<tr>
<th>Histone PTM/protein</th>
<th>Source</th>
<th>Identifier</th>
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<td>RNA polymerase II</td>
<td>Abcam</td>
<td>Cat. no. ab5131; <a href="https://scicrunch.org/resolver/AB_449369">https://scicrunch.org/resolver/AB_449369</a></td>
<td>●</td>
<td></td>
</tr>
<tr>
<td>(phospho S5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Box 3 | Quality control of libraries ● Timing 1.5 h

Procedure
1. Use 1 µl of purified libraries to measure DNA concentration using a Qubit fluorometer and following the manufacturer’s instructions for the Qubit dsDNA HS assay kit.

Troubleshooting
2. Check the fragment size distribution by running 1 µl on an Agilent Bioanalyzer using a high-sensitivity DNA kit.

For ChOR-seq, the expected average size of the library should be between 400 and 500 bp. The second peak of ~500 bp indicates the enrichment of dinucleosome-sized inserts. If dinucleosome-sized fragments are detected in the library, repeat the size selection. If smaller fragments (≤130 bp) are detected, proceed with a 1× purification with AMPure XP beads following the manufacturer’s instructions. If the libraries are sequenced paired-end, it is possible to filter mononucleosome fragments in silico, but this may result in fewer reads of the desired size. This can be an option to avoid further loss of material with an extra size selection.

Troubleshooting

Quality control for library amplification. a, Representative profile of a ChOR-seq library obtained by Agilent Bioanalyzer. An average library size of 400–500 bp is desired. b, Representative profile of a SCAR-seq library obtained by Agilent Bioanalyzer. An average library size of ~280 bp is desired.
library-preparation strategies where indexes are on the PCR primers, the pooling at this stage is not applicable.

We recommend using between 20 and 100 ng of immunoprecipitated DNA per condition for library preparation of ChOR-seq samples. For the input and bulk ChIP samples, we advise starting the library preparation from 10 to 20 ng of DNA. For SCAR-seq, we advise starting the library preparation with 100–300 ng of size-selected immunoprecipitated DNA.

Isolation of replicated DNA

For isolation of EdU-labeled replicated DNA, biotin-triethylene glycol (TEG)-azide is coupled to EdU via copper-catalyzed azide-alkyne cycloaddition click chemistry\(^{21}\). In the most recent version of ChOR-seq\(^{16}\), we used picolyl-azide-PEG4-biotin to increase the efficiency of the click-reaction\(^{62}\). However, we do not recommend using it in SCAR-seq, because it can interfere with the stranded analysis.

The biotinylated DNA fragments are then captured with streptavidin-coated magnetic beads and washed stringently. We recommend testing the specificity of the click reaction and streptavidin pull-down by including a control sample derived from unlabeled cells (see the Troubleshooting section for more details).

Spike-in with chromatin derived from an exogenous reference genome

For quantitative comparison between different ChOR-seq samples (e.g., time-course analysis of PTM restoration), we add to each sample the same amount of chromatin prepared from EdU-labeled *D. melanogaster* S2 cells before ChIP to act as an exogenous reference. By adding a constant amount of the reference genome chromatin to all samples, the sequential ChIP and EdU-mediated pull-down of replicated DNA will result in the same absolute enrichment of the spiked-in DNA from *D. melanogaster*, while the absolute enrichment of mammalian DNA will be sample specific. Therefore, normalization of ChIP read counts from the mammalian genome to the read counts from the *D. melanogaster* genome allows direct comparison across different samples and corrects for the experimental and analytical restrictions of ChIP-seq. The proposed strategy is possible only for proteins in which the antibody epitope is conserved in both species, such as is the case for many histone modifications. For non-conserved proteins, an alternative spike-in strategy may be optimized by using a mammalian-specific antibody in combination with an antibody that exclusively recognizes the *D. melanogaster* histone variant H2Av\(^{63}\). For the preparation of spike-in chromatin, it is crucial to use conditions identical to those used for mammalian cells. This includes fixation, lysis and chromatin shearing. In addition, the fragmentation of the spike-in chromatin needs to be controlled to achieve the same size distribution as for mammalian chromatin samples.

The amount of *D. melanogaster* S2 chromatin to spike must be optimized depending on the protein/modification analyzed. For the histone modifications that we have analyzed, the addition of 0.05–0.1% (wt/wt) *D. melanogaster* chromatin is recommended. When performing ChOR-seq for different histone modifications or non-histone proteins, we recommend using qPCR to estimate the optimal percentage of spike-in chromatin.

Strand separation and purification of nascent and parental strands (SCAR-seq only)

A key step in the SCAR-seq method is the separation of parental unlabeled and newly synthesized EdU-labeled strands with NaOH. For strand separation, the streptavidin magnetic beads with captured biotinylated library fragments are incubated in the alkaline buffer, which denatures double-stranded DNA. After alkaline washes, the newly replicated EdU strands remain bound to the streptavidin beads whereas the unlabeled parental strands are released into the supernatant. The parental strands can be recovered from the supernatant of the first alkaline wash, as indicated in Procedure 2, and analyzed separately by sequencing to retrieve a signal complementary to the EdU-labeled strands\(^{11}\).

Sequencing and data processing

To illustrate the ChOR-seq and SCAR-seq technologies, we have used previously published datasets\(^{11,15}\). For each experiment, at least two independent biological replicates must be included. For both methods, it is important to achieve an appropriate sequencing depth, and this mainly depends on the type of protein/histone modification analyzed. For the histone modifications distributed broadly in the genome, such as H3K27me3, we recommend obtaining ≥15–20 million uniquely mapped reads per sample. For histone modifications with narrower distributions, such as H3K4me3,
10 million reads may suffice. For the analysis of histone modifications covering nearly the entire genome (H4K5ac and K20me2), we recommend ≥40 million unique reads per sample. For general considerations on sequencing depth for ChIP-seq analysis, refer to the Encyclopedia of DNA Elements (ENCODE) recommendations \(^6\). The required sequencing depth may need to be adapted depending on the biological question addressed and the analysis strategy applied. For ChOR-seq, an input control is purified from sheared chromatin as indicated in Procedure 1. The input samples are sequenced and used for correction of potential biases when performing peak-calling analysis (see ‘Sequencing and data analysis’ in Procedure 1). The parameters used to define peaks will have to be adapted to individual chromatin targets, with special attention in the case of histone PTMs with broad distributions.

In SCAR-seq, we assess the enrichment over the input for two genomic strands separately (see Procedure 2, ‘Evaluation of strand-specific enrichment of histone modifications over the strand-specific signal from input’). This is especially important for histone PTMs lacking a well-defined occupancy pattern, such as H4K20me2 or H4K5ac, where the enrichment cannot be evaluated using peak-calling approaches. The inputs in SCAR-seq are prepared from the MNase-digested chromatin (Procedure 2, Box 2), following all steps of the SCAR-seq protocol, including the streptavidin capture and strand separation of newly synthesized DNA strands but excluding the ChIP step, as indicated in Procedure 2.

**Materials**

**Biological materials**

**Cell lines**
- Mouse E14 embryonic stem cells (from the laboratories of Kristian Helin and Joshua Brickman; RRID: CVCL_C320)
- *D. melanogaster* S2-DRSC (Drosophila Genomics Resource Center, cat. no. 181; RRID: CVCL_Z992)
  
  **CAUTION** The cell lines used in your research should be checked regularly to ensure that they are authentic and mycoplasma free.

**Reagents**

**Cell culture reagents for mESCs: 2i medium**
- ‘2i SILAC’ medium (custom-made by Gibco, equivalent to a 1:1 mix of DMEM/F-12 and neurobasal medium, 2 mM Glutamax and 1 mM sodium pyruvate but depleted for l-arginine and l-lysine)
- 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M3148)  
  
  **CAUTION** 2-Mercaptoethanol is toxic if inhaled or absorbed through the skin. Always wear a laboratory coat and gloves and use 2-mercaptoethanol in a fume hood.
- B-27 supplement 50× (Gibco, cat. no. 17504-044)
- Gelatin from bovine skin (Sigma-Aldrich, cat. no. G9391-500G)
- GSK3 inhibitor (CHIR99021) (Sigma-Aldrich, cat. no. SML1046)  
  
  **CAUTION** GSK3 inhibitor is toxic if swallowed or it comes in contact with skin. Wear a laboratory coat and gloves and use GSK3 inhibitor in a fume hood.
- l-arginine monohydrochloride (Sigma-Aldrich, cat. no. A6969)
- l-lysine monohydrochloride (Sigma-Aldrich, cat. no. L8662)
- Leukemia inhibitory factor (LIF; homemade); alternatively, can be purchased from a commercial supplier (Miltenyi Biotec, cat. no. 130-095-779)
- MEK1/2 inhibitor (PD0325901) (Sigma-Aldrich, cat. no. PZ0162)  
  
  **CAUTION** MEK1/2 inhibitor is toxic if swallowed. Wear a laboratory coat and gloves and use MEK1/2 inhibitor in a fume hood.
- N-2 supplement (100×) (Gibco, cat. no. 17502-048)
- Penicillin-streptomycin (10,000 U/ml) (Gibco, cat. no. 15140-122)  
  
  **CAUTION** Penicillin-streptomycin is an irritant if it comes in contact with skin. Always wear gloves and a laboratory coat.
- Soybean trypsin inhibitor (Gibco, cat. no. 17075029)
- Trypsin-EDTA (0.25% (wt/vol)) (Gibco, cat. no. 25200-056)

**Cell culture reagents for mESCs: DMEM-serum-LIF medium**
- 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M3148)  
  
  **CAUTION** 2-Mercaptoethanol is toxic if inhaled or absorbed through the skin; always wear a laboratory coat and gloves and use 2-mercaptoethanol in a fume hood.
- DMEM (Gibco, cat. no. 31966-021)
HyClone research-grade FBS, South American origin (Cytiva, cat. no. SV30160.03) ▲ CRITICAL Each serum batch must be tested for its ability to support embryonic stem cells in an undifferentiated state.65

Gelatin from bovine skin (Sigma-Aldrich, cat. no. G9391-500G)

LIF (homemade); alternatively, can be purchased from a commercial supplier (Miltenyi Biotec, cat. no. 130-095-779)

MEM non-essential amino acids solution (100×) (Gibco, cat. no. 11140-050)

Penicillin-streptomycin (10,000 U/ml) (Gibco, cat. no. 15140-122) ! CAUTION Penicillin-streptomycin is an irritant if it comes in contact with skin. Always wear gloves and a laboratory coat.

Trypsin-EDTA (0.25% (wt/vol)) (Gibco, cat. no. 25200-056)

Cell culture reagents for D. melanogaster S2-DRSC cells

Bactopeptone (Thermo Fisher Scientific, cat. no. 211677)

HyClone research-grade FBS, South American origin (Cytiva, cat. no. SV30160.03) ▲ CRITICAL Each serum batch must be tested for its ability to support embryonic stem cells in an undifferentiated state.65

KHCO₃ (Sigma-Aldrich, cat. no. 60339)

Penicillin-streptomycin (10,000 U/ml) (Gibco, cat. no. 15140-122) ! CAUTION Penicillin-streptomycin is an irritant if it comes in contact with skin. Always wear gloves and a laboratory coat.

Shields and Sang M3 insect medium (Sigma-Aldrich, cat. no. 58398) ! CAUTION Shields and Sang M3 insect medium causes serious eye irritation. Wear appropriate gloves and a laboratory coat when handling.

Yeast extract (Sigma-Aldrich, cat. no. Y-1000)

Common reagents

10× PBS (pH 7.4; Thermo Fisher Scientific, cat. no. 70011044)

EdU (Jena Bioscience, cat. no. CLK-N001-25)

Agencourt AMPure XP (Beckman Coulter, cat. no. A63881)

Aprotinin (Sigma-Aldrich, cat. no. 10981532001) ! CAUTION Aprotinin may cause an allergic skin reaction or breathing difficulties if inhaled. Wear gloves and a laboratory coat and work in a chemical hood.

Click-iT EdU Alexa Fluor 647 flow cytometry assay kit (Thermo Fisher Scientific, cat. no. C10420)

CuSO₄ (Jena Bioscience, cat. no. CLK-MI004-50)

Dynabeads MyOne streptavidin T1 (Thermo Fisher Scientific, cat. no. 65602)

UltraPure EDTA (0.5 M, pH 8.0; Life Technologies, cat. no. 15575-038) ! CAUTION UltraPure EDTA is toxic if swallowed. Wear gloves when handling.

EGTA (0.5 M, sterile solution, pH 8.0; bioWorld, cat. no. 40520008-2) ! CAUTION EGTA is toxic if swallowed or inhaled. Wear gloves when handling.

Ethanol (Sigma-Aldrich, cat. no. 51976) ! CAUTION Ethanol is flammable. Keep away from open flames.

Glycerol (for molecular biology, >99% (vol/vol); Sigma-Aldrich, cat. no. 56-81-5)

High-sensitivity DNA kit for Bioanalyzer (Agilent, cat. no. 5067-4626)

KAPA Hyperprep kit (Kapa Biosystems, Roche, cat. no. KK8504)

Leupeptin (Sigma-Aldrich, cat. no. 11034626001) ! CAUTION Leupeptin is toxic if swallowed or inhaled or if it comes in contact with skin. Wear a laboratory coat and gloves.

Liquid nitrogen ! CAUTION Avoid contact with skin. Always wear isolation gloves and eye shields and handle it in a ventilated room.

MinElute reaction cleanup kit (Qiagen, cat. no. 28204)

Sodium ascorbate (Jena Bioscience, cat. no. CLK-MI005-1G)

NGS indexed PentAdapters (PentaBase, cat. no. SKU 316)

Pepstatin (Sigma-Aldrich, cat. no. 11524488001)

Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, cat. no. 93482-250ML) ! CAUTION PMSF is toxic if swallowed. It may cause a serious burn if it comes in contact with skin or eyes. Wear a laboratory coat, gloves and eye shield when handling.

Propidium iodide (PI) (Thermo Fisher Scientific, cat. no. BMS500PI) ! CAUTION PI is harmful if swallowed. It is irritating to eyes, respiratory system and skin. Wear gloves and protective clothes when handling.

Proteinase K (Sigma-Aldrich, cat. no. P2308-100MG)

Qiagen Qiaquick PCR purification kit (Qiagen, cat. no. 28104)

Qubit double-stranded (ds) DNA broad range (BR) assay kit (2–1,000 ng) (cat. no. Q32853)
- Qubit dsDNA high-sensitivity (HS) assay kit (0.2–100 ng) (cat. no. Q32854)
- RNase A (Sigma-Aldrich, cat. no. R4875-100MG)
- NaCl (Sigma-Aldrich, cat. no. S7653)
- Sodium dodecyl sulfate (SDS) solution, 20% (wt/vol) (Sigma-Aldrich, cat. no. 05030) **CAUTION** SDS is corrosive to the skin and is a respiratory irritant. Wear gloves when handling the solution. Thoroughly wash any skin exposed to this chemical.
- Thymidine (Sigma-Aldrich, cat. no. T1895)
- Tris–HCl buffer (1 M, pH 7.5; Thermo Fisher Scientific, cat. no. 15567027)
- Tris–HCl buffer (1 M, pH 8.0; Thermo Fisher Scientific, cat. no. 15568025)
- Tris-(3-hydroxypropyltriazolylmethyl)amine (THPTA) (Sigma-Aldrich, cat. no. 762342) **CAUTION** THPTA is a skin and eye irritant. Wear gloves when handling.
- Triton X-100 (molecular biology grade; Sigma-Aldrich, cat. no. T8787-100ml) **CAUTION** Triton X-100 is a skin and eye irritant. Wear gloves when handling.
- Trizma base (Sigma-Aldrich, cat. no. T1503)
- Tween 20 (Sigma-Aldrich, cat. no. P9416)
- Water, PCR grade (Sigma-Aldrich, cat. no. 3315959001)

**ChOR-seq reagents**
- 16% (wt/vol) formaldehyde, methanol free (Thermo Fisher Scientific, cat. no. 28908) **CAUTION** Formaldehyde is toxic if inhaled, ingested, or absorbed through the skin; always wear a laboratory coat and gloves and use formaldehyde in a chemical hood.
- (Optional) Aminoguanidine hydrochloride (Sigma-Aldrich, cat. no. 396494) **CAUTION** Aminoguanidine hydrochloride is a skin and eye irritant. Wear gloves when handling.
- BSA (Sigma-Aldrich, cat. no. A4503)
- IGEPAL CA-630 (Sigma-Aldrich, cat. no. 56741) **CAUTION** IGEPAL CA-630 is corrosive to the skin. Wear gloves when handling and thoroughly wash any skin exposed to this chemical.
- LiCl (Sigma-Aldrich, cat. no. L9650) **CAUTION** LiCl is harmful if swallowed. It causes skin and eye irritation. Wear protective gloves and a laboratory coat.
- Picolyl-azide-PEG4-biotin (Jena Bioscience, cat. no. CLK-1167-100)
- Protein A agarose beads (Thermo Fisher Scientific, cat. no. 20333)
- Sodium deoxycholate (Sigma-Aldrich, cat. no. 30970) **CAUTION** Sodium deoxycholate is harmful if swallowed or absorbed through the skin. It may cause respiratory and eye irritation. Wear gloves and avoid breathing dust.
- truChIP chromatin shearing kit (Covaris, SKU: 520155)

**SCAR-seq reagents**
- (Optional for isolation of parental strands; Step 118) Acetic acid (100% (vol/vol); EMD Millipore, cat. no. 100056) **CAUTION** Acetic acid is a flammable liquid and vapor. Keep away from open flames. It causes severe skin burns and eye damage. Work in a chemical hood and wear a laboratory coat and gloves.
- Biotin-TEG-azide (Berry & Associates, cat. no. BT1085) **CRITICAL** We do not recommend picolyl-azide-PEG4-biotin for SCAR-seq.
- Calcium chloride dihydrate (Sigma-Aldrich, cat. no. 1023820250) **CAUTION** Calcium chloride dihydrate is an eye irritant. Wear gloves when handling.
- Dynabeads M-280 sheep anti-mouse IgG (Thermo Fisher Scientific, cat. no. 11201D)
- Dynabeads M-280 sheep anti-rabbit IgG (Thermo Fisher Scientific, cat. no. 11203D)
- HEPES (Sigma-Aldrich, cat. no. H4034)
- MgCl₂ (Sigma-Aldrich, cat. no. M8266) **CAUTION** MgCl₂ is a skin and eye irritant. Wear gloves when handling.
- (Optional for isolation of parental strands; Step 118) Millipore Amicon Ultra-0.5, nominal molecular weight limit (NMWL) 10 kDa (Millipore, cat. no. UFC501096)
- MNase (Worthington, cat. no. LS004797) **CRITICAL** Unit definitions may differ if the enzyme is purchased from a different manufacturer, and additional optimizations may be necessary.
- KCl (Sigma-Aldrich, cat. no. P9333)
- NaOH (VWR, cat. no. 28226.293) **CAUTION** Sodium hydroxide is corrosive and toxic if it comes in contact with skin; always wear a laboratory coat and gloves.
- Sucrose for molecular biology (Sigma-Aldrich, cat. no. S0389)
Equipment

Common equipment

- 2100 Bioanalyzer instrument (Agilent, cat. no. G2939BA)
- BD FACS Calibur (BD Biosciences, discontinued)
- Brand Bürker counting chambers (Fisher Scientific, cat. no. 718905)
- Cell scrapers (TPP, cat. no. 9903)
- CELLSTAR polypropylene tubes, conical bottom, 15 ml (Greiner Bio-One, cat. no. 188271)
- CELLSTAR polypropylene tubes, conical bottom, 50 ml (Greiner Bio-One, cat. no. 227261)
- Centrifuge 5418 R (Eppendorf, cat. no. 5401000010)
- Counting chambers: KOVA Glasstic slide 10 with counting grids (KOVA International, cat. no. 87144)
- DiaMag Rotator (Diagenode, cat. no. B050000001)
- DNA LoBind tubes, 1.5 ml (Eppendorf, cat. no. 022431021)
- DNA LoBind tubes, 2.0 ml (Eppendorf, cat. no. 00300000876)
- DynaMag-2 magnet (Invitrogen, cat. no. 12321D)
- DynaMag-PCR magnet (Invitrogen, cat. no. 492025)
- Eppendorf Dualfilter T.I.P.S. LoRetention (50–1,000 μl) (Eppendorf, cat. no. EP00300000786)
- Eppendorf ThermoMixer C (Eppendorf, cat. no. 5382000015)
- Heracell 150i CO incubator with copper chambers (Thermo Scientific, cat. no. 11636250)
- Integra Biosciences Pipetboy Accu 2 pipette controller (Fisher Scientific, cat. no. 10798252)
- Multifuge X4R Pro (Thermo Scientific, cat. no. 75095915)
- NextSeq 500/550 high-output kit v2.5 (Illumina, cat. no. 20024906)
- PCR tubes, 0.2 ml, flat cap (Thermo Fisher Scientific, cat. no. AB0620)
- Proflex PCR system (Thermo Scientific, cat. no. 4484073)
- Qubit 4 fluorometer (Thermo Fisher Scientific, cat. no. Q33238)
- Rotapure (Rotapure Lab Instruments)
- Sorenson low-binding aerosol barrier tips, MicroGuard G, maximum volume 10 μl (Sigma-Aldrich, cat. no. Z719374)
- Sorenson low-binding aerosol barrier tips, MultiGuard, maximum volume 200 μl (Sigma-Aldrich, cat. no. Z719447)
- Sorenson low-binding aerosol barrier tips, MultiGuard, maximum volume 20 μl (Sigma-Aldrich, cat. no. Z719412)
- Vortex-Genie 2 (Scientific Industries, cat. no. SI-A256)

ChOR-seq equipment

- M220 Focused-ultrasonicator (Covaris, SKU: 500295)
- milliTUBE 1 ml AFA fiber (Covaris, SKU: 520135)
- Thermostatically controlled cabinet TC 140 G (Lovibond, cat. no. 2438210)
- Unimax 1010 orbital shaker (Heidolph, cat. no. 543-12310-00)

SCAR-seq equipment

- BD Microlance stainless steel needles, 21G (BD, cat. no. 304432)
- Plastic syringe (1 ml) (Terumo, cat. no. SS-01H1)

Software

- fetchChromsizes (University of California, Santa Cruz Genome Browser, https://scicrunch.org/resolver/SCR_005780): http://hgdownload.cse.ucsc.edu/admin/exe/linux.x86_64/
Reagent setup

▲ CRITICAL Stock solutions commonly used in laboratory protocols are prepared following standard molecular biology recipes66 and http://cshprotocols.cshlp.org/site/recipes/nav_s.dtl.

Cell culture of mESCs

0.2 % (wt/vol) gelatin. Dissolve 2 g of gelatin in 1 liter of ddH2O by heating at 50 °C. Filter-sterilize and store at 4 °C for ≤1 month.

DMEM-serum-LIF medium. Mix 500 ml of DMEM medium with 88 ml of FBS, 5 ml of penicillin-streptomycin, 5.8 ml of MEM non-essential amino acids solution (100×), 3.5 µl of 2-mercaptethanol and 1 ml of LIF. Filter-sterilize and store at 4 °C for ≤2 weeks. Prewarm to 37 °C before use.

2i medium. Mix 500 ml of ‘2i SILAC’ medium with 2.5 ml of 100× N-2 supplement, 5 ml of 50× B-27 supplement, 5 ml of penicillin-streptomycin, 3.5 µl of 2-mercaptoethanol, 1 ml of LIF, 1 ml of 500× l-arginine, 1 ml of 500× l-lysine, 50 µl of 10,000× MEK1/2 inhibitor and 100 µl of 5,000× GSK3 inhibitor. Filter-sterilize and store at 4 °C for ≤2 weeks. Prewarm to 37 °C before use.

500× l-arginine. Dissolve 577.7 mg of l-arginine hydrochloride in 10 ml of ddH2O. Filter-sterilize, prepare 1-ml aliquots and store at −20 °C for ≤2 years.

500× l-lysine. Dissolve 592.2 mg of l-lysine hydrochloride in 10 ml of ddH2O. Filter-sterilize, prepare 1-ml aliquots and store at −20 °C for ≤2 years.

GSK3 inhibitor 5,000× (15 mM). Dissolve 5 mg of GSK3 inhibitor (CHIRON99021) in 716 µl of dimethyl sulfoxide (DMSO). Prepare 100-µl aliquots and store at −20 °C for ≤1 year.

MEK1/2 inhibitor 10,000× (10 mM). Dissolve 5 mg of MEK1/2 inhibitor (PD0325901) in 1.04 ml of DMSO. Prepare 50-µl aliquots and store at −20 °C for ≤1 year.

Culture medium for D. melanogaster S2-DRSC cells

Refer to the following protocol from Drosophila Genomics Resource Center: https://dgrc.bio.indiana.edu/include/file/TissueCultureMedium.pdf.

Common reagents for ChOR-seq and SCAR-seq

100 mM CuSO4. Dissolve 100 mg in 6.27 ml of ddH2O. Divide into aliquots and store at 4 °C for ≤1 year.

1 M sodium ascorbate. Dissolve 200 mg in 1.01 ml of ddH2O. Divide into aliquots and store at −20 °C for ≤1 year. Each time, prepare a fresh working solution of 100 mM by mixing 10 µL of 1 M stock with 90 µL of ddH2O. ▲ CRITICAL Discard and prepare fresh if the solution has turned yellow.

20 mM EdU. Dissolve 25 mg in 4.956 ml of DMSO. Divide into aliquots and store at −20 °C for ≤1 year.

2× BWT buffer. Prepare as outlined below. 2× BWT buffer can be stored at room temperature (RT; 22 °C) for ≤6 months.
1× BWT buffer. Mix 25 ml of 2× BWT buffer with 25 ml of ddH₂O. This can be stored at RT for ≤6 months.

50 mM THPTA. Dissolve 100 mg in 4.603 ml of ddH₂O. Divide into aliquots and store at 4 °C for ≤1 year.

80% (vol/vol) ethanol. Mix 8 ml of absolute ethanol with 2 ml of ddH₂O. ▲CRITICAL Prepare fresh each time.

AMPure XP beads. Divide bead solution into 2-ml aliquots (can be done in advance) and store at 4 °C up to the expiration date specified on the bottle. ▲CRITICAL It is critical to equilibrate the AMPure XP beads at RT (≥22 °C) for ≥30 min before use for an optimal size selection.

Elution buffer (EB). Prepare as outlined below. Store at RT for ≤1 year.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris-HCl, pH 8.5</td>
<td>1 M Tris-HCl, pH 8.5</td>
<td>0.5</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>≤50</td>
</tr>
</tbody>
</table>

Elution buffer with Tween 20 (EBT). Prepare as outlined below. Store at RT for ≤1 year.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris-HCl, pH 8.5</td>
<td>1 M Tris-HCl, pH 8.5</td>
<td>0.5</td>
</tr>
<tr>
<td>0.05% (vol/vol) Tween 20</td>
<td>10% (vol/vol) Tween 20</td>
<td>0.25</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>≤50</td>
</tr>
</tbody>
</table>

NGS indexed PentAdapters. Dissolve 750 pmol of adapters in 50 µl of ddH₂O to get a concentration of 15 µM. Store at −20 °C for ≤1 year. Adjust concentration when required by following the recommendations below:

<table>
<thead>
<tr>
<th>Starting material (ng)</th>
<th>Adapter stock concentration</th>
<th>Adapter/insert molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>15 µM</td>
<td>100:1</td>
</tr>
<tr>
<td>50</td>
<td>15 µM</td>
<td>200:1</td>
</tr>
<tr>
<td>25</td>
<td>7.5 µM</td>
<td>200:1</td>
</tr>
<tr>
<td>10</td>
<td>3 µM</td>
<td>200:1</td>
</tr>
<tr>
<td>5</td>
<td>1.5 µM</td>
<td>200:1</td>
</tr>
<tr>
<td>2.5</td>
<td>750 nM</td>
<td>200:1</td>
</tr>
<tr>
<td>1</td>
<td>300 nM</td>
<td>200:1</td>
</tr>
</tbody>
</table>
Prepare as outlined below. Store at RT for ≤1 year.

### ChOR-seq Reagents

**10% (wt/vol) sodium deoxycholate.** Dissolve 5 g into 50 ml of ddH₂O. Store at RT for ≤1 year protected from light.

**10 mM thymidine (1,000×).** Dissolve 25 mg in 10.32 ml of DMSO. Divide into aliquots and store at −20 °C for ≤1 year.

*(Optional) 100 mM aminoguanidine.* Dissolve 100 mg in 13.5 ml of ddH₂O. Divide into aliquots and store at −4 °C for ≤1 year.

**100 mM picolyl-azide-PEG₄-biotin.** Dissolve 100 mg in 1.61 ml of DMSO. Store at 4 °C for ≤1 year.

### Dialysis Buffer

Prepare as outlined below. The dialysis buffer can be stored at RT for 1 month. Sterilize by filtration. Before use, supplement with 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin.

### High-salt RIPA Buffer

Prepare as outlined below. High-salt RIPA buffer can be stored at RT for 1 month. Sterilize by filtration. Before use, supplement with 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin.

### Incubation Buffer

Prepare as outlined below. Incubation buffer can be stored at RT for 1 month. Sterilize by filtration. Before use, supplement with 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin.
<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>350 mM NaCl</td>
<td>5 M NaCl</td>
<td>3.5</td>
</tr>
<tr>
<td>10 mM Tris-HCl, pH 8.0</td>
<td>1 M Tris-HCl, pH 8.0</td>
<td>0.5</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-</td>
<td>≤50</td>
</tr>
</tbody>
</table>

**LiCl wash buffer.** Prepare as outlined below. LiCl wash buffer can be stored at RT for 1 month. Sterilize by filtration. Before use, supplement with 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 mM LiCl</td>
<td>5 M LiCl</td>
<td>2.5</td>
</tr>
<tr>
<td>10 mM Tris-HCl, pH 8.0</td>
<td>1 M Tris-HCl, pH 8.0</td>
<td>0.5</td>
</tr>
<tr>
<td>1 mM EDTA, pH 8.0</td>
<td>0.5 M EDTA, pH 8.0</td>
<td>0.1</td>
</tr>
<tr>
<td>0.50% (vol/vol) IGEPAL CA-630</td>
<td>20% (vol/vol) IGEPAL CA-630</td>
<td>1.25</td>
</tr>
<tr>
<td>0.50% (wt/vol) sodium deoxycholate</td>
<td>10% (wt/vol) sodium deoxycholate</td>
<td>2.5</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-</td>
<td>≤50</td>
</tr>
</tbody>
</table>

**Low-salt RIPA buffer.** Prepare as outlined below. Low-salt RIPA buffer can be stored at RT for 1 month. Sterilize by filtration. Before use, supplement with 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>140 mM NaCl</td>
<td>5 M NaCl</td>
<td>1.4</td>
</tr>
<tr>
<td>10 mM Tris-HCl, pH 8.0</td>
<td>1 M Tris-HCl, pH 8.0</td>
<td>0.5</td>
</tr>
<tr>
<td>1 mM EDTA, pH 8.0</td>
<td>0.5 M EDTA, pH 8.0</td>
<td>0.1</td>
</tr>
<tr>
<td>1% (vol/vol) Triton X-100</td>
<td>10% (vol/vol) Triton X-100</td>
<td>5</td>
</tr>
<tr>
<td>0.10% (wt/vol) SDS</td>
<td>20% (wt/vol) SDS</td>
<td>0.25</td>
</tr>
<tr>
<td>0.10% (wt/vol) sodium deoxycholate</td>
<td>10% (wt/vol) sodium deoxycholate</td>
<td>0.5</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-</td>
<td>≤50</td>
</tr>
</tbody>
</table>

**SCAR-seq reagents**

100 mM biotin-TEG azide. Dissolve 25 mg in 0.562 ml of DMSO. Store at 4 °C for ≤1 year.

**Alkaline wash buffer**

▲**CRITICAL** Prepare fresh each time from 10 M NaOH stock.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM NaOH</td>
<td>10 M NaOH</td>
<td>0.1</td>
</tr>
<tr>
<td>0.05% (vol/vol) Tween-20</td>
<td>10% (vol/vol) Tween-20</td>
<td>0.05</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-</td>
<td>≤10</td>
</tr>
</tbody>
</table>

**Buffer A.** Prepare as outlined below. Buffer A can be stored at 4 °C for 1 month. Sterilize by filtration.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM HEPES, pH 7.9</td>
<td>0.5 M HEPES, pH 7.9</td>
<td>1</td>
</tr>
<tr>
<td>10 mM KCl</td>
<td>2 M KCl</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Buffer D. Prepare as outlined below. Buffer D can be stored at 4 °C for 1 month. Sterilize by filtration. Before use, supplement with 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 mM MgCl₂</td>
<td>1 M MgCl₂</td>
<td>0.075</td>
</tr>
<tr>
<td>0.34 M sucrose</td>
<td>1 M sucrose</td>
<td>17</td>
</tr>
<tr>
<td>10% (vol/vol) glycerol</td>
<td>100% (vol/vol) glycerol</td>
<td>5</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>≤50</td>
</tr>
</tbody>
</table>

ChIP low-salt washing buffer. Prepare as outlined below. ChIP low-salt washing buffer can be stored at 4 °C for 1 month. Sterilize by filtration. Before use, supplement with 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM HEPES, pH 7.9</td>
<td>0.5 M HEPES, pH 7.9</td>
<td>2</td>
</tr>
<tr>
<td>0.2 mM EDTA, pH 8.0</td>
<td>0.5 M EDTA, pH 8.0</td>
<td>0.2</td>
</tr>
<tr>
<td>250 mM KCl</td>
<td>2 M KCl</td>
<td>6.25</td>
</tr>
<tr>
<td>20% (vol/vol) glycerol</td>
<td>100% (vol/vol) glycerol</td>
<td>10</td>
</tr>
<tr>
<td>0.20% (vol/vol) Triton X-100</td>
<td>10% (vol/vol) Triton X-100</td>
<td>1</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>≤50</td>
</tr>
</tbody>
</table>

ChIP high-salt washing buffer. Prepare as outlined below. ChIP high-salt washing buffer can be stored at 4 °C for 1 month. Sterilize by filtration. Before use, supplement with 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (ml) for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris-HCl, pH 8.0</td>
<td>1 M Tris-HCl, pH 8.0</td>
<td>1</td>
</tr>
<tr>
<td>2 mM EDTA, pH 8.0</td>
<td>0.5 M EDTA, pH 8.0</td>
<td>0.2</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>5 M NaCl</td>
<td>1.5</td>
</tr>
<tr>
<td>1% (vol/vol) Triton X-100</td>
<td>10% (vol/vol) Triton X-100</td>
<td>5</td>
</tr>
<tr>
<td>0.10% (wt/vol) SDS</td>
<td>20% (wt/vol) SDS</td>
<td>0.25</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>≤50</td>
</tr>
</tbody>
</table>

ChIP elution buffer. Prepare as outlined below. ChIP elution buffer can be stored at RT for 1 year.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock concentration</th>
<th>Volume (µl) for 1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris-HCl, pH 8.0</td>
<td>1 M Tris-HCl, pH 8.0</td>
<td>20</td>
</tr>
<tr>
<td>10 mM EDTA, pH 8.0</td>
<td>0.5 M EDTA, pH 8.0</td>
<td>20</td>
</tr>
</tbody>
</table>
MNase (Worthington). Dissolve a vial in ddH2O to a final concentration of 50 U/µl. Make single-use aliquots of 20 µl and store at −20 °C for ≤3 years. ▲CRITICAL Test the MNase digestion efficiency for each new batch. ▲CRITICAL Avoid multiple freeze–thaw cycles of MNase and use a new aliquot each time.

**Procedure 1: ChOR-seq**

**Chromatin preparation for spike-in ● Timing 39 h**

1. Culture *D. melanogaster* S2 cells following general procedures for maintenance of *D. melanogaster* cell lines from the Drosophila Genomics Resource Center.  
   ▲CRITICAL STEP A typical ChOR-seq experiment requires ~2–5 × 10⁵ S2 cells per condition. However, we recommend growing a big batch of S2 cells (2–5 × 10⁶ cells) and freezing them in small aliquots of 2 × 10⁷ cells after EdU labeling, fixation and sonication (Procedure 1, Step 38). S2 cells can also be grown in spinner flasks, which is a convenient way to work with large volumes of cultured cells. We recommend to grow and label *D. melanogaster* S2 cells well in advance of starting the mammalian cell cultures.

2. Label the nascent chromatin of S2 cells by transferring 10 ml of medium from the culture to a 15-ml conical tube and add EdU (20 mM stock solution) to have a final concentration of 10 µM (e.g., 100 µl for 200 ml of culture). Mix by inverting the tube multiple times and pour 10 ml of EdU-containing medium back to the spinner flask. Incubate at 25 °C for 38 h.  
   ▲CRITICAL STEP It is important to label cells long enough to ensure that the whole genome will be labeled with EdU. The doubling time for S2 cells is 33–38 h in our hands.

**Troubleshooting**

3. Transfer cells from the spinner flask to 50-ml conical tubes and centrifuge for 5 min at RT and 300g. Discard the supernatant.

4. Wash the cells thoroughly with an equivalent volume of ice-cold 1× PBS to stop the EdU pulse. Centrifuge for 5 min at RT and 300g. Discard the supernatant.

5. Proceed to fixation and sonication following the manufacturer’s instructions for truChIP chromatin shearing kit (suspension cells, high cell protocol) instead of Steps 12–23 of Procedure 1. Then, proceed to Steps 24–39 of Procedure 1, which are equally applicable to E14 mESCs and *D. melanogaster* S2 cells.

   ■PAUSE POINT Aliquots can be stored at −80 °C for ≤1 year.

**Culture of adherent cell lines ● Timing 2–7 d**

7. Culture E14 mESCs in DMEM supplemented with 15% (vol/vol) FBS and LIF as previously described.

8. Seed 4 × 10⁶ cells in a 150-mm gelatin-coated plate with 20 ml of medium and grow them for 48 h at 37 °C and 5% CO₂. Prepare one 150-mm plate per ChIP reaction.  
   ▲CRITICAL STEP This protocol describes the procedure for performing ChOR-seq on E14 mESCs. However, we anticipate that it applies to any other proliferating adherent mammalian cell line. We have also performed ChOR-seq on cells in suspension (human Hela S3 cells; see Supplementary Methods and Supplementary Table 1 for details of cell culture, EdU labeling, synchronization and chromatin shearing).

9. 1 d before harvesting, replace culturing medium with fresh prewarmed medium.

**EdU labeling ● Timing 30 min**

10. Transfer 10 ml of the medium from each plate to a 50-ml tube and add 10 µl of 20 mM stock solution of EdU. Mix by inverting the tube multiple times and pour 10 ml of medium containing EdU back into each plate. The final concentration of EdU is 10 µM. Incubate plates at 37 °C for the desired amount of time.
CRITICAL STEP Labeling time must be optimized, because it will determine the resolution and yield of the experiment (see 'Experimental design').

CRITICAL STEP To keep the labeling time consistent between the plates, it is recommended to add and remove the EdU-containing medium in the same order and at a fixed time interval (e.g., 30 s to 1 min) between plates.

TROUBLESHOOTING

To analyze newly replicated chromatin, follow option A. To perform a time course of chromatin maturation, follow option B.

A) For analysis of newly replicated chromatin
   (i) Rapidly aspirate the medium and add 10 ml of ice-cold 1× PBS to stop the EdU pulse. When processing several plates at the same time, they can be stored at 4 °C after adding the PBS until the last plate is processed.
   (ii) Proceed to fixation (Steps 12–23).

B) For time-course analyses of chromatin maturation
   (i) Rapidly aspirate the medium and wash the plates twice with 20 ml of PBS at RT and add 20 ml of fresh prewarmed medium containing 10 µM thymidine (add 1 µl of 10 mM thymidine per ml of culture medium) to chase the EdU pulse.
   (ii) Incubate at 37 °C and chase for the desired amount of time before proceeding to fixation (Steps 12–23).

CRITICAL STEP For long time courses, we recommend replacing medium containing 10 µM thymidine with fresh prewarmed medium after 1 or 2 h.

CRITICAL STEP To avoid cell loss due to the detaching of cell colonies from the plate, always use PBS at RT and carefully pipette medium and washing solutions on the side of the culture dish.

(iii) For quality control of EdU incorporation, proceed to Box 1.

Fixation • Timing 1.5–2 h

CRITICAL The following steps describe how to perform crosslinking for mESCs following the manufacturer’s instructions for the Covaris truChIP chromatin shearing kit (adherent cells, high cell protocol) with minor modifications.

12 Freshly prepare solutions from the Covaris truChIP chromatin shearing kit for the appropriate number of plates being processed. All reagents are included in the kit, except for 1× PBS and ddH₂O.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per 150-mm plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ice-cold 1× PBS</td>
<td>25 ml per plate. Store on ice</td>
</tr>
<tr>
<td>1× fixing buffer A</td>
<td>7 ml per plate</td>
</tr>
<tr>
<td>Fresh 11.1% (wt/vol) formaldehyde</td>
<td>Mix 700 µl of fixing buffer A with 6.3 ml of ddH₂O</td>
</tr>
<tr>
<td>Quenching buffer E</td>
<td>Place in a 55 °C water bath to dissolve crystals and then place at RT</td>
</tr>
</tbody>
</table>

CRITICAL STEP Always use fresh methanol-free formaldehyde to achieve reproducible results.

13 Aspirate medium from the plate and wash once with 5 ml of ice-cold 1× PBS.

14 Aspirate 1× PBS and add 7 ml of 1× fixing buffer A at RT.

15 Add 700 µl of freshly prepared 11.1% (wt/vol) formaldehyde solution to produce a final concentration of 1% (wt/vol) and start timing the crosslinking reaction.

16 Place the plate on a shaking platform at RT for the required time. See 'Experimental design' for advice on fixation time.

17 Quench the crosslinking reaction by adding 420 µl of quenching buffer E to the plate. Place the plate on a shaking platform at RT for 5 min.

CRITICAL STEP Caution should be taken to keep the exact fixation time. To keep the fixation time exact to the recommended time, we advise adding formaldehyde solution and quenching buffer E to plates in the same order with a fixed time interval (30 s to 1 min) between plates.

18 Completely aspirate the solution from the plate.
Add 5 ml of ice-cold 1× PBS to the plate and scrape cells into a 15-ml conical tube. Keep on ice. If you are growing multiple plates for the same condition, combine cells from two plates in one 50-ml tube before proceeding to the next step.

Add 5 ml of ice-cold 1× PBS to the plate to collect the remaining cells and transfer them to the same conical tube from the previous step.

Centrifuge for 5 min at 4 °C and 200g. Discard the supernatant.

Wash cell pellets twice with 5 ml of ice-cold 1× PBS per plate and collect by centrifugation for 5 min at 4 °C and 200g.

Completely aspirate the supernatant from the tube. Keep the tubes on ice until proceeding to nuclei preparation.

**PAUSE POINT** Fixed cell pellets can be snap-frozen in liquid nitrogen and stored at −80 °C for a few days before nuclei preparation.

**Nuclei preparation**

**Timing 1.5–2 h**

**Critical** Nuclei preparation is performed following the manufacturer’s instructions for the truChIP chromatin shearing kit (adherent cells, high cell protocol) with minor modifications as described below.

Freshly prepare solutions from the Covaris truChIP chromatin shearing kit for the appropriate number of tubes being processed. All reagents are included in the kit, except for ddH₂O.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1× lysis buffer B</td>
<td>2.0 ml per plate. Store on ice</td>
</tr>
<tr>
<td></td>
<td>Mix 400 µl of 5× lysis buffer B with 1.6 ml of ddH₂O</td>
</tr>
<tr>
<td></td>
<td>Add 20 µl of 100× buffer F</td>
</tr>
<tr>
<td>1× wash buffer</td>
<td>2.0 ml per plate. Store on ice</td>
</tr>
<tr>
<td></td>
<td>Mix 200 µl of 10× wash buffer C with 1.8 ml of ddH₂O</td>
</tr>
<tr>
<td></td>
<td>Add 20 µl of 100× buffer F</td>
</tr>
<tr>
<td>1× shearing buffer D3</td>
<td>4 ml per plate. Store on ice</td>
</tr>
<tr>
<td></td>
<td>Mix 400 µl of 10× shearing buffer D3 with 3.6 ml of ddH₂O</td>
</tr>
<tr>
<td></td>
<td>Add 40 µl of 100× buffer F</td>
</tr>
</tbody>
</table>

Add 2 ml of 1× lysis buffer B to crosslinked cell pellets (from Step 23) from each plate to lyse the cell membrane and gently resuspend by pipetting up and down four times. If cells were frozen after formaldehyde fixation, thaw cells on ice first.

Transfer the solution to a 2-ml tube and incubate for 10 min on a rocker at 4 °C.

Centrifuge for 5 min at 4 °C and 1,700g to collect the intact nuclei. Decant the supernatant without disturbing the nuclei pellet.

Gently resuspend the nuclei pellet in 2 ml of 1× wash buffer C and incubate for 10 min on a rocker at 4 °C.

Centrifuge for 5 min at 4 °C and 1,700g to collect the intact nuclei. Decant the washing solution without disturbing the nuclei pellet.

Gently rinse the sides of the tube with 1 ml of 1× shearing buffer D3 by slowly dispensing the buffer around the inner wall of the tube, taking care not to disturb the nuclei pellet.

Centrifuge for 5 min at 4 °C and 1,700g to collect the intact nuclei. Decant the supernatant without disturbing the nuclei pellet.

Repeat Steps 30 and 31 an additional time. Carefully remove and discard the supernatant without disturbing the nuclei pellet.

Resuspend the nuclei pellet in 1 ml of 1× shearing buffer D3.

**PAUSE POINT** Nuclei suspension aliquots in shearing buffer D3 can be snap-frozen in liquid nitrogen and stored at −80 °C for ≤1 year. To reduce thawing time, we recommend freezing them in small volumes (e.g., 200 µl) and adding the remaining shearing buffer after thawing.

Before sonication, make a 1:100 dilution in shearing buffer D3 (2 µl of nuclei and 198 µl of 1× shearing buffer D3). Load 10 µl of the diluted nuclei suspension in the chamber of a Kova glastic slide or a hemocytometer and count nuclei manually. Because the nuclei from mESCs are very small, we found manual counting more reproducible than counting with an automated counter.
Chromatin shearing ● Timing 25 min per sample
35 Dilute the nuclei suspension to a concentration of 1.5–2 × 10^7/ml with 1× shearing buffer D3.
  ▲ CRITICAL STEP Do not exceed the concentration of 1.5–2 × 10^7 nuclei/ml when using a Covaris sonicator M220. A higher concentration of nuclei will result in inconsistent chromatin shearing.
36 Shear chromatin using a Covaris M220 with the following settings: duty cycle: 10% intensity; 200 cycles/burst; processing time: 20 min; bath temperature: 7 °C; and water level: full.
  ▲ CRITICAL STEP These settings were applied to E14 mESCs and D. melanogaster S2 cells to obtain fragments between 100 and 500 bp. The maximum number of nuclei per milliliter must be determined empirically for each cell line or when using a different model of Covaris sonicator. Follow the manufacturer’s instructions for optimization of shearing conditions.
37 Centrifuge for 10 min at 4 °C and 14,000g.
38 Transfer the supernatant containing the soluble chromatin fraction to a new 1.5-ml tube and keep it on ice.
  ■ PAUSE POINT Sheared chromatin can be stored at 4 °C for ≤2 d or snap-frozen in liquid nitrogen and stored at −80 °C for 1 year. Avoid multiple freeze–thaw cycles because this can reduce immunoprecipitation efficiency and reproducibility.
39 Transfer 25 μl of sheared chromatin to a PCR tube and proceed to quality control of chromatin shearing (Box 2).

ChIP: preabsorption and antibody incubation ● Timing 2 h and overnight incubation
40 Prepare protein A or protein G agarose beads (50:50 slurry), pooling 30 μl per ChIP reaction in a 1.5-ml tube.
  ▲ CRITICAL STEP The binding properties of protein A and protein G differ between species and subclasses of IgG. We, therefore, recommend carefully checking the binding strength of your antibody to protein A or protein G. Protein A has better binding capacity for rabbit, pig, dog and cat IgG. Protein G is generally preferred for a broad range of mouse and human IgG subclasses.
41 Prepare separate protein A or protein G agarose beads (50:50 slurry, 30 μl per ChIP reaction) for preabsorption.
42 Centrifuge beads from Steps 40 and 41 for 2 min at 4 °C and 1,000g. Discard the supernatant with a 200-μl filter tip.
  ▲ CRITICAL STEP Take care not to transfer the beads with the supernatant.
43 Wash once with an equivalent volume of low-salt RIPA buffer.
44 Centrifuge for 3 min at 4 °C and 1,000g. Carefully remove as much buffer as possible, taking care not to disturb the bead pellet.
45 For beads for preabsorption, resuspend beads in 100 μl of low-salt RIPA buffer per reaction.
46 For beads for ChIP, resuspend beads for all reactions in a total volume of 10 ml of low-salt RIPA buffer and add 100 μl of 100 mg/ml BSA per reaction. Incubate overnight on a rotating platform in a cold room.
47 For each immunoprecipitation, take the equivalent of 30 μg of genomic DNA of soluble chromatin from Step 38. See Box 2, step A: ‘Quantification of genomic DNA and quality control of chromatin shearing (ChOR-seq)’.
  ▲ CRITICAL STEP To improve material recovery, we recommend performing all subsequent steps using low-binding filter tips and low-binding tubes.
48 Mix 30 μg of chromatin with 0.05% (mass/mass of DNA) D. melanogaster S2 chromatin.
  ▲ CRITICAL STEP The amount of D. melanogaster S2 chromatin must be optimized depending on the protein/modification analyzed. Refer to ‘Experimental design’ for guidelines about spike-in normalization.
49 Adjust volume to 500 μl with dialysis buffer supplemented with protease inhibitors.
50 Add 400 μl of incubation buffer supplemented with protease inhibitors.
51 Add 100 μl of beads for preabsorption (from Step 45) and incubate for 1 h on a rotating platform in a cold room.
52 Centrifuge for 2 min at 4 °C and 1,000g. Transfer the supernatant to a new 1.5-ml low-binding tube.
53 Transfer 10 μl of supernatant to a new PCR tube and keep at −20 °C until proceeding to crosslinking reversal and DNA purification (Steps 66–71). This is the input sample.
54 Add an appropriate amount of antibody to each tube and incubate overnight on a rotating platform in a cold room. For histone modifications, we use 8–10 μg of antibody for 30 μg of chromatin.
ChIP: antibody capture and washes  ● Timing 5–7 h

55 Centrifuge beads for ChIP from Step 46 for 2 min at 4 °C and 1,000g. Carefully discard the supernatant with a 200-μl filter tip and resuspend in 100 μl of low-salt RIPA buffer per ChIP reaction.

56 Mix by pipetting and transfer 100 μl of bead suspension to each reaction from Step 54.

57 Incubate for 3 h on a rotating platform in a cold room.

58 Centrifuge for 2 min at 4 °C and 1,000g.

▲ CRITICAL STEP Keep the tubes on ice during the washes while adding and removing washing buffers. When working with multiple tubes, we use a Rotapure rotator, or an analogous system, allowing the mixing and centrifuging of samples in batch to save time. All washing buffers must be supplemented with protease inhibitors (see ‘Reagent setup’).

▲ CRITICAL STEP Carefully discard the supernatant after each wash using a 1,000-μl filter tip, taking care not to disturb the bead pellet.

59 Rinse the beads twice with 500 μl of low-salt RIPA buffer. Centrifuge for 2 min at 4 °C and 1,000g and discard the supernatant.

60 Resuspend the beads in 500 μl of low-salt RIPA buffer and incubate in a rotating platform for 5 min in a cold room. Centrifuge for 2 min at 4 °C and 1,000g and discard the supernatant.

61 Resuspend the beads in 500 μl of high-salt RIPA buffer and incubate in a rotating platform for 5 min in a cold room. Centrifuge for 2 min at 4 °C and 1,000g and discard the supernatant.

62 Repeat Step 61 two more times.

63 Resuspend the beads in 500 μl of LiCl wash buffer and incubate in a rotating platform for 5 min in a cold room. Centrifuge for 2 min at 4 °C and 1,000g and discard the supernatant.

64 Resuspend the beads in 500 μl of TE 1× and incubate in a rotating platform for 5 min in a cold room. Centrifuge for 2 min at 4 °C and 1,000g and discard the supernatant.

65 Repeat Step 64 one more time.

66 Resuspend beads from Step 65 in 100 μl of TE 1× and transfer to a PCR tube. Take out the input samples from Step 53 stored at −20 °C. Add 90 μl of TE 1× to each of the input samples. Then, continue with Step 67, processing ChIP and input samples in parallel.

67 Add 5 μl of RNase A (10 mg/ml). Incubate for 30 min at 37 °C in a thermocycler.

68 Add 1 μl of proteinase K (10 mg/ml), 2.5 μl of 20% (wt/vol) SDS and 2 μl of 5 M NaCl. Incubate for 10 h at 37 °C followed by a 6-h incubation at 65 °C to reverse crosslinking.

■ PAUSE POINT Use a PCR thermocycler with a heated lid for this incubation, adding an infinite hold at 4 °C at the end, and continue with the protocol the following day.

69 Purify DNA using the MinElute reaction cleanup kit following the manufacturer’s instructions.

70 Elute twice in 25 μl of MinElute EB buffer and combine eluates in one tube.

71 Use 1 μl to measure DNA concentration using a Qubit fluorometer and following the manufacturer’s instructions for the Qubit dsDNA HS assay kit.

■ PAUSE POINT Samples can be stored at this point at −20 °C for 1 year.

? TROUBLESHOOTING

Quality control of immunoprecipitated DNA  ● Timing 1.5 h

72 Use 1 μl of purified DNA to check the size distribution of the immunoprecipitated material using a high-sensitivity DNA kit for Bioanalyzer.

▲ CRITICAL STEP We strongly recommend checking the size distribution of immunoprecipitated fragments when performing ChIP with a new antibody. This control can be omitted when working with antibodies that have been already optimized for ChIP and do not exhibit preferential enrichment for large DNA fragments.

? TROUBLESHOOTING

Library preparation: end repair and A-tailing (KAPA Hyperprep kit)  ● Timing 1.5 h

73 Assemble each end repair and A-tailing reaction in a PCR low-binding tube on ice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoprecipitated DNA</td>
<td>X</td>
</tr>
<tr>
<td>PCR-grade ddH₂O</td>
<td>≤50</td>
</tr>
</tbody>
</table>

Table continued
CRITICAL STEP It is important to start with the same amount of DNA for all ChOR-seq samples within the same experiment to ensure a similar number of reads for all samples that will be directly compared, because they will be pooled in a forthcoming step before amplification. Refer to ‘Experimental design’ for advice on the minimum amount of immunoprecipitated DNA to generate ChOR-seq libraries. Using too little material will result in low-complexity libraries and a high proportion of PCR duplicates.

Mix by pipetting.

Incubate in a thermocycler programmed as outlined in the table below.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>End repair and A-tailing</td>
<td>20</td>
<td>30 min</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>30 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

CRITICAL STEP A heated lid is required for this incubation. If possible, set the temperature of the lid at 65 °C instead of the usual ~105 °C.

Proceed immediately to the next step.

Library preparation: adapter ligation (KAPA Hyperprep kit) • Timing 1.5 h

Dilute NGS indexed PentAdapters stocks to the appropriate concentration (see ‘Reagent setup’).

CRITICAL STEP Use different indexes for the different samples. Refer to the Illumina TruSeq guidelines for the optimal strategy for pooling indexed libraries for multiplexing.

In the same tubes in which end repair and A-tailing was performed, assemble each adapter ligation reaction as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>End repair and A-tailing reaction product</td>
<td>60</td>
</tr>
<tr>
<td>Adapter stock (concentration as required)</td>
<td>5</td>
</tr>
<tr>
<td>PCR-grade water</td>
<td>5</td>
</tr>
<tr>
<td>Ligation buffer</td>
<td>30</td>
</tr>
<tr>
<td>DNA ligase</td>
<td>10</td>
</tr>
<tr>
<td>Total volume</td>
<td>110</td>
</tr>
</tbody>
</table>

Mix thoroughly by pipetting and centrifuge briefly.

Incubate in a thermocycler with an opened lid programmed as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adapter ligation</td>
<td>20</td>
<td>60 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

CRITICAL STEP To achieve higher ligation rates and library yields, particularly for low-input samples, consider increasing the ligation time—to a maximum of 4 h at 20 °C or overnight at 4 °C.
Please note that longer ligation times may lead to increased levels of adapter-dimers. Adapter concentration may have to be optimized if ligation times are extended significantly. ■ PAUSE POINT Samples can be stored at 4 °C at this point overnight.

Library preparation: post-ligation cleanup ● Timing 1 h
81 Equilibrate AMPure XP beads at RT for ≥30 min before use.
82 Transfer the ligation from Step 80 to a 1.5-ml DNA low-binding tube and bring to RT for 5 min.
83 Add 88 µl of AMPure XP beads (ratio: 0.8×) and mix thoroughly by vortexing and spin down briefly. Incubate the tubes at RT for 10 min to bind DNA to the beads.
84 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear. Carefully remove and discard the supernatant without disturbing the beads.
85 Keeping the tubes on the magnet, add 200 µl of freshly prepared 80% (vol/vol) ethanol.
86 Incubate the tubes on the magnet at RT for ≥30 s.
87 Carefully remove and discard the ethanol.
88 Repeat Steps 85–87 one more time.
89 Remove all residual ethanol without disturbing the beads.
▲ CRITICAL STEP Do not let the beads dry, because it will result in irreversible DNA binding to the beads.
90 Remove the tubes from the magnet and resuspend the beads in 15 µl of PCR-grade water.
▲ CRITICAL STEP We recommend using PCR-grade water for elution and not any solution containing EDTA, TRIS or other copper-chelating compounds, because they will interfere with the click reaction.
91 Incubate the open tubes in a ThermoMixer for 5 min at 37 °C to elute DNA off the beads and evaporate the residual ethanol. Cover the ThermoMixer with a clean lid or a piece of aluminum foil to protect the tubes from dust.
92 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.
93 Carefully transfer 14.5 µl of the supernatant to a new 1.5-ml low-binding tube.
■ PAUSE POINT Samples can be stored at −20 °C until proceeding with the next step.

Click biotinylation ● Timing 45 min–1 h
▲ CRITICAL These steps are for EdU-labeled samples. For unlabeled samples, proceed directly to size selection (Steps 97–115).
94 Pool together adapter-ligated DNA from all different EdU-labeled samples from the same time-course experiment into one low-binding tube before performing the click reaction.
▲ CRITICAL STEP If the total volume after pooling the samples exceeds 73.4 µl, scale up the click reaction accordingly.
95 Set up the click reaction by adding the following reagents sequentially to the tubes from Step 94:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl) per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>H2O</td>
<td>≤73.4</td>
<td>-</td>
</tr>
<tr>
<td>10× Click-it buffer (or 10× PBS, pH 7.5)</td>
<td>10</td>
<td>1×</td>
</tr>
<tr>
<td>100 mM picolyl-azide-PEG4-biotin</td>
<td>0.5</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>100 mM aminoguanidine (optional)</td>
<td>5</td>
<td>5 mM</td>
</tr>
<tr>
<td>50 mM THPTA</td>
<td>1</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>100 mM CuSO4</td>
<td>0.1</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>100 mM sodium ascorbate</td>
<td>10</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

▲ CRITICAL STEP The THPTA and CuSO4 should be premixed and added in a single pipetting step.
▲ CRITICAL STEP For an optimal click reaction, it is important to strictly follow the order stated above when adding the reagents and use a freshly prepared solution of sodium ascorbate.
96 After adding sodium ascorbate, mix immediately by pipetting and incubate for 30–45 min at RT.
▲ CRITICAL STEP Do not vortex the tubes; mix by pipetting and respect the optimal reaction temperature. If the room temperature is low (<22 °C), place the reactions in a thermoblock at 25 °C without mixing.)
Double size selection  ● Timing 1.5 h

97 Equilibrate AMPure XP beads at RT for ≥30 min before use.
98 For the upper size-selection cutoff (0.56:1), add 56 µl of AMPure XP beads to 100 µl of click reaction and mix thoroughly by vortexing.

▲ CRITICAL STEP Scale-up if using a higher volume for the click reaction.
99 Incubate at RT for 10 min to bind the unwanted, large DNA fragment molecules to the beads.

▲ CRITICAL STEP Using a ratio of 0.56:1, the fragments larger than 700 bp will bind to the beads whereas smaller fragments will remain in the supernatant.
100 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.

▲ CRITICAL STEP Using a ratio of 0.56:1, the fragments larger than 700 bp will bind to the beads whereas smaller fragments will remain in the supernatant.
101 Carefully transfer 156 µl of supernatant containing unbound smaller DNA fragments to a new tube.

▲ CRITICAL STEP Take care not to transfer the beads with the supernatant, because the beads bind the undesired large fragments.
102 Discard the tubes containing the remaining beads.
103 For the lower size-selection cutoff (0.85:1), add 29 µl of AMPure XP beads to 156 µl of the supernatant from the first bead selection and mix thoroughly by vortexing.

▲ CRITICAL STEP Using a ratio of 0.85:1, the fragments larger than 200 bp will bind to the beads whereas smaller fragments will remain in the supernatant.
104 Perform a quick spin of the tubes and incubate at RT for 10 min to bind the desired DNA fragments to the beads.
105 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.
106 Carefully remove and discard the supernatant with a 200-µl filter tip.
107 Keeping the tubes on the magnet, add 200 µl of freshly prepared 80% (vol/vol) ethanol.
108 Incubate the tubes on the magnet at RT for ≥30 s.
109 Carefully remove and discard the ethanol with a 200-µl filter tip.
110 Repeat Steps 107–109 one more time.
111 Remove all residual ethanol without disturbing the beads.

▲ CRITICAL STEP Do not let the beads dry, because it will result in irreversible DNA binding to the beads.
112 Remove the tubes from the magnet and thoroughly resuspend the beads in 21 µl of EB.
113 Incubate the open tubes in a ThermoMixer for 5 min at 37 °C to elute DNA off the beads and evaporate the residual ethanol. Cover the ThermoMixer with a clean lid or a piece of aluminum foil to protect the tubes from dust.
114 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.
115 Transfer 20 µl of the supernatant containing size-selected DNA to a new 1.5-ml low-binding tube.

Streptavidin capture of biotinylated library fragments  ● Timing 1–1.5 h

116 Resuspend the stock of MyOne T1 streptavidin Dynabeads by gentle vortexing.
117 Pipette 10 µl of the bead suspension per reaction into a 1.5-ml low-binding tube. Place the tube on the magnet to capture the beads. Incubate until the liquid is clear.
118 Remove and discard the supernatant with a 200-µl filter tip.
119 Remove the tube from the magnet and add 200 µl of 1× BWT buffer; mix by pipetting.
120 Place the tube on the magnet to capture the beads. Incubate until the liquid is clear.
121 Remove and discard the supernatant with a 200-µl filter tip.
122 Repeat Steps 119–121 two more times.
123 Remove the tube from the magnet and resuspend the beads in 20 µl of 2× BWT buffer per reaction.
124 Add 20 µl of the washed bead suspension into the tube containing the purified ligation reaction from Step 115 and mix by pipetting.
125 Incubate the tube on a rotating platform at 15–20 rpm for 30 min at RT.

▲ CRITICAL STEP Do not quick-spin the samples with the beads before incubation. Beads must remain in suspension throughout the incubation time. Because of the small volume, sideways rotation of the tube is preferred rather than inversion.
126 Spin the tube briefly in a microcentrifuge and place the tube on the magnet to capture the beads.
127 Remove and discard the supernatant with a 200-µl filter tip.

▲ CRITICAL STEP Process one sample at a time and proceed immediately to the next step to avoid overdrying the beads.
128 Remove the tubes from the magnet, add 200 µl of 1× BWT buffer and mix thoroughly by pipetting with a 200-µl low-binding filter tip. Transfer the entire volume to a new 1.5-ml low-binding tube.
129 Place the tube on the magnet to capture the beads. Incubate until the liquid is clear. Remove and discard the supernatant with a 200-µl filter tip.
130 Repeat washing steps (128–129) three more times with 200 µl of 1× BWT buffer without transferring the beads to a new tube.
131 Add 200 µl of EBT and mix thoroughly by pipetting.
132 Place the tube on the magnet to capture the beads. Incubate until the liquid is clear. Remove and discard the supernatant.
133 Resuspend the beads in 20 µl of EB and transfer them to a new low-binding PCR tube and place it on ice.

**PAUSE POINT**  Bead suspensions can be stored for 1–2 d at 4 °C or for several months at −20 °C.

**Library amplification (KAPA Hyperprep kit)**  ● **Timing 1–1.5 h**
134 Assemble each library amplification reaction in a low-binding PCR tube as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2× KAPA HiFi HotStart ReadyMix</td>
<td>25</td>
</tr>
<tr>
<td>10× KAPA library amplification primer mix</td>
<td>5</td>
</tr>
<tr>
<td>Bead suspension with the bound adapter-ligated library</td>
<td>20</td>
</tr>
<tr>
<td>Total volume</td>
<td>50</td>
</tr>
</tbody>
</table>

135 Mix thoroughly by pipetting with a 200-µl low-binding filter tip.
136 Amplify using the following cycling protocol:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>98</td>
<td>45 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>15 s</td>
<td>11</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
<td>-</td>
</tr>
</tbody>
</table>

**CRITICAL STEP**  It is important to use a minimal number of amplification cycles to minimize the generation of PCR duplicates. For unlabeled samples that have not been subjected to click biotinylation or streptavidin pull-down, the number of PCR cycles should be reduced to five to seven.

**PAUSE POINT**  Store the tubes at 4 °C or −20 °C for ≤72 h, or proceed directly to Post-amplification cleanup (below).

**Post-amplification cleanup**  ● **Timing 1 h**
137 Equilibrate AMPure XP beads at RT for ≥30 min before use.
138 Place the PCR tube on a magnet compatible with PCR tubes to collect streptavidin beads and transfer the supernatant to a new 1.5-ml low-binding tube.
139 Wash streptavidin beads once in 200 µl of EBT, resuspend in 20 µl of EB and store at −20 °C for up to several months. Nascent DNA library amplification (Steps 134–139) can be performed one more time if necessary.
140 Perform a 0.8× solid-phase reversible immobilization (SPRI) cleanup to the supernatant that contains the amplified library by combining the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR reaction product</td>
<td>50</td>
</tr>
<tr>
<td>AMPure XP beads</td>
<td>40</td>
</tr>
<tr>
<td>Total volume</td>
<td>90</td>
</tr>
</tbody>
</table>
141 Mix thoroughly by vortexing.
142 Incubate the tubes at RT for 10 min to bind DNA to the beads.
143 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.
144 Carefully remove and discard the supernatant with a 200-µl filter tip.
145 Keeping the tubes on the magnet, add 200 µl of freshly prepared 80% (vol/vol) ethanol.
146 Incubate the tubes on the magnet at RT for ≥30 s.
147 Carefully remove and discard the ethanol with a 200-µl filter tip.
148 Repeat Steps 145–147.
149 Remove all residual ethanol without disturbing the beads.

▲ CRITICAL STEP Do not let the beads dry, because it will result in irreversible DNA binding to the beads.

150 Remove the tubes from the magnet and resuspend the beads in 21 µl of EB.
151 Incubate the open tubes in a ThermoMixer for 5 min at 37 °C to elute DNA off the beads and evaporate the residual ethanol. Cover the ThermoMixer with a clean lid or a piece of aluminum foil to protect the tubes from dust.
152 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.
153 Carefully transfer 20 µl of the supernatant to a new 1.5-ml DNA low-binding tube and proceed to quality control of libraries (Box 3).

▲ PAUSE POINT At this point, samples can be stored at −20 °C for ≤1 year.

? TROUBLESHOOTING

Sequencing and data analysis ● Timing Variable

▲ CRITICAL The ChOR-seq data analysis described in the following steps (Fig. 2a) is performed on single-end reads.

154 Sequence the pooled libraries on an Illumina next-generation sequencing platform in single or paired-end mode.
155 Upload demultiplexed FASTQ files to the Galaxy server (https://usegalaxy.org/)^68 and start the analysis evaluating sequencing quality by running the FASTQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) on the raw sequence data.
156 Trim the FASTQ files using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with default settings to remove low-quality reads.
157 Align the reads to a reference genome by Bowtie^69 using parameters -m1 -- best to obtain uniquely mapped reads. In the current protocol, we aligned reads to mm10 mouse genome assembly and dm3 D. melanogaster genome assembly.
158 Remove PCR duplicates using RmDup from SAMtools^70 in both alignment files.

? TROUBLESHOOTING

159 For the D. melanogaster Binary Alignment Map (BAM) file, count the total number of unique reads. Calculate the spike-in normalization factor for each sample by dividing 10^6 by the total number of unique reads from D. melanogaster.

? TROUBLESHOOTING

160 For the mouse alignment, convert BAM to Browser Extensible Data (BED) and extend the reads to the average size of sonicated chromatin using BAMtoBED and slopBed, respectively, from BEDtools^71. The average size of sonicated chromatin is determined by analysis on an Agilent Bioanalyzer (Box 2).
161 Generate 250-bp bins across the genome using fetchChromSizes to obtain genome sizes and BEDtools MakeWindows to create the bins.
162 To compute reference-adjusted reads per million (RRPM), calculate the coverage of each bin using BEDtools Genome Coverage function, adding in the -scale parameter the spike-in normalization factor calculated in Step 159. The output of this tool is a BedGraph file containing quantitative data that can be used for further data analysis and visualization.

? TROUBLESHOOTING

163 For peak calling, run MACS^72 with default parameters to identify peaks on the BAM files from Step 162, using input as a control file. For histone PTMs distributed in broad domains, we recommend using broad peak calling (--broad).
164 Alternatively, BAM files from Step 157 can be uploaded to SeqMonk (RRID:SCR_001913; https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/), a program that enables the visualization and analysis of mapped sequence data. We routinely use SeqMonk to extend reads and sum them in fix-size bins across the genome. We have successfully performed normalization to RRPM, visualization and quantitative analysis in SeqMonk. Besides, it is a very useful tool to filter data for custom-defined regions of interest such as peaks, as well as standard genomic features.
Procedure 2: SCAR-seq

Cell culture ● **Timing 3–7 d**

1. Culture E14 mESCs in 20 ml of 2i medium supplied with inhibitors, amino acids, LIF, 2-mercaptoethanol and penicillin-streptomycin as described previously. We recommend preparing two 150-mm plates at 70–80% confluence per condition, equivalent to ~1 × 10^8 cells.

   ▲ **CRITICAL STEP** This protocol describes the procedure to perform SCAR-seq on E14 mESCs. However, we anticipate that it applies to any other proliferating adherent mammalian cell line.

2. 1 d before harvesting, replace culturing medium with fresh prewarmed medium.

EdU labeling and cell harvesting ● **Timing 1.5 h**

3. Transfer 10 ml of the medium from each plate to a 50-ml tube and add 10 µl of 20 mM EdU stock solution. Mix by inverting the tube and add 10 ml of EdU-containing medium back to the plate. The final EdU concentration is 10 µM. Incubate plates at 37 °C for the desired amount of labeling time.

   ▲ **CRITICAL STEP** Labeling time must be optimized according to the histone PTM being analyzed because it will determine the resolution and yield of the experiment (see 'Experimental design').

4. Rapidly aspirate the medium and rapidly add 10 ml of ice-cold 1× PBS to immediately stop the EdU pulse.

   ▲ **CRITICAL STEP** To keep the labeling time consistent between the plates, it is recommended to add and remove the EdU-containing medium in the same order and at a fixed time interval (e.g., 30 s to 1 min) between plates.

5. Store the plates at 4 °C until all plates are processed.

6. Collect the cells by scraping with a clean cell scraper in a cold room and transfer the cell suspension to 50-ml conical centrifuge tubes. Keep the tubes on ice. To collect the remaining cells, rinse each plate with 10 ml of ice-cold 1× PBS and collect in the same 50-ml conical tubes. Centrifuge for 10 min at 4 °C and 300g. Discard the supernatant.

Isolation of nuclei ● **Timing 1.5 h**

7. Resuspend the cell pellet from two plates in 1.5 ml of buffer A. Centrifuge for 5 min at 4 °C and 1,300g. Discard the supernatant.

   ▲ **CRITICAL STEP** Supplement buffer A with protease inhibitors (1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin) before use. For analysis of histone acetylation, include trichostatin A at 1 µg/ml. For other types of histone modifications, for example, phosphorylation, add relevant inhibitors at recommended concentrations.

   ▲ **CRITICAL STEP** Volumes should be scaled accordingly when working with different numbers of cells.

8. Resuspend the pellet in 1 ml of buffer A.

9. Add 10 µl of 10% (vol/vol) Triton X-100 (final concentration: 0.1% (vol/vol)) and mix by inverting gently.

10. Lay the tube horizontally on ice for 7 min.

11. Centrifuge for 5 min at 4 °C and 1,300g. Discard the supernatant.

12. Resuspend the pellet in 1 ml of buffer A using a wide-orifice pipette tip. You can make the wide-orifice tips yourself by cutting the tips with a clean scalpel or scissors before use.

13. Centrifuge for 5 min at 4 °C and 1,300g. Discard the supernatant.

14. Resuspend the pellet in 1 ml of buffer A using a wide-orifice tip and distribute two 500-µl aliquots into two 1.5-ml tubes.

15. To count nuclei, prepare a 1:100 dilution in buffer A (1 µl of nuclei and 99 µl of buffer A). Load 10 µl of the diluted nuclei suspension on a Kova glassic slide or hemocytometer and count nuclei manually. In our hands, we found that manual counting of nuclei was more reproducible than counting with an automated counter.

   ▲ **CRITICAL STEP** For accurate counting, it is important to have a homogeneous suspension of intact nuclei.

   ■ **PAUSE POINT** At this step, 500-µl nuclei suspension aliquots can be snap-frozen in liquid nitrogen and stored at −80 °C for ≤1 year.
**MNase digestion and chromatin preparation ● Timing 4–6 h**

16 Thaw nuclei suspension on ice.
17 Pre-warm the nuclei suspension in a ThermoMixer for 5 min at 30 °C and 300 rpm.
18 Add 5 µl of 100 mM CaCl₂ to 500 µl of nuclei suspension and mix by inverting the tube several times.
19 Add an appropriate amount of MNase and immediately mix by vortexing very briefly.

▲ **CRITICAL STEP** The amount of MNase should be empirically determined as described in ‘Experimental design’ and Box 2. For example, for mESCs grown in 2i medium, we used 0.4 µl (20 U) of Worthington MNase (50 U/µl) per 1 × 10⁷ nuclei. It is critical to store MNase in small aliquots and use a new aliquot each time.

20 Immediately place the tube in a ThermoMixer for exactly 20 min, at 30 °C and 300 rpm.
21 Place the tube on ice and stop digestion by immediately adding 10 µl of a premixed 1:1 solution of 0.1 M EGTA (pH 8.0) and 0.5 M EDTA (pH 8.0). Mix by inverting the tube several times.

▲ **CRITICAL STEP** To keep the digestion time consistent between samples, we recommend adding MNase and stopping digestion in the same order, and allowing a fixed interval of time (e.g., 30 s to 1 min) between tubes.

22 Add 5 µl of 10% (vol/vol) Triton X-100 and 75 µl of 2 M KCl. Mix immediately by inverting the tube several times.

23 Add protease inhibitors to the tubes (5 µl of 100 mM PMSF, 0.5 µl of leupeptin, 0.5 µl of pepstatin and 0.5 µl of aprotinin (1 mg/ml each)). Mix by inverting.

▲ **CRITICAL STEP** Add deacetylase inhibitor trichostatin A at 1 mg/ml for the experiments with histone acetylation marks or other appropriate inhibitors when relevant (see ‘Experimental design’).

24 Pass through a 21-gauge needle attached to a 2-ml syringe (up and down 10 times) in a cold room to facilitate resuspension of nuclei and release of digested chromatin.

25 Incubate the tubes by rotating at 20 rpm in a cold room for 2–4 h to solubilize the chromatin.
26 Centrifuge for 10 min at 4 °C and 14,000g.
27 Transfer the supernatant containing the soluble chromatin fraction to a new 1.5-ml tube and keep on ice. Keep the pellet and perform the quality control of the MNase digestion and purification of the input DNA (Box2).

▲ **CRITICAL STEP** The native chromatin cannot be stored or frozen. Proceed immediately with native ChIP.

**? TROUBLESHOOTING**

**Native ChIP: antibody incubation ● Timing 1 h and overnight incubation**

28 For each immunoprecipitation, take an equivalent of 50 µg of DNA (Box 2) of the soluble chromatin from Step 27. Adjust the volume to 500 µl with buffer D. For the ‘no antibody’ control, take 20 µg of chromatin and adjust to 200 µl with buffer D.

29 Add 16 µg of antibody per 50 µg of chromatin. Do not add antibody to the ‘no antibody’ control.

▲ **CRITICAL STEP** The amount of antibody may need to be optimized depending on the antibody and the protein/PTM of interest.

30 Incubate overnight on a rotating platform in a cold room.

**Native ChIP: antibody capture, washes and elution ● Timing 3.5 h including 2-h incubation**

31 Prepare 83 µl of IgG Dynabeads anti-rabbit (or anti-mouse, or other relevant beads, depending on the antibody) per ChIP reaction and 50 µl for the ‘no antibody’ control. Wash beads three times with 500 µl of buffer D. For each wash, incubate for 1 min in a rotating platform at 18 rpm in a cold room at 4 °C. Collect beads on the magnet and discard the supernatant. Resuspend beads in buffer D (83 µl per ChIP reaction plus 50 µl for each ‘no antibody’ control).

32 Add 83 µl of prewashed anti-rabbit (or anti-mouse) IgG Dynabeads from Step 31 to each ChIP reaction from Step 30 and 50 µl to each ‘no antibody’ control. Incubate for 2–3 h on a rotating platform at 18 rpm and 4 °C.

33 Place the tubes on a magnetic rack placed on ice to collect the beads. Remove and discard the supernatant with a 1,000-µl tip.

34 Resuspend the beads in 500 µl of ice-cold ChIP low-salt washing buffer and transfer the suspension to a new DNA low-binding tube prechilled on ice.

35 Bring the tubes to a cold room, invert them several times to gently resuspend the beads and incubate on a rotating platform for 5 min. Collect beads on the magnet and discard the supernatant with a 1,000-µl tip. Place the tubes on ice.
36 Repeat Steps 34 and 35 two more times, except for the transfer step to a new tube.
37 Add 500 µl of ice-cold ChIP high-salt washing buffer to the tubes. Bring the tubes to the cold room, invert them several times to gently resuspend the beads and incubate on a rotating platform for 5 min.
38 Place the tubes on the magnet and remove and discard the supernatant with a 1,000-µl tip. Place the tubes on ice.
39 Repeat Steps 37 and 38 two more times.

▲CRITICAL STEP For optimal recovery of immunoprecipitated chromatin, we recommend performing all subsequent steps using low-binding filter tips and DNA low-binding tubes.

40 Add 100 µl of ChIP elution buffer to each tube to elute the immunoprecipitated chromatin.
41 Incubate in a ThermoMixer for 15 min at 37 °C and 1,400 rpm.
42 Collect beads on the magnet and transfer the supernatant to a new 1.5-ml low-binding tube.
43 Repeat Steps 40–42 and combine the supernatants from the two elutions in one tube.
44 Purify DNA using the MinElute reaction cleanup kit, adding 600 µl of ERC buffer to 200 µl of the sample.
45 Load 400 µl of the mixture on the MinElute column and centrifuge for 30 s at RT and 14,000 g.
46 Discard the flow-through, load the remaining 400 µl of the sample to the same column and centrifuge for 30 s at RT and 14,000 g.
47 Discard the flow-through and proceed to the washing step with PE buffer according to the manufacturer’s protocol.
48 Place the column in a 1.5-ml low-binding tube with a cut cap and elute the purified DNA by adding 25 µl of MinElute EB buffer to the center of the column membrane. Let the column stand for 1 min and centrifuge for 30 s at RT and 14,000 g.
49 Add again 25 µl of MinElute EB buffer to the center of the column membrane without changing the collection tube. Let the column stand for 1 min and centrifuge for 30 s at RT and 14,000 g. Now, the purified ChIP DNA is eluted in 50 µl of MinElute EB buffer.

■PAUSE POINT At this point, samples can be stored at −20 °C for ≤1 year.

Quality control of immunoprecipitated DNA ▶ Timing 1.5 h
50 Use 0.5 µl of ChIP DNA and 20 µl of ‘no antibody’ control to measure DNA concentration with a Qubit fluorometer and following the manufacturer’s instructions for the Qubit dsDNA HS assay kit. No DNA should be detected in the ‘no antibody’ control.

▲CRITICAL STEP It is important to check the specificity and the yield of the ChIP. Refer to ‘Experimental design’ for advice on the minimum amount of purified DNA to generate SCAR-seq libraries. Proceeding with non-optimal-sized fragments or a low amount of DNA may result in low-complexity libraries.

? TROUBLESHOOTING
51 Use 1 µl of purified DNA to check the size distribution of the immunoprecipitated material using a high-sensitivity DNA kit for Bioanalyzer.

▲CRITICAL STEP We strongly recommend checking the size distribution of immunoprecipitated fragments when performing ChIP with a new antibody. This control can be omitted when working with antibodies that have already been optimized for ChIP and do not exhibit preferential enrichment for large DNA fragments.

? TROUBLESHOOTING

Double size selection I: removal of large DNA fragments ▶ Timing 1 h
52 Equilibrate AMPure XP beads at RT for ≥30 min before use.
53 From this step, include the input samples (Box 2, step B(iv)) in parallel with the ChIP samples. For the input, transfer 10 µl of the purified DNA from step iv (Box 2, step B) to a new 1.50-ml low-binding tube and add 40 µl of EB.
54 For the upper size-selection cutoff (0.8:1), add 40 µl of AMPure XP beads to 50 µl of DNA and mix thoroughly by vortexing.

▲CRITICAL STEP Using a ratio of 0.8:1, the fragments larger than 350 bp will bind to the beads, whereas smaller fragments will remain in the supernatant.
55 Perform a quick spin of the tubes and incubate at RT for 10 min to bind the unwanted, large DNA fragments to the beads.
56 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.
57 Carefully transfer 90 µl of the supernatant to a new tube.  
**CRITICAL STEP** Avoid transferring the beads with the supernatant, because the undesired large fragments are bound to the beads.

58 Discard the tubes containing the beads.

59 For the lower size-selection cutoff (3:1), add 110 µl of AMPure XP beads to 90 µl of the supernatant from Step 57 and mix thoroughly by vortexing.  
**CRITICAL STEP** Using a ratio of 3:1, the desired fragments will bind to the beads.

60 Perform a quick spin of the tubes and incubate at RT for 10 min to bind DNA fragments to the beads.

61 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.

62 Carefully remove and discard the supernatant with a 200-µl filter tip.

63 Keeping the tubes on the magnet, add 200 µl of freshly prepared 80% (vol/vol) ethanol.

64 Incubate the tubes on the magnet at RT for 30 s.

65 Carefully remove and discard the ethanol with a 200-µl filter tip.

66 Repeat Steps 63–65 one more time.

67 Remove all residual ethanol without disturbing the beads.  
**CRITICAL STEP** Do not let the beads dry, because it will result in irreversible DNA binding to the beads.

68 Remove the tubes from the magnet and thoroughly resuspend the beads in 80 µl of PCR-grade water.  
**CRITICAL STEP** We recommend using PCR-grade water for elution but not any solution containing EDTA, TRIS or other copper-chelating compounds, because they will interfere with the click reaction.

69 Incubate the open tubes in a ThermoMixer for 5 min at 37 °C to elute DNA off the beads and to evaporate the residual ethanol. Cover the ThermoMixer with a clean lid from a tip box or a piece of aluminum foil to protect the tubes from dust.

70 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.

71 Transfer 79 µl of clear supernatant containing size-selected DNA to a new 1.5-ml low-binding tube.  
**PAUSE POINT** At this point, samples can be stored at −20 °C for ≤1 year.

**TROUBLESHOOTING**

Click biotinylation  
**Timing 1 h**

72 Set up the click reaction by adding the following reagents sequentially to the tubes containing the size-selected DNA from Step 71.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl) per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>78.4</td>
<td>-</td>
</tr>
<tr>
<td>10× Click-it buffer (or 10× PBS, pH 7.5)</td>
<td>10</td>
<td>1×</td>
</tr>
<tr>
<td>100 mM biotin-TEG-azide</td>
<td>0.5</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>50 mM THPTA</td>
<td>1</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>100 mM CuSO₄</td>
<td>0.1</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>100 mM sodium ascorbate</td>
<td>10</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

**CRITICAL STEP** The THPTA and CuSO₄ should be premixed and added in a single pipetting step.

**CRITICAL STEP** For an optimal click reaction, it is important to strictly follow the order stated above when adding the reagents and use a freshly prepared solution of sodium ascorbate.

73 After adding sodium ascorbate, mix immediately by pipetting and incubate for 30–45 min at RT.  
**CRITICAL STEP** Do not vortex the tubes; mix by pipetting and respect the optimal reaction temperature. If the RT is low (<22 °C, place the reactions in a thermoblock at 25 °C without mixing).

74 Purify DNA using the MinElute reaction cleanup kit following the manufacturer’s instructions. To elute the purified DNA, place the column in a new low-binding 1.5-ml Eppendorf tube and elute the purified DNA by adding 25 µl of MinElute EB buffer to the center of the column membrane. Let the column stand for 1 min and centrifuge for 30 s at RT and 14,000g.
Add again 25 µl of MinElute EB buffer to the center of the column membrane without changing the collection tube. Let the column stand for 1 min and centrifuge for 30 s at RT and 14,000 g. Now, the purified DNA is eluted in 50 µl of MinElute EB buffer.

**PAUSE POINT** Samples can be stored at –20 °C at this point for ≤1 year.

**Library preparation: end repair and A-tailing (KAPA Hyperprep kit)**

Assemble each end repair and A-tailing reaction in a low-binding PCR tube on ice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated DNA (Step 75)</td>
<td>50</td>
</tr>
<tr>
<td>End repair &amp; A-tailing buffer</td>
<td>7</td>
</tr>
<tr>
<td>End repair &amp; A-tailing enzyme mix</td>
<td>3</td>
</tr>
<tr>
<td>Total volume</td>
<td>60</td>
</tr>
</tbody>
</table>

Timing 1.5 h

Mix by pipetting.

Incubate in a thermocycler programmed as outlined below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>End repair and A-tailing</td>
<td>20</td>
<td>30 min</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>30 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

▲ **CRITICAL STEP** A heated lid is required for this incubation. If possible, set the temperature of the lid to 65 °C or leave the lid open during the first step of incubation at 20 °C.

Proceed immediately to the next step.

**Library preparation: adapter ligation (KAPA Hyperprep kit)**

Dilute NGS indexed PentAdapters stocks to the appropriate concentration (see ‘Reagent setup’).

▲ **CRITICAL STEP** Use different indexes for the different samples. Refer to the Illumina TruSeq guidelines for the optimal strategy for pooling indexed libraries for multiplexing.

In the same tubes in which end repair and A-tailing was performed, assemble each adapter ligation reaction as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>End repair and A-tailing reaction product</td>
<td>60</td>
</tr>
<tr>
<td>Adapter stock</td>
<td>5</td>
</tr>
<tr>
<td>PCR-grade water</td>
<td>5</td>
</tr>
<tr>
<td>Ligation buffer</td>
<td>30</td>
</tr>
<tr>
<td>DNA ligase</td>
<td>10</td>
</tr>
<tr>
<td>Total volume</td>
<td>110</td>
</tr>
</tbody>
</table>

Mix thoroughly by pipetting and centrifuge briefly.

Incubate in a thermocycler with an open lid programmed as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adapter ligation</td>
<td>20</td>
<td>60 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

▲ **CRITICAL STEP** To achieve higher conversion rates, particularly for low-input samples, consider increasing the ligation time to a maximum of 4 h at 20 °C or overnight at 4 °C.
Adapter concentrations may need to be optimized if ligation times are extended significantly.

**PAUSE POINT** Samples can be stored at 4 °C at this point overnight.

**Library preparation: post-ligation cleanup** ● **Timing 1 h**

84 Equilibrate AMPure XP beads at RT for ≥30 min before use.
85 Transfer the ligation from Step 83 to a 1.5-ml low-binding tube and bring to RT for 5 min.
86 Add 88 µl of AMPure XP beads (ratio: 0.8×) and mix thoroughly by vortexing and spin down briefly.
87 Incubate the tubes at RT for 10 min to bind DNA to the beads.
88 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.
89 Carefully remove and discard the supernatant with a 200-µl filter tip without disturbing the beads.
90 Keeping the tubes on the magnet, add 200 µl of freshly prepared 80% (vol/vol) ethanol.
91 Incubate the tubes on the magnet at RT for 30 s.
92 Carefully remove and discard the ethanol with a 200-µl filter tip.
93 Repeat Steps 90–92 once.
94 Remove all residual ethanol without disturbing the beads.

▲ **CRITICAL STEP** Do not let the beads dry, because it will result in irreversible DNA binding to the beads.
95 Remove the tubes from the magnet and resuspend the beads in 20.5 µl of EB.
96 Incubate the open tubes in a ThermoMixer for 5 min at 37 °C to elute DNA off the beads and to evaporate the residual ethanol. Cover the ThermoMixer with a clean lid from a tip box or a piece of aluminum foil to protect the tubes from dust.
97 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.
98 Carefully transfer 20 µl of the supernatant to a new 1.5-ml low-binding tube. Keep on ice.

**Streptavidin capture of biotinylated library fragments** ● **Timing 1–1.5 h**

99 Resuspend the stock of MyOne T1 streptavidin Dynabeads by gentle vortexing.
100 Pipette 10 µl of the bead suspension per reaction into a 1.5-ml low-binding tube. Place the tube on the magnet to capture the beads. Incubate until the liquid is clear.
101 Remove and discard the supernatant with a 200-µl tip.
102 Remove the tube from the magnet and add 200 µl of 1× BWT buffer; mix by pipetting.
103 Place the tube on the magnet to capture the beads. Incubate until the liquid is clear.
104 Remove and discard the supernatant with a 200-µl filter tip.
105 Repeat Steps 102–104 two more times.
106 Remove the tube from the magnet and resuspend the beads in 20 µl of 2× BWT buffer per reaction.
107 Add 20 µl of the washed bead suspension into the tube containing the purified ligation reaction from Step 98 and mix by pipetting.
108 Incubate the tube on a rotating platform at 15–20 rpm for 30 min at RT.

▲ **CRITICAL STEP** Do not quick-spin the samples with the beads before incubation. Beads must remain in suspension throughout the incubation time. Because of the small volume, sideways rotation of the tube is preferred rather than inversion.
109 Spin the tube briefly in a microcentrifuge and place the tube on the magnet to capture the beads.
110 Remove and discard the supernatant with a 200-µl filter tip.

▲ **CRITICAL STEP** Process one sample at a time and proceed immediately to the next step to avoid overdrying the beads.
111 Remove the tubes from the magnet, add 200 µl of 1× BWT buffer and mix thoroughly by pipetting with a 200-µl low-binding filter tip. Transfer the entire volume to a new 1.5-ml low-binding tube.
112 Place the tube on the magnet to capture the beads. Incubate until the liquid is clear. Remove and discard the supernatant with a 200-µl filter tip.
113 Repeat washing steps (Steps 111 and 112) two more times with 200 µl of 1× BWT buffer without transferring the beads to a new tube.

**Strand separation and isolation of the newly synthesized strands** ● **Timing 30 min**

114 Resuspend the beads in 200 µl of 2× BWT buffer by pipetting with a 200-µl low-binding filter tip.
115 Place the tube on the magnet to capture the beads. Incubate until the liquid is clear. Remove and discard the supernatant.
Add 200 µl of alkaline wash buffer and mix thoroughly by pipetting with a 200-µl low-binding filter tip. Keep at RT for exactly 1 min. **CRITICAL STEP** It is critical to incubate exactly for 1 min and then place the tube immediately back on the magnet. The three alkaline washes should not exceed 5 min in total, including the time for bead capture on the magnet, because longer incubation may interfere with the biotin-streptavidin interaction and result in loss of DNA. We recommend not processing more than four tubes in parallel during this step, leaving a fixed interval of time (20–30 s) between the tubes.

Place the tube on the magnet to capture the beads and remove the supernatant with a 200-µl filter tip.

(Optional) If performing an analysis of the parental unlabeled strands, keep the supernatant of the first alkaline wash. Neutralize pH by adding 4 µl of 2.5 M acetic acid. Purify using Millipore Amicon columns (cutoff: 10 kD) following the manufacturer’s instructions. Proceed with PCR amplification in Step 126.

Repeat the alkaline wash (Steps 116 and 117) two more times and discard the supernatants.

Remove the tubes from the magnet, add 200 µl of 1× BWT buffer and mix thoroughly by pipetting with a 200-µl low-binding filter tip.

Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear. Remove and discard the supernatant.

Repeat Steps 120 and 121 once.

Add 200 µl of EBT and mix thoroughly by pipetting with a 200-µl low-binding filter tip.

Place the tube on the magnet to capture the beads. Incubate until the liquid is clear. Remove and discard the supernatant.

Resuspend the beads in 20 µl of EB and transfer to a new low-binding PCR tube and place it on ice. **PAUSE POINT** Bead suspensions can be stored for 1–2 d at 4 °C or ≤1 year at −20 °C.

### Nascent DNA library amplification (KAPA Hyperprep kit) ● Timing 1.5 h

Assemble each library amplification reaction in a low-binding PCR tube as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2× KAPA HiFi HotStart ReadyMix</td>
<td>25</td>
</tr>
<tr>
<td>10× KAPA library amplification primer mix</td>
<td>5</td>
</tr>
<tr>
<td>Bead suspension with the bound adapter-ligated library</td>
<td>20</td>
</tr>
<tr>
<td>Total volume</td>
<td>50</td>
</tr>
</tbody>
</table>

Mix thoroughly by pipetting with a 200-µl low-binding filter tip.

Amplify using the following cycling protocol:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>98</td>
<td>45 s</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>15 s</td>
<td>11</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

**CRITICAL STEP** It is important to use a minimal number of amplification cycles to minimize the generation of PCR duplicates. **PAUSE POINT** Store the tubes at 4 °C or −20 °C for ≤72 h, or proceed directly with Post-amplification cleanup and size selection II: isolation of mononucleosome-sized library inserts (below).

### Post-amplification cleanup and size selection II: isolation of mononucleosome-sized library inserts ● Timing 1.5 h

Equilibrate AMPure XP beads at RT for ≥30 min before use.

Place the PCR tube on a magnet compatible with PCR tubes to collect streptavidin beads and transfer the supernatant to a new 1.5-ml low-binding tube.
131 Wash streptavidin beads once in 200 µl of EBT, resuspend in 20 µl of EB and store at −20 °C for up to several months. Nascent DNA library amplification (Steps 126–128) can be performed one more time if necessary.

132 For the upper cutoff size selection (0.77:1), add 38.5 µl of AMPure XP beads to 50 µl of DNA and mix thoroughly by vortexing.

**CRITICAL STEP** At a bead ratio of 0.77:1, the undesired library fragments larger than 300 bp will bind to the beads, and the desired smaller fragments will remain in the supernatant.

133 Incubate at RT for 10 min to bind the unwanted, large DNA fragments to the beads.

134 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.

135 Carefully transfer 88.5 µl of the supernatant containing smaller DNA fragments to a new tube.

**CRITICAL STEP** Take care not to transfer the beads with the supernatant, because the beads bind the undesired large fragments. Discard the tubes containing the beads.

136 Prepare 3× concentrated AMPure XP beads for the second size selection step as follows. For each reaction, pipette 30 µl of AMPure XP beads to a new 1.5-ml low-binding tube. Place the tube on the magnet, incubate until the liquid is clear and remove 20 µl of the supernatant. Resuspend the beads in 10 µl of the remaining supernatant. The result is 3× concentrated AMPure XP beads.

**CRITICAL STEP** 3× concentration of AMPure XP beads for the second size selection increases the number of bead particles for easier subsequent collection/washing steps while preserving PEG/salt concentration in the buffer.

137 For the lower cutoff size selection (0.9:1), add 6.5 µl of 3× concentrated AMPure XP solution from Step 136 to 88.5 µl of the supernatant from the first size selection (Step 135) and mix thoroughly by vortexing.

**CRITICAL STEP** At a bead ratio of 0.9:1, the library fragments smaller than 300 bp but larger than 100 bp will bind to the beads, and the undesired smaller fragments and primers will remain in the supernatant.

138 Perform a quick spin of the tubes and incubate at RT for 10 min to bind the desired library fragments to the beads.

139 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.

140 Carefully remove and discard the supernatant.

141 Keeping the tubes on the magnet, add 200 µl of freshly prepared 80% (vol/vol) ethanol.

142 Incubate the tubes on the magnet at RT for 30 s.

143 Carefully remove the supernatant with a 200-µl filter tip and discard.

144 Repeat Steps 141–143 once.

145 Remove all residual ethanol without disturbing the beads.

**CRITICAL STEP** Do not allow the beads to dry, because it will result in irreversible DNA binding to the beads and decreased yield.

146 Remove the tubes from the magnet and resuspend the beads in 21 µl of EB.

147 Incubate the open tubes in a ThermoMixer for 5 min at 37 °C to elute DNA off the beads and to evaporate the residual ethanol. Cover the ThermoMixer with a clean lid from a tip box or a piece of aluminum foil to protect the tubes from dust.

148 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.

149 Carefully transfer 20 µl with size-selected DNA to a new 1.5-ml low-binding tube and proceed to quality control of libraries (Box 3).

**PAUSE POINT** At this point, samples can be stored at −20 °C for ≤1 year.

**TROUBLESHOOTING**

### Sequencing

150 Pool the mononucleosome-sized libraries for multiplexing according to standard Illumina protocols.

**CRITICAL STEP** Depending on the histone modification analyzed, distinct libraries may result in very different concentrations (e.g., H4K20me2 <1 ng/µl and H4K5ac >5 ng/µl). To achieve a precise pooling proportion of the libraries in the run, we recommend diluting the libraries to a similar range of concentration before calculating the molarity and pooling. For precise library loading, we also recommend checking the concentration and recalculating the molarity of the final pool. Library size is on average 280 bp. This is slightly shorter than the Illumina recommendation for
TruSeq sequencing; yet, for NextSeq500 and 550 series, we recommend loading a standard 1.8 pM denatured library pool for sequencing to achieve an optimal cluster density.

Sequence the pooled libraries on an Illumina NGS platform in single or paired-end mode.

**Data processing ● Timing Variable**

▲CRITICAL The SCAR-seq data analysis described in the following steps (Fig. 2b) is performed on single-end reads.

152 Download the stable release (v1.0.0) Replication_SCARseq-v1.0.0.zip from Zenodo at https://zenodo.org/record/4719235#.YN0mzhNudN0, containing the necessary Bash, Perl and R scripts for the following analysis steps. Alternatively, clone release v1.0.0 directly via: git clone --depth 1 --branch v1.0.0 (https://github.com/anderssonlab/Replication_SCARseq.git).

153 Map the raw reads from FASTQ files to reference mouse genome (mm10) using Burrows-Wheeler Aligner (BWA) aln version 0.7.13 with the following parameters (quality threshold = 5, seed length = 32, maximum differences in seed = 2 and MAPping Quality (MAPQ) = 30).

154 Mark and remove duplicated reads with Picard-tools (version 2.8.2) and mask out Encyclopedia of DNA Elements mm10 blacklist regions74.

? TROUBLESHOOTING

155 Compute the read counts by samtools flagstat (version 1.5) before and after removal of duplicated reads.

156 Compute genome coverage by normalizing to Reads Per Millions (RPM) using genomecov from bedtools setting in the -scale parameter a factor corresponding to the total number of reads divided by 1 million.

? TROUBLESHOOTING

157 Using the bam files from Step 154, split the single-end reads into forward and reverse strands according to the SAM flag, using samtools view (version 1.5) -F 20 and -f 16, respectively. Importantly, SAM flags should be changed when dealing with paired-end reads.

158 Convert bam files to bigWig using BEDtools GenomeCov with BEDgraph (-bg) and -g genome file set to mouse genome (mm10).

159 Generate non-overlapping bins of 1 kb across the genome using BEDtools MakeWindowsBed.

160 Count forward and reverse reads from Step 157 in 1-kb non-overlapping bins from Step 158 using bigWigAverageOverBed.

161 Normalize the stranded, aggregated read counts to counts per million on the basis of forward and reverse strands with a pseudocount of 1.

162 Calculate the partitioning score in 1-kb windows using the following formula: \[ \text{Partition} = \frac{(F - R)}{(F + R)} \]

\(F\) and \(R\) correspond to the number of mapped reads to the forward and the reverse strand, respectively.

163 To remove windows with low coverage, filter windows that contained a counts per million value >0.3. This filtered file will be used for data analysis and statistics.

164 The data can be smoothed for visualization purposes (optional) by using partition_smooth_find_-breakpoints.sh. Perform the smoothing across consecutive 1-kb bins by computing a uniform blur considering the neighboring 30 bins on each side. The bin sizes can be adjusted according to noise level and desired visual resolution.

**Evaluation of strand-specific enrichment of histone modifications over the strand-specific signal from input**

165 To deduct strand-specific input nucleosome partition signal and potential technical biases from SCAR-seq signal, SCAR-seq data are paired with an input sample to evaluate the signal above the input ratio for each genomic strand separately. Do this separately for the forward and reverse strands by feeding a pileup signal with fragment extension of 75 bp to MACS2 bdgcmp72 (version 2.1.1) and using the -m log10 likelihood (logLR) command.

166 Evaluate the logLR between the ChIP-enriched model and open chromatin model as instructed in replication_SCARseq.R: use the resulting logLR signal averaged across 1-kb windows to evaluate the partitioning skew significance. For this, only the regions with positive average logLR on either strand have to be tested in the same statistical tests as the SCAR-seq signals.
# Troubleshooting

Troubleshooting advice can be found in Table 2.

<table>
<thead>
<tr>
<th>Section</th>
<th>Procedure 1 (ChOR-seq) step</th>
<th>Procedure 2 (SCAR-seq) step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeling</td>
<td>2 and 10</td>
<td>3</td>
<td>The number of EdU-positive cells is lower than the number of cells expected to undergo S-phase (e.g., in ES14 60–80% of cells are expected to be in S-phase and EdU positive)</td>
<td>The click reaction did not work well</td>
<td>Check EdU concentration (10–20 μM)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Check the quality of reagents for the click reaction</td>
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<td></td>
<td>Check the cell cycle by FACS-PI staining</td>
</tr>
<tr>
<td>Chromatin fragmentation</td>
<td>39 and Box 2</td>
<td>27 and Box 2</td>
<td>Unexpected size distribution of DNA fragments</td>
<td>Suboptimal sonication or MNase set-up</td>
<td>Perform a pilot experiment for sonication or MNase treatment. For sonication, try changing cell concentration and time and eventually adjusting the intensity of energy transferred to the water bath to find the correct sonication conditions for your cells. For MNase digestion, try different incubation times and/or different MNase concentrations</td>
</tr>
<tr>
<td>ChIP</td>
<td>71</td>
<td>50</td>
<td>No or low DNA yield after ChIP</td>
<td>Overfixation of chromatin</td>
<td>Reduce fixation time</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Antibody incompatible with ChIP</td>
<td>Use a different antibody, preferably a validated ChIP-grade antibody</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>Not enough starting material</td>
<td>Check chromatin concentration and eventually start experiment with a higher number of cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Not enough antibody</td>
<td>Increase the amount of antibody up to 10 μg or according to the manufacturer’s recommendations</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>Incompatible antibody-bead combination</td>
<td>Refer to the manufacturer’s recommendation</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Problems with ChIP reagents and washing stringency</td>
<td>Try to use a different carrier of the secondary antibody</td>
</tr>
<tr>
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<td></td>
<td>DNA detected in ‘no antibody’ control</td>
<td>Include a positive control antibody to confirm that the procedure is working well</td>
</tr>
<tr>
<td></td>
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<td>The washing steps were not stringent enough</td>
<td>Use the ChIP protocol recommended by the antibody manufacturer</td>
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</tr>
<tr>
<td>Library preparation</td>
<td>153</td>
<td>149</td>
<td>No/low amount of DNA after library preparation</td>
<td>The click/streptavidin pull-down did not work</td>
<td>Perform a control on genomic DNA from cells continuously labeled with EdU</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Library amplification is inhibited because of an excess of magnetic beads</td>
<td>Reduce the amount of magnetic streptavidin beads used in the streptavidin pull-down</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>Ligation of adapters did not work</td>
<td>Perform qPCR control on the library before the click reaction and streptavidin pull-down (or on the supernatant of streptavidin pull-down) using primers for library amplification</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DNA is lost during size selection</td>
<td>Make sure to equilibrate the AMPure XP beads at RT before use and to use the correct bead concentration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low input material for PCR</td>
<td>Take the beads after Step 139 (ChOR-seq) or 131 (SCAR-seq) and repeat Steps 134–153 (ChOR-seq) or 126–149 (SCAR-seq)</td>
</tr>
</tbody>
</table>

Table continued
Table 2 (continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Procedure 1 (ChOR-seq) step</th>
<th>Procedure 2 (SCAR-seq) step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>153 and Box 3</td>
<td>149 and Box 3</td>
<td>Unexpected library size</td>
<td>Incorrect size selection</td>
<td>DNA degraded after the click reaction</td>
<td>Alternatively, increase the number of PCR cycles in Step 136 (ChOR-seq) or 131 (SCAR-seq)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enrichment in undesired large fragments (&lt;125 bp)</td>
<td>Make sure to equilibrate the AMPure XP beads at RT before use and adjust the ratio of beads according to the manufacturer’s instructions</td>
<td>Copper may induce degradation of DNA. We recommend checking the integrity of the DNA sample after the click reaction before and after the click reaction using an Agilent Bioanalyzer</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Primer dimers (80 bp)</td>
<td>Make sure not to transfer beads from the first size selection with the supernatant</td>
<td>Make sure not to transfer beads from the first size selection with the supernatant</td>
<td>Reduce the primer concentration for library amplification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adapter dimers (125 bp)</td>
<td>Perform an additional 1× SPRI cleanup</td>
<td>Perform an additional 1× SPRI cleanup</td>
<td>Reduce the adapter concentration</td>
</tr>
<tr>
<td>Sequencing and data analysis</td>
<td>158</td>
<td>154</td>
<td>PCR overamplification</td>
<td>Reduce the number of PCR cycles in Step 136 (ChOR-seq) and 128 (SCAR-seq)</td>
<td>Reduce the number of PCR cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High levels of PCR duplicates</td>
<td>Low library complexity</td>
<td>Alternatively, multiplex different samples and mix them at Step 94 (ChOR-seq)</td>
<td>Use the same amount of immunoprecipitated DNA when multiplexing samples that are obtained with the same antibody</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unequal distribution of reads between multiplexed samples in ChOR-seq</td>
<td>Multiplexing incorrect amount of chromatin per sample</td>
<td>Adapt the quantity of DNA to multiplex depending on the size of the fragments purified by each antibody. Small fragments will be amplified more efficiently than big ones</td>
<td>Adapt the quantity of DNA to multiplex depending on the size of the fragments purified by each antibody. Small fragments will be amplified more efficiently than big ones</td>
</tr>
<tr>
<td>162</td>
<td>156</td>
<td>Unexpected genomic distribution of signal/high background</td>
<td>High background during ChIP step</td>
<td>Troubleshoot the specificity of ChIP experiments according to standard ChIP protocols</td>
<td>Run the click reaction and streptavidin pull-down on 500 ng of fragmented genomic DNA derived from unlabeled cells. After the washes, perform the elution by heating the beads at 70 °C for 5 min in 10 µl of 95% (wt/vol) formamide and quantify the entire eluted volume with a Qubit single-stranded DNA kit (no DNA should be detected)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-specific click reaction</td>
<td>See also the ChIP troubleshooting section</td>
<td>See also the ChIP troubleshooting section</td>
<td>Run the click reaction and streptavidin pull-down on 500 ng of fragmented genomic DNA derived from unlabeled cells. After the washes, perform the elution by heating the beads at 70 °C for 5 min in 10 µl of 95% (wt/vol) formamide and quantify the entire eluted volume with a Qubit single-stranded DNA kit (no DNA should be detected)</td>
</tr>
<tr>
<td>Data analysis (spike-in normalization)</td>
<td>159</td>
<td>NA</td>
<td>Low number of reads aligning to mammalian genome</td>
<td>Reduce the amount of D. melanogaster chromatin that is mixed with mammalian chromatin at Step 48. See ‘Experimental design’ for guidance on spike-in optimization</td>
<td>Reduce the amount of D. melanogaster chromatin that is mixed with mammalian chromatin at Step 48. See ‘Experimental design’ for guidance on spike-in optimization</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low number of reads aligning to D. melanogaster genome</td>
<td>Excess of spike chromatin</td>
<td>Change the antibody. Use an antibody that reacts with the D. melanogaster epitope or use an antibody reacting with the D. melanogaster histone variant H2Av in parallel with the antibody specific for the protein analyzed</td>
<td>Change the antibody. Use an antibody that reacts with the D. melanogaster epitope or use an antibody reacting with the D. melanogaster histone variant H2Av in parallel with the antibody specific for the protein analyzed</td>
</tr>
</tbody>
</table>

NA, not applicable.
Timing

**Procedure 1: ChOR-seq**
Steps 1–6, chromatin preparation for spike-in: 39 h
Steps 7–9, culture of mESCs: 2–7 d
Steps 10 and 11, mESC EdU labeling: 30 min
Steps 12–23, fixation: 1.5–2 h
Steps 24–34, nuclei preparation: 1.5–2 h
Steps 35–39, chromatin shearing: 25 min per sample
Steps 40–54, ChIP—preabsorption and antibody incubation: 2 h and overnight incubation
Steps 55–71, ChIP—antibody capture and washes: 5–7 h, including a 3-h incubation
Step 72, quality control of immunoprecipitated DNA: 1.5 h
Steps 73–76, library preparation—end repair and A-tailing: 1.5 h
Steps 77–80, library preparation—adapter ligation: 1.5 h
Steps 81–93, library preparation—post-ligation cleanup: 1 h
Steps 94–96, click biotinylation (only for EdU-labeled samples; for unlabeled samples, proceed directly to size selection): 45 min–1 h
Steps 97–115, double size selection: 1.5 h
Steps 116–133, streptavidin capture of the biotinylated library fragments: 1–1.5 h
Steps 134–136, library amplification: 1–1.5 h
Steps 137–153, post-amplification cleanup: 1 h
Steps 154–166, sequencing and data processing: variable, depending on the sequencing platform used, number of analyzed samples, the computational skills of the user and the familiarity with the indicated software

**Procedure 2: SCAR-seq**
Steps 1 and 2, cell culture: 3–7 d
Steps 3–6, EdU labeling and cell harvesting: 1.5 h
Steps 7–15, isolation of nuclei: 1.5 h
Steps 16–27, MNase digestion and chromatin preparation: 4–6 h
Steps 28–30, native ChIP—antibody incubation: 1 h and overnight incubation
Steps 31–49, native ChIP—antibody capture, washes and elution: 3.5 h including a 2-h incubation
Steps 50 and 51, quality control of immunoprecipitated DNA: 1.5 h
Steps 52–71, double size selection I—removal of large DNA fragments: 1 h
Steps 72–75, click biotinylation: 1 h
Steps 76–79, library preparation—end repair and A-tailing: 1.5 h
Steps 80–83, library preparation—adapter ligation: 1.5 h
Steps 84–98, library preparation—post-ligation cleanup: 1 h
Steps 99–113, streptavidin capture of the biotinylated library fragments: 1–1.5 h
Steps 114–125, strand separation—isolation of newly synthesized strands: 30 min
Steps 126–128, library amplification: 1.5 h
Steps 129–149, post-amplification cleanup and size selection II—isolation of mononucleosome-sized fragment library inserts: 1.5 h
Steps 150–166, sequencing and data processing: variable, depending on the sequencing platform used, number of analyzed samples, the computational skills of the user and the familiarity with the indicated software

**Anticipated results**

**Chromatin preparation and fragmentation**
For ChOR-seq, correct chromatin preparation and shearing should result in fragments ranging from 100 to 500 bp. In SCAR-seq, the MNase digestion should render predominantly mononucleosome-sized fragments (~147 bp). Chromatin fragmentation is a critical step and needs to be controlled to achieve reproducible results (Box 2). In both methods, the presence of large fragments will negatively affect subsequent steps.
Fig. 3 | Representative results for ChOR-seq and SCAR-seq. a and b, Comparison of H3K27me3 ChOR-seq profiles at nascent (T0) and mature chromatin 10 and 24 h after EdU labeling (T10 and T24, respectively) in HeLa S3 cells normalized to reads per million (RPM, a) or to RRPM using exogenous spike-in chromatin from D. melanogaster S2 cells (RRPM, b). c, Representative SCAR-seq profiles showing RFD (OK-seq, purple) and partitioning of old (H4K20me2, orange) and new (H4K5ac, green) histones between sister chromatids in wild-type (WT) and MCM2-2A mutant cells. d, Average RFD and partition of old (H4K20me2) and new (H4K5ac) histones around initiation zones in WT (solid lines) and MCM2-2A (dashed lines) mESCs. Panels a and b show data from replicate 1 from ref. 15, whereas panel c shows data from ref. 11: H4K20me2 mESC wt_r3; H4K5ac mESC wt_r1, H4K20me2 mESC MCM2_2#1 r2, H4K5ac MCM2_2A#1 r2, OK-seq mESC wt_r1. Panel d is adapted from ref. 11 as permitted under the American Association for the Advancement of Science (AAAS) author license to publish and represents the averaged partitioning signal of all replicates.
ChIP
The amounts of immunoprecipitated DNA may differ, depending on the abundance of protein/PTM of interest, the quality of the antibody and the pull-down efficiency. A high yield is not always a sign of a good immunoprecipitation and may very well reflect high unspecific binding by the antibody. It is crucial to validate antibody specificity and control for the size of purified immunoprecipitated DNA because some antibodies preferentially capture large chromatin fragments, which may influence downstream steps and compromise reproducibility.

Library preparation
It is critical to control for the fragment size of the libraries. The expected average size of a ChOR-seq library should be 400–500 bp, whereas in SCAR-seq, an average size of 278 bp is expected for a mononucleosome-sized library (Box 3). Abundant fragments of ~130 bp usually reflect primer dimers that should be removed before sequencing, because they can subtract a significant portion of sequencing reads from the desired library fragments. Conversely, enrichment in large fragments poses the opposite challenge, because they will be inefficiently sequenced.

Sequencing results
Examples of sequencing results from ChOR-seq and SCAR-seq experiments in mESCs are presented in Fig. 3.

In ChOR-seq, the analysis of nascent samples provides information about the distribution and abundance of the protein/PTM of interest right after replication fork passage. Standard normalization to RPM (Fig. 3a) can be used to compare the occupancy of the analyzed factors between the nascent sample and a steady-state ChIP sample, which for histone PTMs will provide details on the accuracy of the recycling of modified old histones. Steady-state ChIP-seq reflects population-averaged occupancy in asynchronous cells; to specifically determine the pre-replication occupancy pattern, we recommend synchronizing the cells and harvesting a ChIP-seq sample 1 h before ChOR-seq analysis15. Spike-in normalization allows quantitative comparison, making it possible to address changes in protein/PTM abundance as chromatin matures after replication (Fig. 3b).

SCAR-seq measures a relative partitioning of histone PTMs between the replicated sister chromatids (Fig. 3c). Correlating the partitioning score with the measurements of RFD by OK-seq allows for assessment of the old and new histone distribution to the leading and lagging strands (Fig. 3d). For example, we compared the old and new histone partitioning in wild-type mESCs and mESCs carrying an MCM2-2A mutation that abolishes MCM2-H3-H4 interactions75,76. The defect in MCM2-histone binding results in an exacerbated distribution of the old histones toward the leading strand and the new histones toward the lagging strand, respectively, compared to the wild type, indicating that in the MCM2-2A mutant histone, recycling to the lagging strand is impaired (Fig. 3c,d).

Reporting Summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Published available sequencing raw and processed datasets analyzed in this work are available in GSE110354 for ChOR-seq and GSE117274 for SCAR-seq. Data analyzed in Fig. 3a,b correspond to GSM2988387, GSM2988389 and GSM2988390 from ref. 15. Data shown in Fig. 3c are GSM3290321, GSM3290334, GSM3290324, GSM3290344 and GSM3290342. Data used in Fig. 3d correspond to the average signal of all replicates described in ref. 11.

Code availability
Code used to analyze SCAR-seq data is available at https://zenodo.org/record/4719235#.YN5p0BNuclDU (GitHub: https://github.com/anderssonlab/Replication_SCARseq, release v1.0.0).

References


Acknowledgements

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Author contributions

C.G.-A., N.R.-G. and A.G. conceived the ChOR-seq project. C.G.-A. and N.R.-G. developed the ChOR-seq method with input from N.P. and supervision from A.G. C.G.-A. and N.R.-G. built the ChOR-seq analysis pipeline. N.P. and A.G. conceived the SCAR-seq project. N.P. developed the SCAR-seq method with supervision from A.G. M.D. built the SCAR-seq analysis pipeline, with support from N.P. and supervision from R.A. N.P., N.R.-G. and C.G.-A. wrote the manuscript with input from all authors.

Competing interests

A.G. is co-inventor on a patent covering the therapeutic targeting of Tonsoku-like protein (TONSL) for cancer therapy. A.G. is a co-founder and chief strategy officer of Ankrin Therapeutics.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-021-00585-3.

Correspondence and requests for materials should be addressed to A.G.

Peer review information *Nature Protocols* thanks Frederic Berger, Angelika Feldmann, Robert Klose, and the other, anonymous, reviewer(s) for their contributions to the peer review of this work.

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Related links

**Key references using this protocol**


Reporting Summary

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- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
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- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Published available sequencing raw and processed datasets analyzed in this work are available in GSE110354 for ChOR-seq and GSE117274 for SCAR-seq.

Data analyzed in Figure 3 a and b correspond to GSM2988387, GSM2988389 and GSM2988390. Data shown in Figure 3c are: GSM3290321, GSM3290334, GSM3290324, GSM3290344 and GSM3290342. Data used in Figure 3d correspond to the average signal of all relevant replicates (GSE117274). Figure 3d is adapted from (Petryk et al., 2018, PMID: 30115746), as permitted under the AAAS’s license to publish.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No sample-size calculation was performed. The sample size is determined by the number of reads obtained by the sequencer machine. The differences between samples are corrected by normalization by million of reads (RPM).

Data exclusions
No data exclusion was applied

Replication
In the original ChOR-seq and SCAR-seq studies (Reverón-Gómez N. et al 2018, and Petryk N. et al 2018, respectively) a minimum of two replicates per condition were used showing a high degree of reproducibility. For ChOR-seq analysis done in synchronized HeLa S3 cells, although very similar, replicated regions of the genome were not identical in the different replicates due to slight differences in the release after synchronisation. Thus, it is preferable to analyze each replicate separately. In the figure 3 of this work, one representative replicate was shown except for the Figure 3d, which represents the averaged partitioning signal of all replicates and is adapted from (Petryk et al., 2018, PMID: 30115746), as permitted under the AAAS’s license to publish.

Randomization
Not applicable. This a protocols paper with no biological conclusion intended

Blinding
Not applicable. This a protocols paper with no biological conclusion intended

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Materials & experimental systems

- n/a
- Involved in the study
- Antibodies
- Eukaryotic cell lines
- Palaeontology and archaeology
- Animals and other organisms
- Human research participants
- Clinical data
- Dual use research of concern

Methods

- n/a
- Involved in the study
- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

- Antibodies used
  Described in Table 2
- Validation
  The commercial antibodies were validated by the manufacturers. For H4K20me2 antibody (Diagenode, C15200205) were additionally validated by dot-blot (described in Petryk et al 2018 (PMID: 30115746)).

Eukaryotic cell lines

Policy information about cell lines

- Cell line source(s)
  Human HeLa S3 cells (Cat. No. CCL-2-2; RRID: CVCL_0058); Mouse E14 ES Cells (Laboratories of Kristian Helin and Joshua Brickman; RRID:CVCL_C320); D. melanogaster S2-DRSC (Drosophila Genomics Resource, Center, Stock No. 181)
- Authentication
  None were authenticated
- Mycoplasma contamination
  Negative for mycoplasma
- Commonly misidentified lines
  None used.
ChIP-seq

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- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
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May remain private before publication.

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To review GEO accession GSE117274 (SCAR-seq) go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi

Files in database submission

In GSE110354:
- GSM2988386 H3K27me3_ChIP-seq_Parental_rep1
- GSM2988387 H3K27me3_CHOR-seq_T0_rep1
- GSM2988388 H3K27me3_CHOR-seq_T4_rep1
- GSM2988389 H3K27me3_CHOR-seq_T10_rep1
- GSM2988390 H3K27me3_CHOR-seq_T24_rep1
- GSM2988391 H3K27me3_CHOR-seq_T0+EZH2i_rep1
- GSM2988392 H3K27me3_CHOR-seq_T4+EZH2i_rep1
- GSM2988393 H3K27me3_CHOR-seq_T10+EZH2i_rep1
- GSM2988394 H3K27me3_CHOR-seq_T24+EZH2i_rep1
- GSM2988395 H3_CHIP-seq_Parental_rep1
- GSM2988396 H3_CHOR-seq_T0_rep1
- GSM2988397 Streptavidin_pull-down_midS_rep1
- GSM2988398 H3K27me3_CHIP-seq_Parental_rep2
- GSM2988399 H3K27me3_CHOR-seq_T0_rep2
- GSM2988400 H3K27me3_CHOR-seq_T4_rep2
- GSM2988401 H3K27me3_CHOR-seq_T10_rep2
- GSM2988402 H3K27me3_CHOR-seq_T24_rep2
- GSM2988403 H3K27me3_CHOR-seq_T0+EZH2i_rep2
- GSM2988404 H3K27me3_CHOR-seq_T4+EZH2i_rep2
- GSM2988405 H3K27me3_CHOR-seq_T10+EZH2i_rep2
- GSM2988406 H3K27me3_CHOR-seq_T24+EZH2i_rep2
- GSM2988407 H3_CHIP-seq_Parental_rep2
- GSM2988408 H3_CHOR-seq_T0_rep2
- GSM2988409 Streptavidin_pull-down_midS_rep2
- GSM2988418 Input_biotin_rep1
- GSM2988419 H3_CHIP-seq_biotin_Parental_rep1
- GSM2988420 H3_CHIP-seq_biotin_Parental_rep2
- GSM2988421 H3K27me3_CHIP-seq_biotin_Parental_rep1
- GSM2988422 H3K27me3_CHIP-seq_biotin_Parental_rep2
- GSM2988423 Streptavidin_pull-down_biotin_midS_rep1
- GSM2988424 Streptavidin_pull-down_biotin_midS_rep2
- GSM2988425 H3_CHOR-seq_biotin_T0_rep1
- GSM2988426 H3_CHOR-seq_biotin_T0_rep2
- GSM2988427 H3K27me3_CHOR-seq_biotin_T0_rep1
- GSM2988428 H3K27me3_CHOR-seq_biotin_T0_rep2
- GSM3227882 H3K4me3_CHIP-seq_Parental_rep1
- GSM3227883 H3K4me3_CHIP-seq_Parental_rep2
- GSM3227884 H3K4me3_CHIP-seq_T0_rep1
- GSM3227885 H3K4me3_CHIP-seq_T0_rep2
- GSM3227886 H3K4me3_CHOR-seq_T1_rep1
- GSM3227887 H3K4me3_CHOR-seq_T1_rep2
- GSM3227888 H3K4me3_CHOR-seq_T6_rep1
- GSM3227889 H3K4me3_CHOR-seq_T6_rep2
- GSM3227890 H3K4me3_CHOR-seq_T12_rep1
- GSM3227891 H3K4me3_CHOR-seq_T12_rep2
- GSM3227892 H3K36me3_CHIP-seq_Parental_rep1
- GSM3227893 H3K36me3_CHIP-seq_Parental_rep2
- GSM3227894 H3K36me3_CHOR-seq_T0_rep1
- GSM3227895 H3K36me3_CHOR-seq_T0_rep2
- GSM3227896 H3K36me3_CHIP-seq_Parental_rep1
- GSM3227897 H3K36me3_CHIP-seq_Parental_rep2
- GSM3227898 H3K36me3_CHOR-seq_T0_rep1
- GSM3227899 H3K36me3_CHOR-seq_T0_rep2
- GSM3227900 INPUT_earlyS_rep1
- GSM3227901 INPUT_earlyS_rep2
- GSM3227902 Streptavidin_pull-down_earlyS_rep1
- GSM3227903 Streptavidin_pull-down_earlyS_rep2

In GSE117274:
- GSM3290319 SCAR_seq_mESC_K20me2_r1
- GSM3290320 SCAR_seq_mESC_K20me2_r2
- GSM3290321 SCAR_seq_mESC_K20me2_r3
GSM3290322 SCAR_seq_mESC_K20me2_parental
GSM3290323 SCAR_seq_mESC_K20me2_MCM2_2A#1_r1
GSM3290324 SCAR_seq_mESC_K20me2_MCM2_2A#1_r2
GSM3290325 SCAR_seq_mESC_K20me2_MCM2_2A#2_r1
GSM3290326 SCAR_seq_mESC_K20me2_MCM2_2A#2_r2
GSM3290327 SCAR_seq_mESC_K36me3_r1
GSM3290328 SCAR_seq_mESC_K36me3_r2
GSM3290329 SCAR_seq_mESC_K36me3_r3
GSM3290330 SCAR_seq_mESC_K36me3_MCM2_2A#1_r1
GSM3290331 SCAR_seq_mESC_K36me3_MCM2_2A#1_r2
GSM3290332 SCAR_seq_mESC_K36me3_MCM2_2A#2_r1
GSM3290333 SCAR_seq_mESC_K36me3_MCM2_2A#2_r2
GSM3290334 SCAR_seq_mESC_K5ac_r1
GSM3290335 SCAR_seq_mESC_K5ac_r2
GSM3290336 SCAR_seq_mESC_K5ac_parental
GSM3290337 SCAR_seq_mESC_input_EdU30min_r1
GSM3290338 SCAR_seq_mESC_input_EdU15min_r2
GSM3290339 SCAR_seq_mESC_input_EdU15min_r3
GSM3290340 SCAR_seq_mESC_input_MCM2_2A#1_r1
GSM3290341 SCAR_seq_mESC_input_MCM2_2A#2_r1
GSM3290342 OK_seq_mESC
GSM3290343 SCAR_seq_mESC_K5ac_MCM2_2A#1_r1
GSM3290344 SCAR_seq_mESC_K5ac_MCM2_2A#1_r2
GSM3290345 SCAR_seq_mESC_input_K5ac_MCM2_2A#1_r1
GSM3290346 SCAR_seq_mESC_K5ac_MCM2_2A#2_r1
GSM3290347 SCAR_seq_mESC_K5ac_MCM2_2A#2_r2
GSM3290348 SCAR_seq_mESC_input_K5ac_MCM2_2A#2_r1

Genome browser session
(e.g. UCSC)
n/a

Methodology

Replicates
At least two replicates of each condition were performed except for the OK-seq data where only one replicate was done.

Sequencing depth
All experiments were single-end reads of 75bp length. Sequencing depth of the ChOR-seq datasets for this work:
- GSM2988387 H3K27me3_ChOR-seq_T0_rep1: Total number of reads: 73.3 Millions; Unique mapped (hg19): 41.62 Millions; Unique mapped after PCR duplicates (dm3): 10.3 Millions.
- GSM2988389 H3K27me3_ChOR-seq_T10_rep1: Total number of reads: 126.17 Millions; Unique mapped (hg19): 86.27 Millions; Unique mapped after PCR duplicates (dm3): 10.29 Millions.
- GSM2988390 H3K27me3_ChOR-seq_T24_rep1: Total number of reads: 132.56 Millions; Unique mapped (hg19): 97.73 Millions; Unique mapped after PCR duplicates (dm3): 7.41 Millions.

Sequencing stats of the SCAR-seq samples (GSE117274) are listed in Table S1 of Petryk et al 2018 (PMID: 30115746)

Antibodies
All antibodies used are described in Table2

Peak calling parameters
Not applicable in this study

Data quality
It is reported in the manuscript

Software
For ChOR-seq, public tools (Galaxy server and SeqMonk) are reported in the manuscript. For SCAR-seq, a link to custom code is provided.

Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.
**Methodology**

**Sample preparation**
Described in the manuscript

**Instrument**
BD FACS Calibur

**Software**
Data were collected with CellQuest Pro software and analyzed by FlowJo software version 10.7.1.

**Cell population abundance**
2565 cells were acquired.

**Gating strategy**
The FSC/SSC gates defined the single-cell population. The EdU-positive cells reveal AF647 signal above the G1 cells. Gating strategy of EdU-positive cells is described in the Box 1.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.